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Abstract

Antimicrobial photodynamic treatment (APDT) is a promising light based approach to control diseases caused by plant-pathogenic fungi. In the present study, we evaluated the in planta effects of APDT with the phenothiazinium photosensitizer methylene blue (MB) under solar radiation on the germination and viability of conidia of the pathogenic fungus Colletotricum abscissum (former Colletotrichum acutatum sensu lato). Experiments were performed both on leaves and petals of sweet orange (Citrus sinensis) in different seasons and weather conditions. Conidial suspensions were deposited on the leaves and petals surface, treated with the PS (25 or 50 μM) and exposed to solar radiation for only 30 minutes. The effects of APDT on conidia were evaluated by counting the colony forming units (CFU) recovered from leaves and petals and by direct evaluating conidial germination on the surface of these plant organs after the treatment. To better understand the mechanistic of conidial photodynamic inactivation, the effect of APDT on the permeability of the conidial plasma membrane was assessed using the fluorescent probe propidium iodide (PI) together with flow cytometry and fluorescence microscopy. APDT with MB and solar exposure killed C. abscissum conidia and prevented their germination on both leaves and petals of citrus. Reduction of conidial viability was up to 3 logs and a complete photodynamic inactivation was achieved in some of the treatments. APDT damaged the conidial plasma membrane and increased its permeability to PI. No damage to sweet orange flowers or leaves was observed after APDT. The demonstration of the efficacy of APDT in planta represents a further step towards the use of the method for control phytopathogens in the field.

Keywords	antimicrobial photodynamic treatment; antifungal photodynamic treatment; photoantimicrobials; plant-pathogenic fungi;

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Dr. Antonio L. B. Pinheiro
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Dear Dr. Pinheiro,

Colletotrichum abscissum is a major plant-pathogenic fungus. Previous studies have reported that antimicrobial photodynamic treatment (APDT) with different photosensitizers efficiently kills conidia of several genera of plant-pathogenic fungi, including *Colletotrichum*. However, as all of them were conducted *in vitro* there is no data regarding the efficacy of APDT *in planta* under solar radiation. In the present study we assessed the effects of APDT with methylene blue (MB) and solar radiation on the germination and viability of *C. abscissum* conidia both on petals and leaves of citrus. In an attempt to improve the understanding of the mechanisms involved in conidial photoinactivation, the effect of APDT on conidial plasma membrane permeability was investigated. Results showed that photodynamic treatment with MB under solar radiation kills *C. abscissum* conidia both on petals and leaves of sweet orange without damaging the plant host. APDT impaired the conidial plasma membrane increasing its permeability to propidium iodide. This opens the interesting perspective of using APDT to control a major plant-pathogenic fungus.

Yours sincerely,

Gilberto U. L. Braga

Mark Wainwright

In planta photodynamic inactivation of the fungus *Colletotrichum abscissum* with methylene blue under solar radiation

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ABSTRACT

Antimicrobial photodynamic treatment (APDT) is a promising light based approach to control diseases caused by plant-pathogenic fungi. In the present study, we evaluated the *in planta* effects of APDT with the phenothiazinium photosensitizer methylene blue (MB) under solar radiation on the germination and viability of conidia of the pathogenic fungus *Colletotricum abscissum* (former *Colletotrichum acutatum sensu lato*). Experiments were performed both on leaves and petals of sweet orange (*Citrus sinensis*) in different seasons and weather conditions. Conidial suspensions were deposited on the leaves and petals surface, treated with the PS (25 or 50 μM) and exposed to solar radiation for only 30 minutes. The effects of APDT on conidia were evaluated by counting the colony forming units (CFU) recovered from leaves and petals and by direct evaluating conidial germination on the surface of these plant organs after the treatment. To better understand the mechanistic of conidial photodynamic inactivation, the effect of APDT on the permeability of the conidial plasma membrane was assessed using the fluorescent probe propidium iodide (PI) together with flow cytometry and fluorescence microscopy. APDT with MB and solar exposure killed *C. abscissum* conidia and prevented their germination on both leaves and petals of citrus. Reduction of conidial viability was up to 3 logs and a complete photodynamic inactivation was achieved in some of the treatments. APDT damaged the conidial plasma membrane and increased its permeability to PI. No damage to sweet orange flowers or leaves was observed after APDT. The demonstration of the efficacy of APDT *in planta* represents a further step towards the use of the method for control phytopathogens in the field.

1. Introduction

Postbloom fruit drop (PFD) or blossom blight is one of the most important diseases of citrus caused by fungi in the Americas (Timer and Peres 2015; Peres et al 2005; Timmer et al 1994). The disease is caused by the ascomycetes fungi *Colletotrichum acutatum sensu lato* and *Colletotrichum gloeosporioides sensu lato* (Lima et al 2011). A recent phylogeny study has proposed several cryptic species within the *C. acutatum* complex (Damm et al 2012; Bragança et al 2016) and one of these species, *Colletotrichum abscissum*, was identified by molecular analyses as a PFD causal agent (Crous et al 2015; Pinho et al 2015). PFD causes serious economic production losses of different citrus species and varieties (Timmer et al 1994). In Brazil, the reduction of sweet orange yield can reach 80% when the flowering coincides with periods of heavy rainfall in orchards without disease control (De Goes et al 2008; Silva-Junior et al 2014a). Typical symptoms of PFD are orange-brown lesions on petals and small peach-brown to dark-brown necrotic spots on the stigma and style (Lin et al 2001; Marques et al 2013). Flower infection leads to hormonal changes that cause fruit abscission (Li et al 2003; Lahey et al 2004). *C. abscissum* (former *C. acutatum*) produces acervuli (asexual fruiting bodies) on both sides of the petals with abundant unicellular orange conidia surrounded by a mucilaginous matrix (Marques et al 2013; de Menezes et al 2015). The conidia are dispersed by rain splash after the mucilage has been dissolved by water (Peres et al 2005; Timmer et al 1994). *C. abscissum* can survive on surface of citrus and weed leaves in the form of appressoria for up to three months (Zulfiqar et al 1996; Frare et al 2016). During the blooming, different compounds are washed from citrus flowers to leaves, and induce the appressoria to germinate and produce secondary conidia without acervuli (Timmer et al 1994). As the

conidium is an essential developmental phase in the fungal life cycle and critical for plant infection, most conventional antifungal agents have been developed to target conidial germination and early developmental stages (Deng et al 2015).

Current management strategies for *Colletotrichum* spp. are mainly based on preventive fungicide sprays during the blooming period, particularly when rain events occur (Silva-Junior et al 2014a). However, the control of *Colletotrichum* species as well as other plant-pathogenic fungi faces some of the problems that have been reported in the related clinical area, including the selection of antifungal-tolerant strains and the relatively few classes of currently available and effective antifungal agents (Peres et al 2004; Deising et al 2008; Wong et al 2008; Silva-Junior et al 2014a). There are only two groups of antifungal agents (strobilurins and triazoles) available for PFD control in sweet orange commercial orchards to juice production in São Paulo state, Brazil (Silva-Junior et al 2014a). The limited availability of antifungal agents and the selection of tolerant strains have stimulated the development of new strategies for the control of plant-pathogenic fungi, including the light-based approach antimicrobial photodynamic treatment (de Menezes et al 2014; de Menezes et al 2014; Facarolli et al 2016; de Menezes et al 2016).

APDT is a promising antifungal alternative to conventional antifungal agents that can be used to kill fungi causing diseases in animals or plants (Dai et al 2012; de Menezes et al. 2014a,b; Baltazar et al 2015; de Menezes et al 2016; Wainwright et al 2016). The approach is based on the application of a photosensitizer (PS) that binds to the surface or preferentially accumulates in the target microbial cell (de Menezes et al 2014a,b; de Menezes et al 2016; Vandresen et al 2016). Subsequent exposure of the PS to light of an appropriate wavelength starts a photochemical process that produces several reactive oxygen species (ROS), such as peroxides and singlet oxygen, leading to

non-specific oxidative damage and causing the subsequent death of the fungi without significant harm to the plant host tissues (de Menezes et al 2014a,b; Facarolli et al 2016). When produced at the plant surface, either via natural plant-produced PS or via applied PS, reactive species can interact with and kill pathogenic fungi and bacteria (Flors and Nonell 2006; Berenbaum and Larson 1988). In comparison with the currently used fungicides, the multiple and variable targets of reactive oxygen species reduce the chance of selecting tolerant microorganisms. Also, the PS used in APDT are usually less toxic to humans and animals and are less aggressive to the environment than most of the currently used antifungal agents (de Menezes et al 2014a,b; Facarolli et al 2016; Buchovec et al 2016). Unlike some of the conventional antifungal agents that are fungistatic and/or act only on metabolically active cells (Hof 2001; Bartlett et al 2002; Inoue et al 2012), APDT it is able to kill both metabolically active and dormant or quiescent structures such as fungal (Gonzales et al 2010; Gomes et al 2011; de Menezes et al 2014a,b; de Menezes et al 2016; Facarolli et al 2016; Vandresen et al 2016; Bouchovec et al 2016) and bacterial spores (Eichner et al 2015; Luksiene et al 2010). The disadvantages of APDT compared to conventional antifungal agents are that the treatment does not work in the dark, and most of the photosensitizers remain effective against the pathogen for a shorter period than the conventional antifungals on the plant surface.

Previous studies have reported that APDT with different PS efficiently kills conidia of several genera of plant-pathogenic fungi, including *Alternaria*, *Rhizopus* (Lukisiene et al 2004; Likisiene et al 2005), *Colletotrichum* (Facarolli et al 2016; de Menezes et al 2014a,b; Vandresen et al 2016; DiCosmo et al 1982), *Fusarium* (de Menezes et al 2016; Gao et al 2016; Vorobey and Pinchuk, 2008; Asthana and Tuveson 1992) and *Botrytis* (Kairyte et al 2013; Tegegne et al 2008; Mares et al 2004). However,

as all the previous studies were conducted *in vitro* there is no data regarding the efficacy of APDT *in planta* under solar radiation. Also, only a few studies evaluated the effect of the application of PS on the plant host (de Menezes et al 2014a,b; Facaroli et al 2016; Guillaumont et al 2016). We demonstrated that *in vitro* APDT with phenotiazinium PS such as MB, toluidine blue O (TBO), new methylene blue N (NMBN), and the novel pentacyclic derivative S137 efficiently killed conidia of *Colletotrichum* spp. reducing conidial viability up to five logs (de Menezes et al 2014a). The applications of these PS at concentrations much higher than the necessary to kill the conidia did not damage the plant host *Citrus sinensis* (de Menezes et al 2014a).

The aim of this study was to assess the effects of *in planta* APDT with MB and solar radiation on the germination and viability of *C. abscissum* conidia. In an attempt to improve the understanding of the mechanisms involved in conidial photoinactivation, the effect of APDT with MB on conidial plasma membrane permeability was investigated. The effect of APDT on the leaves and flowers of *C. sinensi*s was also determined.

2. Material and Methods

2.1. Source of *Colletotrichum abscissum and Production of Conidial Suspensions*

Colletotrichum abscissum isolate 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. The isolate is stored at the Plant-Pathogenic Fungi Collection of the Department of Phytopathology and Nematology (Escola Superior de Agricultura "Luiz de Queiroz", University of São Paulo, Piracicaba, Brazil). The fungus was grown on 25 mL potato dextrose agar (Acumedia Manufacturers, Lansing, MI, USA) supplemented with 1 g L^{-1} Bacto™ Yeast Extract (Becton, Dickinson and Company,

Sparks, MD, USA) (PDAY) in petri dishes (90 × 10 mm) at 28 °C for 5 days under a 12:12h (dark:light) photoperiod. Conidia were carefully scrapped from the colonies and suspended in sterile distilled water or 0.01% (v/v) Tween 80 (Sigma-Aldrich, Inc. St. Louis, MO, USA). The concentration of conidia suspension was determined by counting with a hemocytometer (Improved Neubauer, Boeco, Germany) and appropriate dilutions were made.

2.2. Photosensitizer

Methylene blue (MB, catalog number M9140) was purchased from Sigma-Aldrich. MB displays a strong aqueous absorption band at 660 nm. The chemical structure of MB and its absorption spectrum were shown previously (de Menezes et al 2014a). Stock solutions of MB were prepared by dissolving the powder in water at concentrations 10-fold higher than the highest concentration used. The solutions were stored in the dark at -20 °C for up to 2 weeks. Dilutions were prepared with a 0.01% (v/v) Tween 80 solution.

2.3. Solar Radiation Measurements

Both solar spectral irradiance and solar UV irradiance were measured using a cosine-corrected irradiance probe (CC-3-UV, Ocean Optics, Dunedin, FL, USA) screwed onto the end of an optical fiber coupled to an USB4000 spectroradiometer (Ocean Optics). Solar spectral irradiance was measured under clear sky at midday in all seasons of the year. As examples, the integrated irradiances in the UV spectrum (290-390 nm) were approximately: 45.8; 37.1; 20.1 e 40.0 W m^{-2}, and in the visible spectrum (400-790 nm) the irradiances were approximately : 579.2; 482.2; 299.7 and 401.7 W m^{-2} on November 17th, 2014 (spring), February 26th, 2015 (summer), April 30th, 2015

(autumn) and August 19th, 2014 (winter), respectively. Midday solar spectral irradiances in different seasons are shown in Supplementary Fig. S1.

2.4. Effect of APDT with MB and Solar Radiation on Conidia Viability

The effects of APDT with MB and solar radiation on the viability of *C. abscissum* conidia were estimated based on the number of colony forming units (CFU) recovered from the citrus plants. Experiments were conducted on both petals and leaves of 'Valencia' sweet orange plants (*C. sinensis*). Potted trees were approximately 1.5 m tall and were obtained from the Fundo de Defesa da Citricultura (Fundecitrus, Araraquara, SP, Brazil). Conidia were suspended in sterile distilled water and 5 µL of the suspension (4×10^6 conidia mL^{-1}) were deposited on the adaxial surfaces of petals and leaves. Then, on the same spot, 5 µL of MB solution (50 or 100 µM) were added. Final concentrations of conidia and MB in the mixture were 2×10^6 conidia mL^{-1} and 25 or 50 µM of MB. After spotting of the photosensitizer, plants were exposed to sunlight for 30 min (+L +PS 25 µM and +L +PS 50 µM). As control, MB solution was replaced by 5 µL of 0.01% Tween 80 solution (+L –PS). Experiments were conducted in Ribeirão Preto, SP, (latitude: 21º 10' 16.2" S, longitude 47º 50' 51.5" W altitude: 560 m). Dark controls were also prepared. In these cases, petals and leaves were treated as previously described (with or without PS) but kept in the dark (–L –PS and –L +PS). After exposure to solar radiation, fragments of petals or leaves which received the treatments were excised from the plant. Each fragment was placed in a 0.5-mL microtube (polypropylene, Axygen Scientific, USA), covered with 200 µL of 0.01% Tween 80 solution and tubes were vortexed. Conidial suspensions were serially diluted 10-fold to give dilutions of 10^{-1} and 10^{-2} times the original concentration, and 50 µL aliquots were spread on the surface of 5 mL PDAY containing 0.08 g L^{-1} deoxycholic

acid (sodium salt) (Fluka, Italy) and 0.5 g L^{-1} chloramphenicol (Sigma) in petri dishes (60 × 15 mm). At this concentration, the salt makes the colonies grow more slowly and compactly, allowing the evaluation of fungal growth for several days (de Menezes et al 2014a,b). Three replicate dishes were prepared for each treatment in each experiment. The dishes were incubated in the dark at 28 °C. After 24 h, CFU were counted daily at a ×8 magnification for up to 5 days. Four experiments were conducted on petals and four on leaves in all seasons encompassing the years of 2014 and 2015. Experiments on petals were performed on August 28 and 29th, September 2nd 2014 and January 23rd 2015, and on leaves they were performed on September 28 and 29th 2014, and January 27 and 28th 2015. Cloud cover, temperature and relative humidity were recorded in each experiment (see Figs. 1 and 2). Temperatures on petals and leaves surfaces were measured using a TI-870 infra red thermometer (Instrutherm, São Paulo, SP, Brazil).

2.5. Effect of APDT with MB and Solar Radiation on Conidia Germination

Petals or leaves of sweet orange were fixed on the bottom of petri dishes using double-sided tape. Ten microliters of conidia suspension were deposited on the adaxial surface of petals (4×10^6 conidia mL^{-1}) or leaves (2×10^5 conidia mL^{-1}). Subsequently, on this drop, 10 µL of MB solution (50 or 100 µM) were applied. Final concentrations in the mixture were 2×10^6 or 10^5 conidia mL^{-1} on petals and leaves, respectively and 25 or 50 µM of PS. As a control, MB solution was replaced by 10 µL 0.01% Tween 80 (+L –PS). Petals and leaves were exposed to full-spectrum solar radiation for 30 min. Dark controls, not exposed to sunlight, were prepared as described previously. After the exposure, petals and leaves were kept in a humid chamber at 28 °C in the dark for 24 h (petals) or 36 h (leaves). Then, 50 µL of a lactophenol-cotton blue solution were applied to the petal surface or 50 µL of a fuchsine solution were applied to the leaf surface.

After 5 min, the excess of dye was washed out and fragments of the petals were mounted between the slide and coverslip. Because of the dark color of the leaves, a drop of super glue was placed on a slide and the leaf fragment was pressed against the slide surface. After 5 min, the fragment was carefully removed from the slide. This process removes conidia and germlings from the leaves. To determine the percentage of conidia germination, 300 conidia were evaluated in each treatment at a ×40 magnification by using an Olympus CX31 optical microscope. A conidium was considered germinated if the germ tube was at least equal in length to the conidium. Germlings were photographed using a digital camera (Camedia C-5060, Olympus) attached to the microscope. Three experiments were performed on petals and leaves. Experiments on petals were performed in spring on August 31st and September 4th and 9th 2015 and on leaves in winter on July 1st, 2nd and 3rd 2016. All experiments were performed under a clear sky between 11 and 12 a.m. Temperatures on petals and leaves surfaces were measured using a TI-870 infra red thermometer (Instrutherm, Brazil).

2.6. Effect of APDT on Conidia Plasma Membrane Permeability

The permeability of the conidial plasma membrane to propidium iodide (PI) after APDT was assessed by flow cytometry and fluorescence microscopy analyses. PI is a fluorescent dye that only penetrates conidia with impaired plasma membrane (de Menezes et al 2016). A mixture of 500 µL of the conidial suspension and 500 µL of the MB solution were added to a 24-well flat-bottomed plate. Final concentrations of conidia and PS in the mixture were 2×10^6 conidia mL^{-1} and 50 µM, respectively. To avoid contamination, plates were covered with a solar radiation-transparent film (0.13-mm-thick premium cellulose triacetate, Liard Plastics, Salt Lake City, UT, USA) and exposed to full-spectrum solar radiation for 30 and 60 min. Exposures were performed

in summer under clear sky on February 25th and March 6th 2015 between 11 a.m. and 2 p.m. To avoid heating during the exposures, plates were kept floating in water at 22 ± 2°C. Three different types of control plates were prepared in all experiments: (i) control plates in which conidia were exposed to solar radiation but not treated with MB (+L – PS), (ii) control plates in which conidia were treated with MB but protected from solar radiation by wrapping in aluminum foil (–L +PS), and (iii) control plates in which conidia were not treated with MB and protected from solar radiation (–L –PS). As a positive control, conidia were also treated with 70% (v/v) ethanol for 30 min. After treatments, 650 µL of the suspension were collected for cytometric analyses and the remainder of the volume (350 µL) was used to evaluate the effect of different treatments on conidia viability as described previously. Conidial suspensions were washed twice with 0.01% (v/v) Tween 80 solution (8000 *g* for 2 min) and resuspended in 650 µL of a 1.5 µM PI solution in 0.01% (v/v) Tween 80. Cytometric analysis was performed on a BD FACSCanto I flow cytometer with the aid of BD FACSDiva software (BD Biosciences). For each treatment, 10000 conidia were analyzed by dye excitation at 488 nm and detection at 670 nm.

Fluorescence microscopic analyses of the conidia after the different treatments were also performed. For fluorescence microscopy imaging, in addition to treatment with PI, conidia were stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) for a better visualization of nuclei. After APDT, 650 µL of the conidial suspension were collected and 1.3 µL of a DAPI (catalog number D9542, Sigma-Aldrich) solution prepared in 0.01% (v/v) Tween 80 was added. Final concentration of the dye was 5.7 µM. The mixture was incubated in the dark with shaking (450 rpm) for 2 h at 28 °C. Subsequently, the suspension was washed three times (8000 *g* for 2 min) with 0.01% Tween 80 solution and resuspended in 650 µL of a 1.5 µM PI (Sigma-

Aldrich) solution in 0.01% Tween 80. The mixture was centrifuged and 600 µL of the supernatant were removed. The remainder 50 µL were agitated, and 3 µL of the conidial suspension were placed on a microscopic slide (Bioslide, 25 × 75 mm) together with 3 µL of low melting point agarose (Ultra PureTM low melting point agarose, Invitrogen) and 3 µL of Fluoromount (Fluoromount aqueous mounting medium, Sigma-Aldrich). The mixture was gently mixed using a micropipette tip and covered with a coverslip (Knittel Glass, 22 × 22 mm). Microscopy inspection was carried out at a 630× magnification by using a confocal microscope (Leica DMI 6000 CS, TCS SP8 scanner, Microsystems GmbH, Germany) operating at simple fluorescence mode. For PI excitation, an OPSL laser was set to 488 nm and detection was set to 535-617 nm. For DAPI excitation, a diode laser was set to 405 nm and detection was set to 430-550 nm.

2.7. Evaluation of APDT Effects on Sweet Orange Leaves and Flowers

The evaluation of APDT effects on sweet orange leaves was performed as described previously (de Menezes et. al., 2014b; Facarolli et al., 2016). Additionally, the effect of APDT was also evaluated on flowers. Using a spray bottle 1.4 mL of MB solutions at 25 or 50 µM was sprayed to flowers. The PS was reapplied daily until natural petal abscission. During the experiments, plants were kept outdoors under a natural sunlight regimen. Experiments were conducted from September 30th to October 14th 2015. Plants were visually evaluated for damage to the leaves and flowers and photographed daily for up to 15 days (see Supplementary Fig. S2).

3. Results

3.1. Effect of APDT with MB and Solar Radiation on Conidia Viability

APDT with MB at 50 μM and solar radiation for 30 min reduced the conidia viability on sweet orange petals in all the four experiments irrespective of the weather conditions or the day the experiments were conducted ($P < 0.05$). The reductions varied from 1.9 to 3.1 $\log_{10}$ (98.9 to 99.9%) in comparison with controls (+L –PS) (Fig. 1).

As observed in experiments conducted on petals, APDT with MB at 25 or 50 μM and solar radiation for 30 min on leaves also reduced the viability of the conidia in all the four experiments ($P < 0.05$). Reduction on conidial viability was up to 3 $\log_{10}$, and in one experiment all conidia were killed. APDT with MB at 25 μM was as effective as with the higher concentration of 50 μM ($P > 0.05$ for all treatment comparisons) (Fig. 2).

3.2. Effect of APDT with MB and Solar Radiation on Conidia Germination

Conidia germination on petal surface in the control group (+L –PS) ranged from 94 to 100% in the three experiments during August and September 2015. APDT (+L +PS) with MB at 25 μM was as effective as with the higher concentration of 50 μM ($P > 0.05$) and both treatments resulted in a decrease in conidia germination to values ranging from 0 to 1% (Fig. 3A and 3B). For leaves, conidia germination in the control group (+L –PS) ranged from 77 to 86% in the three experiments conducted in July 2016. APDT with MB at 25 μM and solar radiation for 30 min was as effective as with the higher concentration of 50 μM ($P > 0.05$) and both treatments resulted in a decrease in conidial germination to values ranging from 5 to 13% (Fig. 4A and 4B).

3.3. Effect of APDT on Conidial Plasma Membrane Permeability

Conidial viability and permeability to PI after APDT with MB and 30 min of exposure to solar radiation are shown Fig. 5. There was no difference in viability among

untreated conidia, conidia treated only with MB, and conidia exposed only to sunlight for 30 min ($P < 0.05$) (Fig. 5A). APDT reduced conidial viability to less than 0.4 % both on the experiments conducted on February 25th and March 6th 2015. Flow cytometric analyses showed that exposure only to solar radiation for 30 min or treatment only with MB at 50 μM did not increase conidial permeability to PI (PI labeling was always below 6%) (Fig. 5A). APDT as well as the treatment with 70% ethanol (positive control) stained close to 100 % of the conidia (Fig. 5A), indicating that APDT damaged the conidial plasma membrane, increasing its permeability to PI. Conidial viability and PI labeling were inversely proportional. DAPI was used in fluorescence microscopy experiments for a better visualization of conidia nuclei. As expected from the flow cytometry analyses, PI penetrates the conidia after APDT, but labeling was not observed in conidia treated only with MB or exposed only to solar radiation for 30 min (Fig. 5B).

3.4. Evaluation of APDT Effects on Sweet Orange Leaves and Flowers

APDT with MB at 25 or 50 μM did not damage leaves or flowers or compromised the formation of the fruits (Supplementary Figs. S2).

4. Discussion

The search for new strategies to control pathogenic microorganisms rekindled the interest in using antimicrobial photodynamic treatment in medicine, agriculture and food microbiology (Rodrigues et al 2012a,b; Rodrigues et al 2013; de Menezes et al 2014a,b; Smijs et al 2014; Baltazar et al 2015; de Menezes et al 2016; Gao et al 2016; Brancini et al 2016). In the agricultural area, several *in vitro* studies have demonstrated

that the APDT with different PS are able to effectively kill conidia of plant-pathogenic fungi of several genera, including *Colletotrichum* (de Menezes et al 2014a,b; Facarolli et al 2016; Vandresen et al 2016). Unfortunately, all the previous studies were performed *in vitro* and, as far as we know, none of them evaluated the effect of APDT *in planta*. In the present study, for the first time, the effects of APDT with MB on the viability and germination of *C. abscissum* (former *C. acutatum*) conidia were evaluated *in planta*. Experiments were performed both on petals and leaves under natural solar radiation. The effect of APDT on conidial viability was evaluated indirectly by counting the UFC recovered from plants and also by the direct evaluation of conidial germination after the different treatments. The two approaches are complementary since the first one allows the evaluation of the effect of APDT on conidial viability with several orders of magnitude and the second one the direct evaluation of conidia. APDT with MB under only 30 min of solar exposure killed *C. abscissum* conidia and prevented their germination on both sweet orange leaves and petals. Reduction on conidial viability was up to 3 logs and a complete kill of conidia was achieved in some of the experiments. The demonstration of APDT efficacy *in planta* is important since it is well established that media could strongly influence the effects of APDT on conidia and yeasts (Shimizu et al 1979; Gonzales et al 2010).

The main blooming period of sweet orange in the São Paulo and Minas Gerais citrus belt occurs during the spring. However, more two or three bloom periods may be observed mainly until summer (Fundecitrus, 2016). The PFD epidemics are observed only when the blooming period coincides with consecutive rainy days (Silva-Junior et al 2014b). In the present study, APDT experiments were conducted in different seasons and under different cloud and environmental conditions. In all the situations evaluated APDT for only 30 min was able to reduce conidial viability in at least two orders of

magnitude. These results indicated that in equatorial or tropical sites, irradiances (light intensities) and fluences (light doses) will not be limiting factors for APDT.

APDT with MB did not damage leaves or flowers or compromised the formation of the fruits in *Citrus sinensis*. This result is consistent with a previous study performed by our group that demonstrated that APDT with MB does not damage the plant tissues (de Menezes et al 2014a). As photodamage to other *Colletotrichum* plant hosts may occur, the adverse effect of APDT should be carefully determined for each pathosystem (de Menezes et al 2014b).

Due to its initial stage of development and intrinsic limitations, we believe that APDT may be used to complement but not substitute the conventional antifungals for control of *Colletotrichum*, since the antifungals probably have a longer period of protection compared to APDT. However, APDT may reduce the viability of ungerminated conidia on petal lesions or on leaves, which is not achieved by the current commercial antifugals, such as strobilurins, that are particularly effective against highly energy-demanding stages, such as spore germination (Bartlett et al 2002; Inoue et al 2012). As a light-based approach, APDT would not work during the night, which is a favorable period for conidial germination and host infection. In addition, PS usually do not have a prolonged residual effect under solar radiation since they can photobleach when exposed to high light intensities. The exposure of the photosensitizers new methylene blue (NMBN) and S135 to solar radiation reduced their effectiveness for this type of APDT. However despite the PS photobleaching, NMBN was still able to kill 90% of *C. abscissum* conidia even after 12 h of exposure to full spectrum sunlight (de Menezes et al 2014a).

The effect of the APDT with MB on *C. abscissum* conidia plasma membrane was assessed by evaluating changes in membrane permeability to PI. The propidium

iodide staining-based assay has been used to evaluate damage to the plasma membrane in fungal cells, including conidia (Davey and Hexley, 2011; de Menezes et al 2016). PI is a membrane-impermeable fluorescent dye that only penetrates cells with impaired plasma membranes. APDT with MB increased the plasma membrane permeability of conidia causing the PI staining of approximately 100% of the conidia (see Fig. 5A). Previous studies with fungi have shown that the plasma membrane is the initial target of ROS generated during APDT with phenothiazinium photosensitizers. APDT with the singlet oxygen sensitizer toluidine blue O (TBO) caused the rapid oxidation of ergosterol and accumulation of oxidized ergosterol derivatives in the plasma membrane, leading to a gradual alteration of the membrane structure and function in *Saccharomyces cerevisiae* (Böcking et al 2000). APDT with MB, TBO, NMBN and S137 also increased the plasma membrane permeability of conidia of *Fusarium oxysporum*, *F. moniliforme* and *F. solani* but only NMBN and S137 caused peroxidation of the membrane lipids (de Menezes et al 2016).

Besides its pre-harvest use for control of plant-pathogenic fungi, such as *Colletotrichum*, other applications for APDT in agriculture are being investigated, such as its post-harvest use to control fungi that cause fruit rotting (de Menezes et al 2016; Buchovec et al 2016) and to kill foodborne pathogens on fruits and seeds (Luksiene et al 2007; Buchovec et al 2016). Although the demonstration of the efficacy of APDT *in planta* represents a further step towards the use of the method for control phytopathogens in the field, some important issues still need to be further investigated. For example, appropriate formulations and best timing of application for each PS and pathosystem of interest, and the environmental impact of these new approaches.

5. Conclusion

In planta photodynamic treatment with MB under solar radiation kills *C. abscissum* conidia both on petals and leaves of sweet orange without damaging the plant host. APDT impaired the conidial plasma membrane increasing its permeability to propidium iodide.

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Figure legends

Fig 1. Effect of antimicrobial photodynamic treatment (APDT) on the viability of *Colletotrichum abscissum* conidia on petals. (**A**) Colony forming units (CFU) recovered from petals after APDT with methylene blue (MB) at 50 μM and solar radiation for 30 min (+L +PS) as compared to control exposed only to solar radiation (+L –PS). Experiments were carried out during different seasons and environmental conditions. Whiskers indicate the standard deviation of the mean of three replicates in the same day. Different lower-case letters indicate that means of controls and APDT are different ($P < 0.05$). **(B)** Plates with CFU from conidia exposed to the two treatments (+L –PS or +L +PS). Images were obtained 96 h after APDT from experiments performed on August 28th, 2014.

Fig. 2. Effect of antimicrobial photodynamic treatment (APDT) on the viability of *Colletotrichum abscissum* conidia on leaves. (**A**) Colony forming units (CFU) recovered from leaves after APDT with two different methylene blue (MB) concentrations (+L +PS 25 μM and +L +PS 50 μM) as compared to control exposed only to solar radiation (+L –PS). Experiments were carried out during different seasons and environmental conditions. Whiskers indicate the standard deviation of the mean of three replicates in the same day. Different lower-case letters indicate that means of controls and APDT are different ($P < 0.05$). † No viability was observed. (**B**) Plates with CFU from conidia exposed to the three treatments (+L –PS, +L +PS 25 μM, or +L +PS 50 μM). Images were obtained 96 h after APDT from experiments performed on September 28th, 2014.

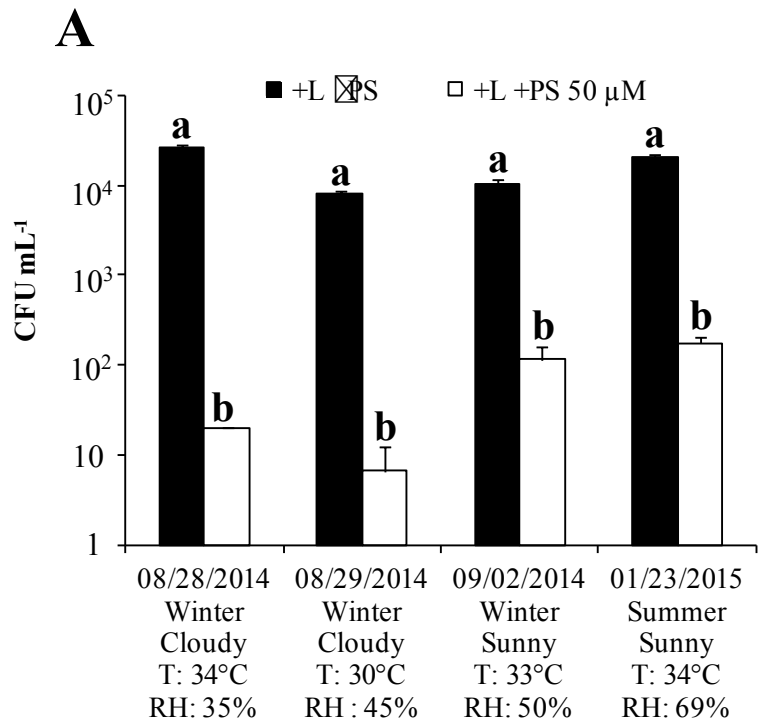
Fig. 3. Effect of antimicrobial photodynamic treatment (APDT) on the germination of *Colletotrichum abscissum* conidia on petals. Conidia exposed only to solar radiation (+L –PS) served as control and APDT was performed with MB at 25 μM (+L +PS 25 μM) or 50 μM (+L +PS 50 μM). In **(A),** whiskers indicate the standard deviation of the mean of three replicates in the same day. Different lower-case letters indicate that means of control and APDT are different ($P < 0.05$). All the experiments were carried out during spring on sunny and clear days. **(B)** Conidial germination after APDT on petals. Micrographs were obtained 24 h after APDT.

Fig. 4. Effect of antimicrobial photodynamic treatment (APDT) on the germination of *Colletotrichum abscissum* conidia on leaves. Conidia exposed only to solar radiation (+L –PS) served as control and APDT was performed with methylene blue (MB) at 25 μM (+L +PS 25 μM) or 50 μM (+L +PS 50 μM). In **(A),** whiskers indicate the standard deviation of the mean of three replicates in the same day. Different lower-case letters indicate that means of control and APDT are different ($P < 0.05$). All the experiments were carried out during winter on sunny and clear days. **(B)** Conidial germination after APDT on leaves. Micrographs were obtained 24 h after APDT.

Fig. 5. (A) Percentage of *Colletotrichum abscissum* conidia stained with fluorescent probe propidium iodite (PI) (grey bars) and conidia viability measured by number of colony forming units (CFU) (dotted line) after antimicrobial photodynamic treatment (APDT) with methylene blue (MB) and solar radiation performed on February 25th (top) and March 6th 2015 (bottom). Whiskers indicate the standard deviation of the mean within each experiment. Different lower- and upper-case letters indicate that means are different ($P < 0.05$). (B) Fluorescence images of conidia exposed only to solar radiation

(+L –PS), treated with APDT (+L +PS), and treated with 70% ethanol. DAPI was used to indicate nuclei and PI was used to evaluate membrane damage.

Fig. 1


A
■ +L ☒PS
□ +L +PS 50 µM
CFU mL-1
10^5
10^4
10^3
10^2
10
1
a
b
a
b
a
b
a
b
08/28/2014
Winter
Cloudy
T: 34°C
RH: 35%
08/29/2014
Winter
Cloudy
T: 30°C
RH : 45%
09/02/2014
Winter
Sunny
T: 33°C
RH: 50%
01/23/2015
Summer
Sunny
T: 34°C
RH: 69%

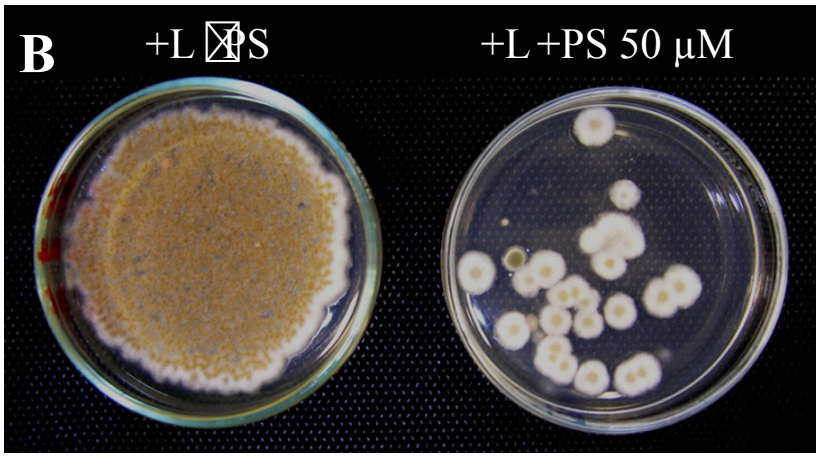
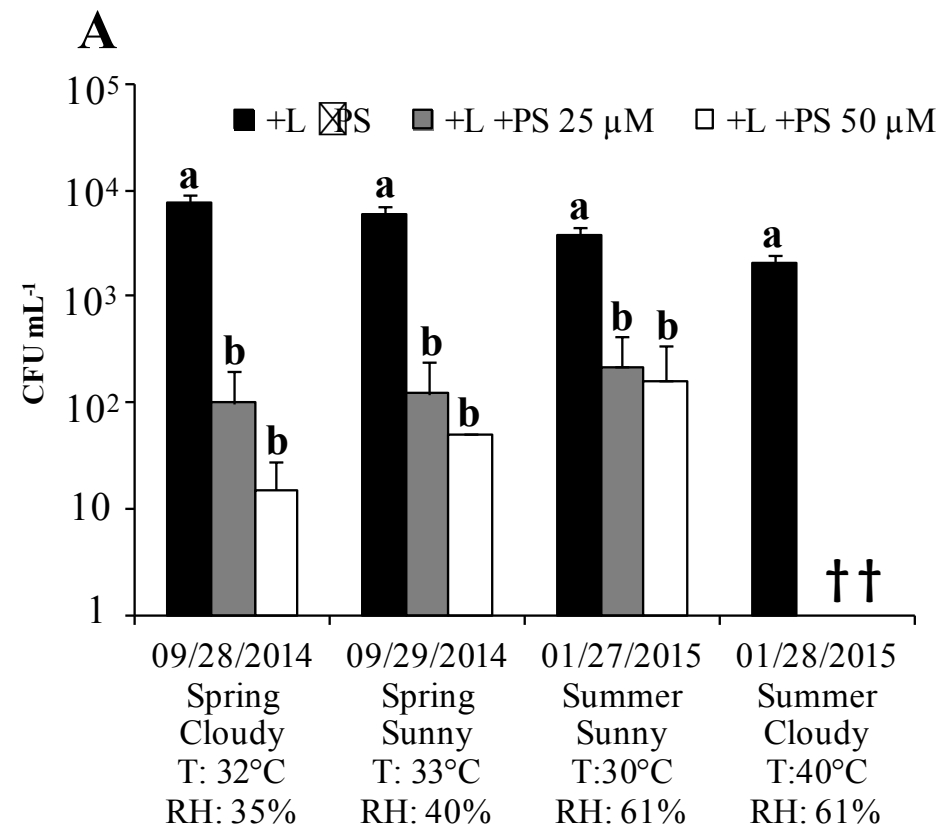

B
+L ☒PS
+L +PS 50 µM

Fig. 2


A
+L −PS
+L +PS 25 µM
+L +PS 50 µM
CFU mL-1
10^5
10^4
10^3
10^2
10
1
a
b
b
a
b
b
a
b
b
a
††
09/28/2014
Spring
Cloudy
T: 32°C
RH: 35%
09/29/2014
Spring
Sunny
T: 33°C
RH: 40%
01/27/2015
Summer
Sunny
T:30°C
RH: 61%
01/28/2015
Summer
Cloudy
T:40°C
RH: 61%

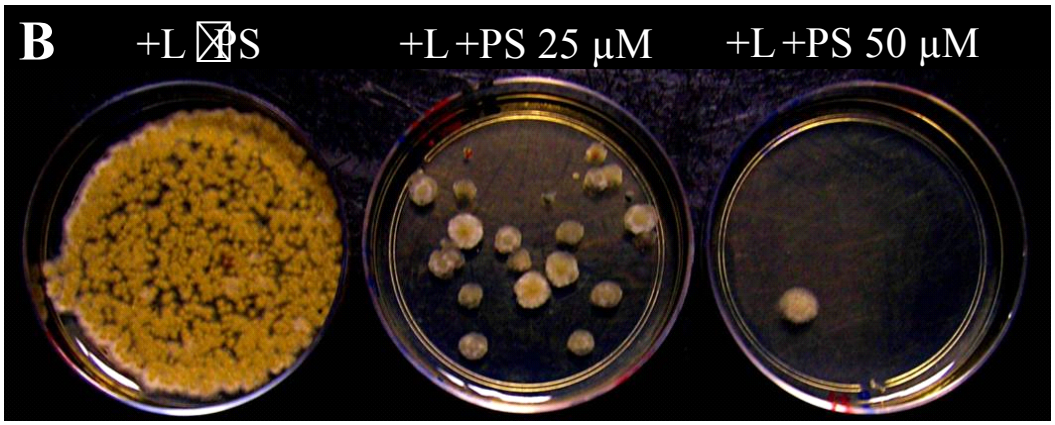
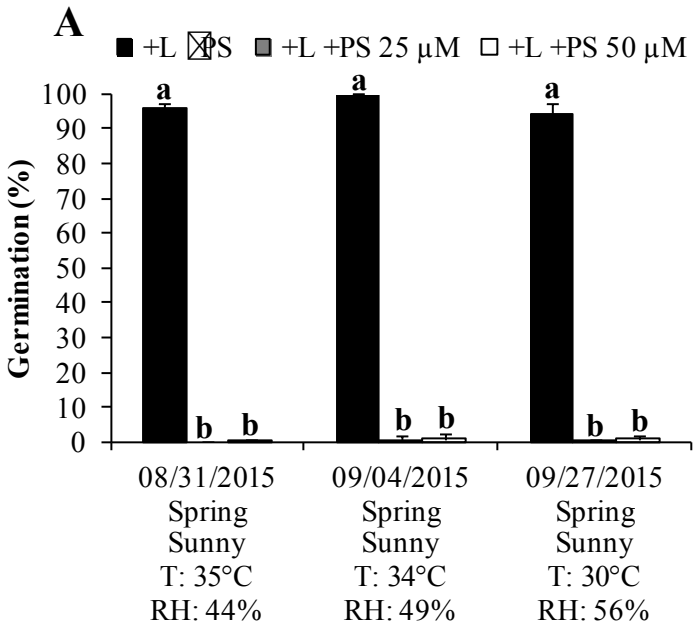

B
+L −PS
+L +PS 25 µM
+L +PS 50 µM

Fig. 3


A
+L -PS
+L +PS 25 µM
+L +PS 50 µM
Germination (%)
100
90
80
70
60
50
40
30
20
10
0
a
b
b
a
b
b
a
b
b
08/31/2015
Spring
Sunny
T: 35°C
RH: 44%
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Spring
Sunny
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09/27/2015
Spring
Sunny
T: 30°C
RH: 56%

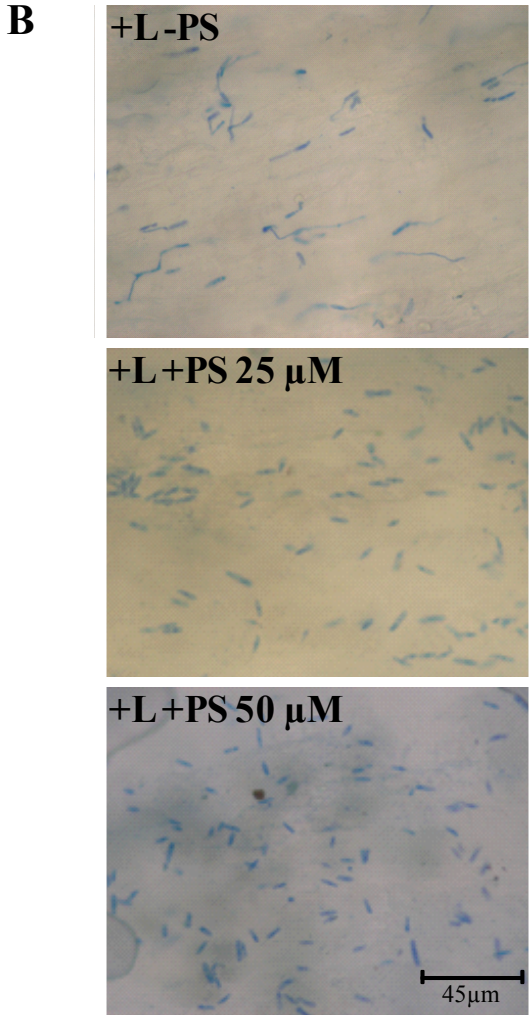
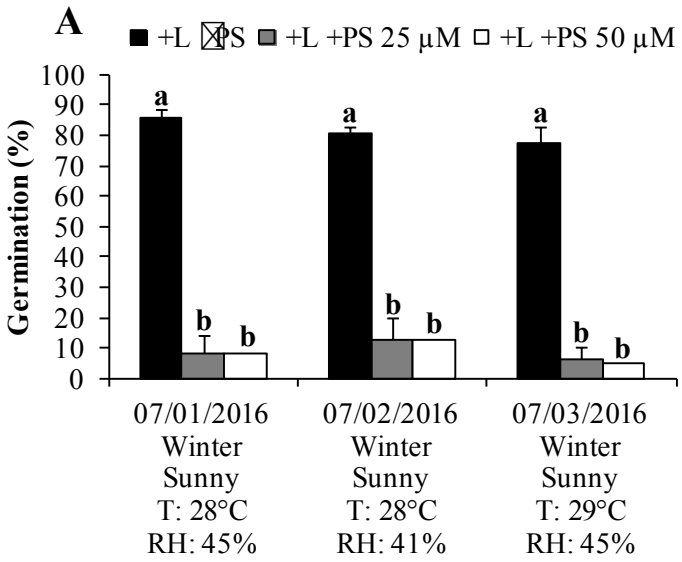

B
+L -PS
+L +PS 25 µM
+L +PS 50 µM
45µm

Fig. 4


A
+L -PS
+L +PS 25 µM
+L +PS 50 µM
Germination (%)
100
90
80
70
60
50
40
30
20
10
0
a
b
b
a
b
b
a
b
b
07/01/2016
Winter
Sunny
T: 28°C
RH: 45%
07/02/2016
Winter
Sunny
T: 28°C
RH: 41%
07/03/2016
Winter
Sunny
T: 29°C
RH: 45%

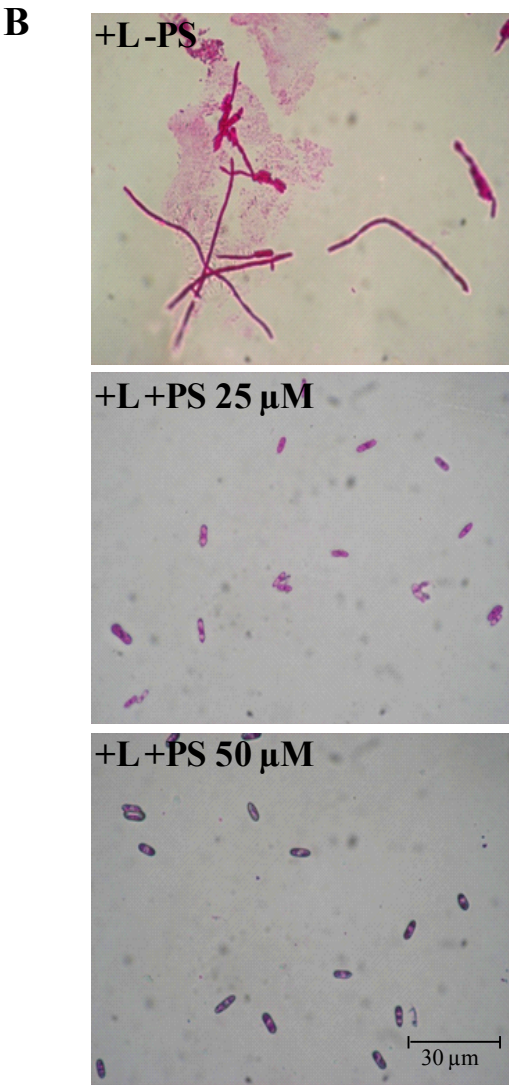
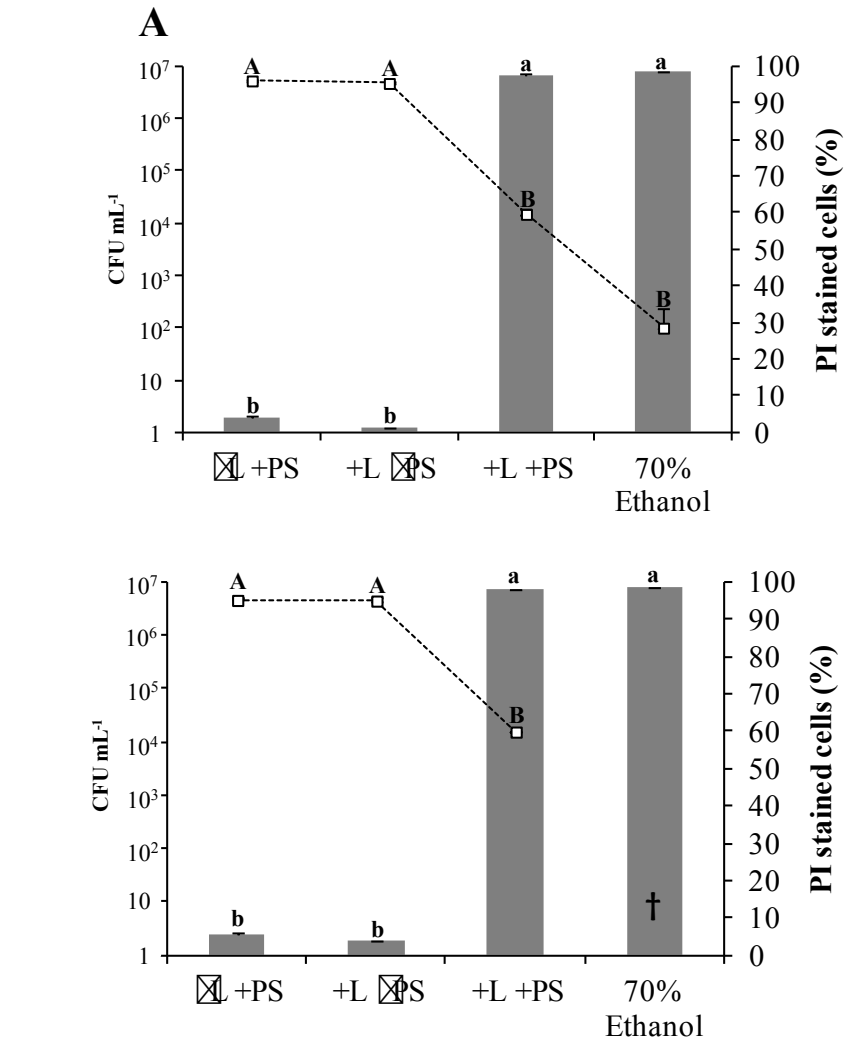
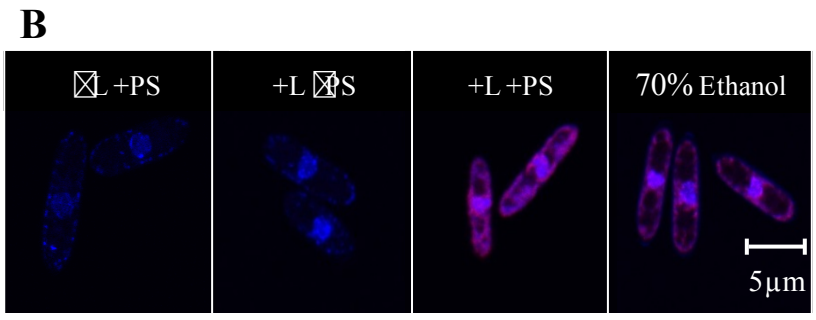

B
+L -PS
+L +PS 25 µM
+L +PS 50 µM
30 µm

Fig. 5


A
CFU mL-1
PI stained cells (%)
A
A
a
a
B
B
b
b
+L +PS
+L +PS
70% Ethanol
†


B
+L +PS
+L +PS
+L +PS
70% Ethanol
5µm

TERMOMETRO INFRAVERMELHO

Instrutherm – Brasil, modelo TI-870

High Temperature

InfraRed thermomether

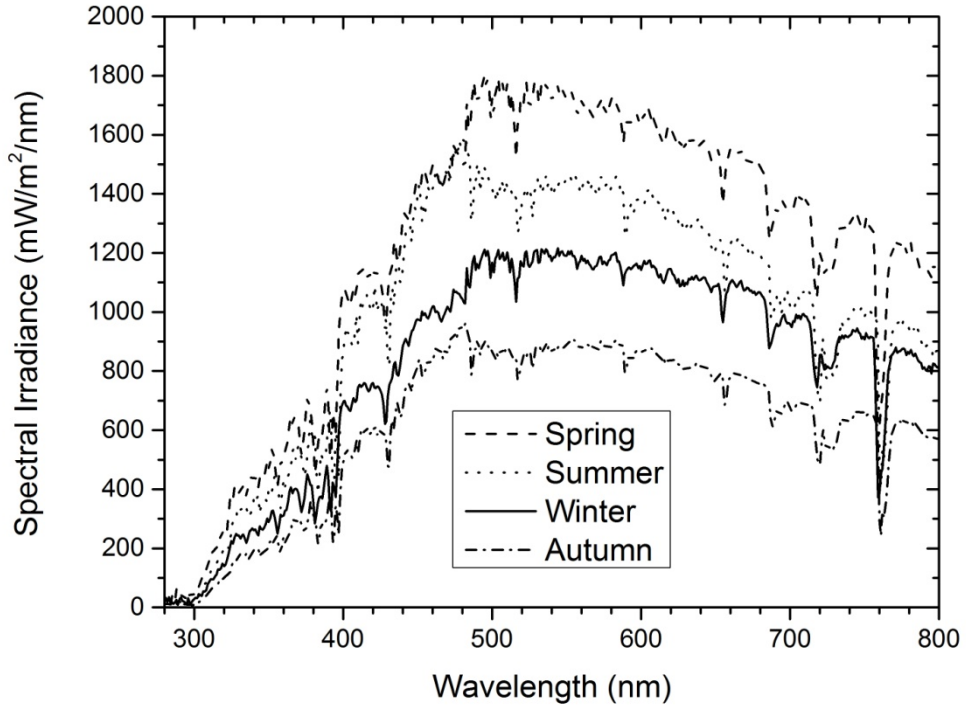
Photodynamic treatment with methylene blue under solar radiation kills *Colletotrichum abscissum* conidia both on petals and leaves of sweet orange.

Photodynamic treatment impairs the conidial plasma membrane.

Photodynamic treatment does not damage the plant host.

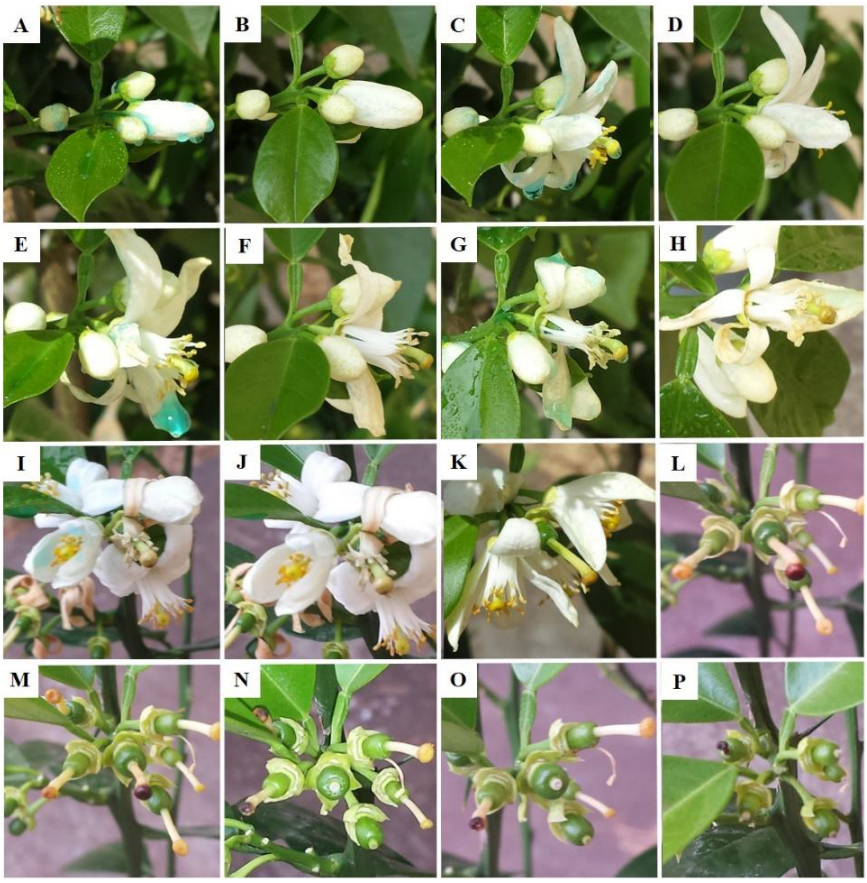
Photodynamic treatment can be used to control plant-pathogenic fungi.

Supplementary Figure S1



Supplementary Figure S1. Midday solar visible irradiance (400-790 nm) and UV irradiance (290-390 nm) recorded in different seasons.

Supplementary Figure S2



Supplementary Figure S2. Evaluation of the effects of APDT with methylene blue at 50 μM under solar radiation on *Citrus sinensis* flowers. The photosensitizer was applied daily for five days. First day: (**A**) immediately after application and (**B**) 2 h later. Second day: (**C**) immediately after application and (**D**) 2 h later. Third day: (**E**) immediately after application and (**F**) 2 h later. Fourth day: (**G**) immediately after and (**H**) 2 h later. Fifth day: (**I**) immediately after application and (**J**) 2 h later. After the fifth day, petals started falling off and MB was no longer applied. Nonetheless, evaluation continued on the (**K**) sixth, (**L**) seventh, (**M**) eighth, (**N**) ninth, (**O**) tenth, and (**P**) eleventh days.