de Menezes, HD, Tonani, L, Bachmann, L, Wainwright, M, Braga, GÚ and von Zeska Kress, MR

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Photodynamic treatment with phenothiazinium photosensitizers kills both ungerminated and germinated microconidia of the pathogenic fungi Fusarium oxysporum, Fusarium moniliforme and Fusarium solani

Henrique Dantas de Menezes[a], Ludmilla Tonani[a], Luciano Bachmann[b], Mark Wainwright[b], Gilberto Úbida Leite Braga[a] and Marcia Regina von Zeska Kress[a*]

[a]Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, 14040-903 Brazil

[b]Departamento de Física, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, 14040-903 Brazil

[c]School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool L3 3AF, United Kingdom

Running title: Photodynamic inactivation of Fusarium spp.

*Corresponding author: Phone: +55 1633150240; Fax: +55 1633154725; E-mail address: kress@fcfrp.usp.br (Marcia Regina von Zeska Kress)

ABSTRACT

The search for alternatives to control microorganisms is necessary both in clinical and agricultural areas. Antimicrobial photodynamic treatment (APDT) is a promising light-based approach that can be used to control both human and plant pathogenic fungi. In the present study, we evaluated the effects of photodynamic treatment with red light and four phenothiazinium photosensitizers (PS): methylene blue (MB), toluidine blue O (TBO), new methylene blue N (NMBN) and the phenothiazinium derivative S137 on ungerminated and germinated microconidia of Fusarium oxysporum, F. moniliforme, and F. solani. APDT with each PS killed efficiently both the quiescent ungerminated and metabolically active germinated microconidia of the three Fusarium species. Washing away the unbound PS from the microconidia (both ungerminated and germinated) before red light exposure reduced but did not prevent the effect of APDT. Subcelullar localization of PS in ungerminated and germinated microconidia and the effects of photodynamic treatment on cell membranes were also evaluated in the three Fusarium species. APDT with MB, TBO, NMBN or S137 increased the membrane permeability and APDT with NMBN or S137 increased the lipid peroxidation in microconidia of the three Fusarium species. These findings expand the understanding of photodynamic inactivation of filamentous fungi with phenothiazinium PS.

Keywords: antimicrobial photodynamic inactivation; methylene blue derivatives; photoantimicrobial; Fusarium oxysporum; Fusarium moniliforme; Fusarium solani.

1. Introduction

Fusarium is a large genus of filamentous fungi widely distributed and easily found in soil. Fusarium species are important pathogens of plants, animals and humans [1-3]. Crop diseases caused by Fusarium sp. generate great economic losses in world production of fruits, vegetables, cereals, and cellulose [4-6]. Another important characteristic of the species of this genus is their ability to produce mycotoxins that contaminate many agricultural commodities affecting both human and animal health [7,8]. Currently, Fusarium oxysporum and F. solani are important emerging opportunistic pathogens that cause keratitis, onychomycoses and invasive fungal infection both in immunocompromised and immunocompetent patients [4,9,10].

During their asexual life cycle, Fusarium species produce a great number of microconidia [1]. These are quiescent structures produced by several fungal species for dispersion and environmental persistence. Conidia are dispersed by wind or rain and when deposited on the host plants they germinate and initiate the infectious cycle [1,11,12]. Germination is generally triggered by nutrient sensing and is characterized by conidial swelling, adhesion, nuclear reorganization, and formation of a germ tube that is often used to define the limit between conidial germination and vegetative growth [13,14]. In addition to the marked difference in metabolic activity, ungerminated conidia differ from germlings in their biochemical composition, redox status, cell organization and structure and composition of the cell wall [15-19]. As conidium and germling are essential developmental phases in the fungal life cycle and critical for plant colonization, most conventional fungicides have been developed to target the conidial germination and early developmental stages [19].

The use of resistant plant varieties is the main and most effective choice [5] in the control of the colonization by Fusarium spp. However, these plants level of tolerance depends

on the environmental conditions under which they grow [20]. Colonization by Fusarium spp. may be more severe in regions where temperatures are elevated, even when resistant plant varieties are used [20]. The control of Fusarium spp. also includes the use of seeds and seedlings treated with fungicides prior to planting, as well as the intensive use of fungicides during the development of the crop [5,20,21]. Unfortunately, failure in fungal control is common because of the selection of tolerant fungal strains. Furthermore, the intensive use of the currently available fungicides is a potential risk for humans and to the environment [21-23]. The control of fungal inoculum in the post-harvest (fruits and vegetables) is frequently carried out with chlorine and acids, even though these chemical sanitizers can be harmful to the environment [24,25].

The increased tolerance to currently used fungicides has stimulated the development of novel and more effective technologies to control pathogenic fungi. A promising alternative is antimicrobial photodynamic treatment (APDT) which can be used to kill fungi both in animal hosts and in the environment [25-27]. APDT is based on the combination of three elements: photosensitizer (PS), light, and molecular oxygen. Usually, the chosen PS accumulates preferentially in the target cell or has affinity for the microbial cell surface; the light should be at the appropriate wavelength to activate the photochemical process, which in the presence of molecular oxygen produces several reactive oxygen species (ROS), such as singlet oxygen [26,28,29]. ROS can cause oxidative damage to practically all biomolecules (proteins, lipids, nucleic acids and carbohydrates) leading to death of the fungal cell without causing significant damage to the host cell [26,30,31]. The multi-targeting feature of ROS reduces the chance of selecting resistant microorganisms. Another advantage of APDT is its ability to kill both metabolically active and quiescent structures such as ungerminated microconidia, in contrast to currently used fungicides that kill only metabolically active cells [30,32,33].

The efficacy of the photodynamic treatment with phenothiazinium PS to kill fungi has been described in different fungal genus such as Candida, Trichophyton, Aspergillus and Metarhizium [30,32,33]. We have recently demonstrated that phenothiazinium PS can easily kill conidia of Colletotrichum acutatum and C. gloeosporioides without causing damage to the plant host [31]. We also demonstrated that phototreatment with natural PS such as coumarins and furanocoumarins can kill conidia of C. acutatum [27]. However, data concerning the APDT of plant-pathogenic fungi with synthetic PS are still scarce. Natural compounds (thiophene derivatives and phenylheptatriyne from the plant Bidens pilosa) have been used as PS to photoinactivate conidia of different genus including Alternaria, Aspergillus, Cladosporium, Colletotrichum, Fusarium, Rhizopus, Pythium and Saprolegnia [34,35]. The hematoporphyrin dimethyl ether was used to photoinactivate conidia of Aspergillus flavus, Fusarium avenaceum, Trichothecium roseum, and Rhizopus oryzae [36].

Most of the studies that evaluated the effects of APDT on fungi were conducted with conidia. Little is known about the effects of APDT on other important fungal structures and developmental phases, such as germlings. Thus, the aim of this study was to evaluate the efficacies of APDT with methylene blue (MB), toluidine blue O (TBO), new methylene blue N (NMBN), and the phenothiazinium derivative S137, both on ungerminated and germinated microconidia of the fungi F. oxysporum, F. moniliforme, and F. solani. In order to understand better the mechanisms involved in microconidia photodynamic inactivation, we evaluated the effect of APDT with the phenothiaziniums PS on microconidial cell membranes by investigating lipid peroxidation and changes in membrane permeability. The localization of the PS inside the ungerminated and germinated microconidia was also determined.

2. Materials and Methods

2.1. Fusarium species and strains

Fusarium oxysporum INCQS 40144 (ATCC 48112), F. moniliforme INCQS 40151 (ATCC 38159), and F. solani INCQS 40099 (ATCC 36031) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA).

2.2. Photosensitizers and visible light sources

Methylene blue (MB) (Catalog number M9140), toluidine blue O (TBO) (Catalog number 202096), and new methylene blue N zinc chloride double salt (NMBN) (Catalog number T3260) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The pentacyclic phenothiazinium PS S137 was synthesized as previously described [37]. All stock solution and all dilutions were prepared with phosphate-buffered saline (PBS), pH 7.4. The stock solutions were stored in the dark at -20 °C up to 6 months and the dilutions of each PS were prepared at the experiment time. Chemical structures of all the PS are shown in Fig S1 in the supplemental material.

Light was provided by an array of 96 light-emitting diodes (LED) with emission peak at 635 nm. The array was made in house using the Cree® 5-mm Round LED (Model number LC503UHR1 – 15Q-Q, CREE Inc., Durham, NC, USA). Light measurement was performed by 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 USB spectroradiometer (Ocean Optics). Light was measured at the sample level (inside the well) to reduce interference. The integrated irradiance (570 to 670 nm) was 9.8 mW cm^{-2}.

2.3. Fungal growth and microconidia production

Fungal cultures were grown on potato dextrose agar medium (PDA) (Acumedia Manufactures, Inc., Lansing, MI, USA) at 28 °C for 5 days. After growth, 10-mL of sterile PBS, pH 7.4, were added to the surface of the culture, microconidia were gently scraped with a glass rod and the resulting suspension filtered through a sterile glass wool (Dinâmica Química Contemporânea Ltda., Diadema, SP, Brazil) to remove residual hyphae. Microconidia concentration was determined with a hemocytometer and appropriate dilutions were made with PBS, pH 7.4.

2.4. Evaluation of metabolic activities of ungerminated and germinated microconidia of F. oxysporum, F. moniliforme and F. solani by FUN-1 staining and flow cytometry

The metabolic activity of the fungal cell can be measured by the conversion of the fluorescent probe FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide] (green-fluorescent form) to the cylindrical intravacuolar structures (CVIS, red-fluorescent form) during endogenous biochemical processing, such as ATP production. Two hundred µL of F. oxysporum, F. moniliforme, and F. solani microconidia suspension (8×10^6 cells mL^{-1}), and 200 µL of the RPMI 1640 culture medium (twofold concentrate) were added to a 1.5-mL tube. Tubes were incubated at 28 °C for 4 h under shaking (150 rpm). After that, tubes were centrifuged 3 times at 5000g for five min to remove the RPMI 1640 culture medium. The germinated microconidia were resuspended in 400 µL of 10 mM sodium HEPES (Sigma-Aldrich, Inc., St. Louis, MO, USA), pH 7.2, supplemented with 2% D-(+)-glucose (Sigma-Aldrich, Inc., St. Louis, MO, USA) and 10 µM FUN-1 (Molecular Probes, Eugene, OR, USA). Tubes were held in the dark for 30 min at 28 °C and the cells were analyzed by flow cytometry (FACSCanto – Becton Dickinson, Sunnyvale, CA, USA). Both the forward angle light scatter (FSC-H) and the side scatter

(SSC-A) signal were used for gating, and a logarithmic amplifier was used. Ten thousand events were analyzed by FACSDiva software (BD, San Jose, CA, USA). Three independent experiments were performed. The conversion of FUN-1 to CVIS by the red (585 ± 40 nm)/green (530 ± 30 nm) fluorescence ratio was also evaluated on ungerminated microconidia, and on ungerminated microconidia exposed to the temperature of 95 °C for 1 h (death control). Two independent experiments were performed.

2.5. Evaluation of APDT effect on ungerminated microconidia of F. oxysporum, F. moniliforme and F. solani based on PS minimal inhibitory concentration (MIC) values

The determination of optimal conditions for the APDT with the phenothiazinium PS MB, TBO, NMBN and S137 were performed as previously described [31,33]. MIC-based experiments were performed in 96-well flat-bottomed microtiter plates (TPP, Switzerland). Fifty µL of the microconidia suspension (4×10^5 cells mL^{-1}) and 50 µL of the PS solution were added to the each well. The final concentration of MB, TBO and NMBN were 0, 1, 2.5, 5, 10, 12.5, 25, 50, 75, 100 and 200 µM and the final concentration of S137 were, 0, 0.5, 1, 2.5, 5, 10, 12.5, 20, 25, 30 and 40 µM. Plates were held in the dark for 30 min at 28 °C and exposed to light fluences of 10, 15 and 20 J cm^{-2} using the LED array as a light source, or alternatively kept in the dark (dark controls). After exposure, 100 µL of RPMI 1640 culture medium (Sigma-Aldrich, Inc., St. Louis, MO, USA) (twofold concentrate) were added to each well, and plates were incubated at 28 °C. Growth was evaluated daily by visual inspection up to 4 days when the MICs were determined. The MIC was considered the minimal PS concentration for each fluence (10, 15 or 20 J cm^{-2}) in which total growth inhibition was achieved. Three independent experiments were performed.

2.6. Effects of APDT with MB, TBO, NMBN and S137 on the survival of ungerminated and germinated microconidia of F. oxysporum, F. moniliforme and F. solani

The survival of F. oxysporum, F. moniliforme and F. solani ungerminated and germinated microconidia was evaluated after APDT with MB, TBO, NMBN and S137. In order to obtain germinated microconidia, 5 mL of microconidia suspension (8×10^6 cells mL^{-1}) and 5 mL of the RPMI 1640 culture medium (twofold concentrate) were added to a 15-mL tube (Corning Incorporated, NY, USA). Tubes were incubated during 4 h at 28 °C under shaking at 150 rpm. After that, tubes were centrifuged at 4000g for 5 min and germlings were resuspended in 5 mL of PBS, pH 7.4. This process was repeated 3 times to remove the RPMI 1640 culture medium. Three hundred µL of ungerminated and germinated microconidia suspension, and 300 µL of the PS solution (MB, TBO, NMBN and S137) were added to a 1.5-mL tube (polypropylene; Axygen Scientific, CA, USA). The final cell concentration in the mixtures was 4×10^6 cells mL^{-1} and the final concentrations of MB, TBO, NMBN and S137 were 50, 75, 5, and 10 µM, repectively for F. oxysporum; 75, 75, 10 and 10 µM for F. moniliforme and 75, 75, 12.5 and 10 µM for F. solani. Tubes were held in the dark for 30 min at 28 °C, and after that, they were divided into 2 groups. In one group, ungerminated and germinated microconidia were washed to remove unbound PS, and in the other group, cells were not washed before light exposure. To remove unbound PS, cells were centrifuged at 5000g for five min and resuspended in PBS, pH 7.4. This process was repeated 3 times. Non-washed and washed ungerminated and germinated microconidia were placed into a 24-well flat-bottomed microtitre plate (TPP, Switzerland) and exposed to fluence of 0 (dark control), 10 and 15 J cm^{-2}. After exposures, suspensions were removed and serially diluted 10-fold in PBS to give dilutions of 10^{-1} to 10^{-3} times the original concentration, and 50 µL of each dilution were spread on the surface of 5 mL PDA medium containing 0.08 g L^{-1} of

deoxycholic acid sodium salt (Fluka, Italy) and incubated at 28 °C in the dark. Three replicates dishes were prepared for each treatment. Colony forming units (CFU) were counted daily at 8× magnification up to seven days. The effects of the different treatments on ungerminated and germinated microconidia survival were determined based on CFU counting, as previously described [31,33]. Survival fractions were expressed as ratio of CFU of fungal cells treated only with light (light effect), only with PS (dark toxicity), and light and PS (APDT effect) to CFU treated with neither. Three independent experiments were performed.

2.7. Microscopy studies of ungerminated and germinated microconidia

Microscopic studies were performed to determine the subcellular localization of the PS in both ungerminated and germinated microconida of F. oxysporum, F. moniliforme, and F. solani. To produce germlings, microconidia (4×10^6 cells mL^{-1}) were incubated in RPMI 1640 culture medium at 28 °C for 4 h. Both the ungerminated and germinated microconidia were treated with 100 µM of MB, TBO, NMBN, and S137 and washed three times with PBS (5000g for 5 min) before being observed at 1000× magnification (Coleman N-120 microscope, Coleman, Santo André, SP, Brazil). In attempt to identify the cellular structures in which the PS accumulated, ungerminated microconidia and germlings were also stained with the fluorescent dyes 4´,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) (3 µg mL^{-1} in distilled water) and FM4-64 (Invitogen™) [1 µg mL^{-1} in 1% (vol/vol) dimethyl sufoxide (DMSO) solution], which have affinity for genetic material, and cellular membranes, respectively. Ungeminated and germinated microconidia were treated with each of the 2 dyes, kept in the dark for 1 h, and washed three times with PBS before being observed with a Leica TCS SP8 Confocal laser microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 630×

magnification and digital zoom (2×). The samples were illuminated at 405 nm (DAPI) and 552 nm (FM4-64) and the signal were captured in two channels, one for acquisition of the DAPI fluorescence (410-540 nm), and the other, of the FM4-64 fluorescence (570-700nm). Transmitted light images were also obtained.

2.8. Evaluation of APDT effect on cell membrane permeability

Cell membrane permeability after APDT was assessed by flow cytometry analyses of propidium iodide (PI) staining microconidia. PI only penetrates cells with severe membrane damage. Three hundred µL of the microconidia suspension and 300 µL of the PS solution (MB, TBO, NMBN and S137) were added into a 24-well flat-bottomed microtitre plate (TPP, Switzerland). The final concentration of microconidia were 4×10^6 cells mL^{-1} and of MB, TBO, NMBN and S137 were 50, 75, 5, and 10 µM, respectively for *F. oxysporum*; 75, 75, 10 and 10 µM for *F. moniliforme*, and 75, 75, 12.5 and 10 µM for *F. solani*. Plates were kept in the dark for 30 min at 28 °C, and exposed to fluences of 0 (dark control) and 15 J cm^{-2}. Microconidia not treated with PS neither exposed to light were used as control of cell viability, and microconidia exposed to 95 °C for 1 h were used as "death control". After APDT, microconidia were recovered from the wells to 1.5-mL tube, washed once with PBS and 1 µL of PI solution (1 g L^{-1}) was added immediately before data acquisition by flow cytometry (FACSCanto – Becton Dickinson, Sunnyvale, CA, USA). Both the forward angle light scatter (FSC-H) and the side scatter (SSC-A) signal were used for gating, and logarithmic amplifier was used. Ten thousand events were analyzed by FACSDiva software (BD, San Jose, CA, USA). We measured the percentage of cells stained with PI at each APDT condition. Three independent experiments were performed.

2.9. Lipid peroxidation assay

Polyunsaturated lipids are easily oxidized by reactive oxygen species resulting in a chain of reactions that has malondialdehyde (MDA) as a major product. Lipid peroxidation after APDT was evaluated with the Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich), according to the manufacturer's protocol, with modifications. After the photodynamic treatment performed as described above, microconidia were recovered from the wells to 1.5-mL tube, centrifuged at 5000g for 5 min. Microconidia were resuspended in 300 µL of MDA lysis buffer, containing 3 µL of BHT (Catalog number MAK085C Sigma-Aldrich) (100× concentrate), and homogenized on ice. Samples were centrifuged at 13000g for 10 min to remove insoluble material and the supernatant from each samples were placed into a new 1.5-mL tube with the addition of 600 µL of thiobarbituric acid solution [0.250 g thiobarbituric acid reconstitute with 7.5 mL glacial acetic acid (J.T.Baker® Chemicals, Center Valley, USA), then adjusted the final volume to 25 mL with water] and incubation at 95 °C for 1 h. After, the samples were cooled in an ice bath for 10 min and 200 µL from each sample were added into a 96-well flat-bottomed microtitre plate for analysis. The absorbance was measured at 532 nm with a Spectramax® Paradigm® Multi-Mode Detection Platform (Molecular Device LLC, Sunnyvale, CA, USA). The concentration of MDA was calculated based in the standard curve.

2.10. Statistical analysis

Data were submitted to one-way analysis of variance (one-way ANOVA) followed by Tukey's post-test to compare the effects of the different treatments. No data transformations were

required in any experiment. P values of < 0.05 were considered significant. All computations were done using Graph pad Prism Software (v 5.0; Graph pad Software, La Jolla, CA, USA).

3. Results

3.1. Evaluation of metabolic activities of ungerminated and germinated microconidia of F. oxysporum, F. moniliforme and F. solani by FUN-1 staining and flow cytometry

The conversion of FUN-1 stain from a green-fluorescent to a red-fluorescent form (cylindrical intra vacuolar structures - CIVS) occurs via the endogenous biochemical processes for ATP production. We evaluated the conversion of FUN-1 to CVIS, in ungerminated and germinated microconidia of F. oxysporum, F. moniliforme and F. solani. Dead microconidia were used as control. The red/green fluorescence ratio in the death control was lower than in ungerminated and germinated microconidia. The red/green fluorescence ratios in F. oxysporum, F. moniliforme and F. solani germinated microconidia were approximately 2 times higher than the red/green ratios in ungerminated micorconidia ($P < 0.05$ for all comparison) (Fig 1).

3.2. Evaluation of APDT effect on F. oxysporum, F. moniliforme and F.solani ungerminated microconidia based on PS minimal inhibitory concentration (MIC)

The efficacy of APDT with each PS (MB, TBO, NMBN, and S137) combined with different fluences of red light (0, 10, 15, 20 J cm^{-2}) was initially evaluated by determining the MIC values. The treatments with the highest concentration tested (200 µM) of MB, TBO and NMBN in the absence of light did not inhibit the growth of F. oxysporum, F. moniliforme and

F. solani (Table 1). Only the PS S137 presented dark toxicity wherein the MIC ranges in the dark were 25-30 µM for F. oxysporum, and F. moniliforme, and 12.5-25 µM for F. solani (Table 1). The exposure of microconidia to red light (at all fluences) in the absence of the PS did not inhibit the growth of F. oxysporum, F. moniliforme and F. solani (data not shown). APDT with each of the PS at all fluences inhibited the growth of all species. The MIC for all PS and fluences are shown in Table 1. Although the MIC values varied among both the PS and species, the MIC values decreased with increasing fluence. NMBN and S137 were the most effective PS for all the species at all fluences. APDT with NMBN (15 J cm^{-2}) inhibited the growth of F. oxysporum, F. moniliforme, and F.solani at 5, 10 and 12.5 µM, respectively. APDT with S137 (15 J cm^{-2}) inhibited the growth of F. oxysporum, F. moniliforme, and F.solani at 10 µM.

3.3. Effects of APDT with MB, TBO, NMBN and S137 on survival of ungerminated and germinated microconidia of F. oxysporum, F. moniliforme and F. solani

The effects of APDT on the survival of ungerminated and germinated microconidia of F. oxysporum, F. moniliforme and F. solani are shown in Fig 2 and 3. Exposures only to red light (light effect) and treatment only with MB, TBO, NMBN, and S137 (dark toxicity) did not kill microconidia of any species ($P > 0.05$ for all species) (Fig 2 and 3). Photodynamic killing of ungerminated microconidia (Fig 2) and germinated microconidia (Fig 3) were observed for all PS at all fluences ($P < 0.05$ for all treatment comparisons). APDT with MB and fluences of 10 and 15 J cm^{-2} resulted in an approximately 4 and 5-log reduction in the survival of ungerminated microconidia of F. oxysporum (MB 50 µM); 1 and 3-log of F. moniliforme (MB 75 µM) and 3.5 and 5-log of F. solani (MB 75 µM) (Fig 2A). APDT with TBO (75 µM) and fluences of 10 and 15 J cm^{-2} resulted in an approximately 4 and 5-log

reduction of F. oxysporum; 1 and 2-log reduction of F. moniliforme, and 1.5 and 3-log reduction of F. solani (Fig 2B). APDT with NMBN (5, 10, and 12.5 µM) and fluences of 10 and 15 J cm^{-2} resulted in approximately 5-log reduction in the survival of microconidia of all species (Fig 2C). APDT with S137 (10 µM) and fluences of 10 and 15 J cm^{-2} resulted in an approximately 3 and 5-log reduction of F. oxysporum, 1 and 3-log reduction of F. moniliforme, and 5-log redution of F. solani (Fig 2D). Washing of the microconidia to remove unbound PS before light exposure reduced the effect of APDT at fluence of 10 J cm^{-2} for MB (F. oxysporum and F. solani), NMBN (F. oxysporum, F. moniliforme, and F. solani), and S137 (F. solani). At the incread fluence of 15 J cm^{-2} washing reduced the effect of APDT for MB (F. oxysporum and F. solani), TBO (F. oxysporum), and NMBN (F. moniliforme) ($P < 0.05$ for all treatments) (Fig 2).

The effects of APDT on germinated microconidia of Fusarium species are presented in Fig 3. APDT with MB and fluences of 10 and 15 J cm^{-2} resulted in an approximately 5-log reduction in the survival of germlings of F. oxysporum (MB 50 µM); 3 and 4-log reduction of F. moniliforme (MB 75 µM); and 4 and 5-log reduction of F. solani (MB 75 µM) (Fig 3A). APDT with TBO (75 µM) and fluences of 10 and 15 J cm^{-2} resulted in an approximately 5-log reduction of F. oxysporum, and F. moniliforme; and 3-log reduction of F. solani (Fig 3B). APDT with NMBN and fluences of 10 and 15 J cm^{-2} resulted in approximately 5-log reduction of F. oxysporum (5 µM); and F. moniliforme (10 µM); and 4.5-log reduction of F. solani (12.5 µM) (Fig 3C). APDT with S137 (10 µM) and fluences of 10 and 15 J cm^{-2} resulted in approximately 5-log reduction of F. oxysporum, and F. solani; and 4.5 and 5-log reduction of F. moniliforme (Fig 3D). The washing of the germinated microconidia to remove unbound PS before light exposure reduced the effect of APDT for MB at fluence of 10 J cm^{-2} for F. oxysporum ($P < 0.05$). For the other treatments, the reductions were not significant ($P > 0.05$) (Fig 3).

3.4. Subcellular localization of the PS in ungerminated and germinated microconidia of F. oxysporum, F. moniliforme and F. solani

Microscopic studies were performed to determine the subcellular localization of each PS in ungerminated and germinated microconidia of all Fusarium species. The internalization of TBO and NMBN was observed in ungerminated microconidia of F. oxysporum, F. moniliforme and F. solani, whereas the germinated microconidia of these fungi species showed the internalization of MB, TBO, and NMBN (Fig 4, 5 and 6). The localization of FM4-64 with the PS TBO and NMBN in ungerminated microconidia and with MB, TBO and NMBN in germinated microconidia of F. oxysporum, F. moniliforme and F. solani, indicated the possible accumulation of the PS in membranous structures such as small vacuoles present in the cytoplasm of the microconidia and germlings (Fig 4, 5 and 6). Additionally, it was observed by FM4-64 cell staining that the treatment with S137 at 100 µM caused cytoplasmic disorganization and the formation of large vesicles in the cytoplasm of the germlings (Fig 4 and 6).

3.5. Evaluation of APDT effect on cell membrane permeability

Propidium iodide (PI) is a membrane-impermeable compound that is not retained inside viable cells. Damage in cell membrane increases PI uptake. The effect of APDT on microconidia cell membrane permeability was evaluated in all Fusarium species. Exposures only to red light did not change the cell membrane permeability of F. oxysporum, F. moniliforme and F. solani microconidia ($P > 0.05$ for all Fusarium species and fluences) (Fig 7, 8, and 9). In the absence of light, only treatment with S137 increased the percentage of cells

stained with PI. The percentages of cells stained were 81.5%, 97.3% and 96.8 % for the microconidia of F. oxysporum, F. moniliforme and F. solani, respectively ($P < 0.05$ for all Fusarium species). Despite the increase in the permeability of the membrane, such treatment killed less than 7% of the conidia of the three species. APDT with all the PS and fluences increased cell membrane permeability for the three Fusarium species ($P < 0.05$ for all treatment comparisons). The greatest membrane permeability increase was observed after APDT with NMBN and S137 (Fig 7, 8, and 9). APDT with MB and TBO increased the membrane permeability of F. solani microconidia (Fig 9). The percentage of cells stained with PI in the death controls was at least 98 % for all Fusarium species ($P < 0.05$).

3.6. Effects of APDT on lipid peroxidation of F. oxysporum, F. moniliforme and F. solani microconidia

Reactive oxygen species (ROS), such as singlet oxygen, produced during the photochemical process of APDT, may result in oxidative damage of lipids present in the microconidia. We evaluated the lipid peroxidation (MDA product) after APDT experiments with all PS at fluence of 15 J cm^{-2}. Exposures only to red light, and treatments only with the PS in the absence of red light did not increase lipid peroxidation in microconidia of any Fusarium species ($P > 0.05$ for all Fusarium species) (Fig 10). Increases in lipid peroxidation in microconidia of F. oxysporum, F. moniliforme and F. solani were observed only after APDT with NMBN and S137 ($P < 0.05$) (Fig 10).

4. Discussion

Phenothiazinium dyes are among the most commonly used PS in APDT against fungal species and novel derivatives, such as NMBN, the pentacyclic S137, and derivatives with basic side chains, which are more effective than the lead compound MB, are still being identified [30,33,37-40]. APDT with the PS MB and TBO have been used to kill fungi of several genera including Kluyveromyces [41], Candida [33,42], Metarhizium and Aspergillus [32]. To our knowledge, there are no data regarding the effect of APDT with phenothiazinium PS such as MB, TBO, NMBN and S137 on F. oxysporum, F. moniliforme and F. solani.

As the MIC based experiments were less time-consuming and allowed the evaluation of several treatments simultaneously, this approach is very convenient to establish the best conditions for APDT [30,31,33] and was used in the present work. NMBN and S137 were the most effective PS, being associated with the lowest MIC for all Fusarium species. Only S137 presented dark toxicity to the fungal cells. The improved performance of NMBN and S137 in comparison with other phenothiazinium PS was also observed in vitro for the human-pathogenic fungi Candida spp. and Trichophyton spp., and for the plant-pathogenic fungi Colletotrichum spp. [30,31,33].

After the establishment of optimized conditions for APDT based on initial MIC experiments, we evaluated the effect of APDT with all PS (MB, TBO, NMBN, and S137) on the survival of ungerminated and germinated conidia. APDT was very effective in killing both ungerminated and germinated microconidia of the three Fusarium species. Treatment with NMBN and red light (15 J cm^{-2}) resulted in approximately 5-log reduction in survival of the ungerminated and germinated microconidia, which is the maximum reduction that could be determinated with the experimental design. APDT with S137 also resulted in the same reduction (5-log) to F. oxysporum and F. solani. However, for F. moniliforme treatment APDT with S137 resulted in approximately 3-log reduction in survival both to ungerminated and germinated microconidia.

As opposed to some currently used fungicides that act only on metabolically active cells and/or exhibit only fungistatic effects, APDT with phenothiazinium PS (MB, TBO, NMBN and S137) killed both the quiescent ungerminated microconidia and the metabolic active germlings of all Fusarium spp. The use of APDT to kill microconidia of Fusarium spp. on seeds, seedling, fruits and vegetables would reduce the inoculums in the treated area. Reduction of the initial inoculum is among the strategies used to control diseases caused by Fusarum species [5,6,20,43].

Washing the conidia to remove unbound PS from the ungerminated microconidia before red light exposure (15 J cm^{-2}) reduced the effect of APDT with phenothiazinium PS. However, the reduction was smaller for S137. This effect was previously observed in APDT with MB and TBO on Aspergillus nidulans conidia [32]. As opposed to what was observed in ungerminated microconidia, washing the germinated microconidia did not reduce the effect of the APDT (at fluence of 15 J cm^{-2}) with all PS on the three Fusarium species.

Conidial germination can be divided into three stages. The first is the activation of the resting conidia by appropriate environmental factors such as exogenous low-molecular nutrients (i.e. sugar and amino acids). Activation is followed by isotropic growth which is the first morphological change that can be observed during germination and involves water uptake and cell wall growth. It occurs simultaneously with the resumption of numerous metabolic activities including respiration, and RNA and protein synthesis and results in a cell whose diameter is two to several times that of the resting conidia [15,16,18]. The last stage is the polarized growth which ends with the formation of a germ tube [13]. After 4 h in culture media, microconidia of all the three Fusarium species are in the second phase of the germination because isotropic growth is evident but no nuclear division or germ tube emergence can be observed (see Fig 4, 5 and 6). Microscopic studies showed that ungerminated and germinated microconidia interact differently with the PS. TBO and NMBN

but not MB were taken up by undergerminated microconidia and accumulated in uncharacterized cytoplasmatic vesicles in all Fusarium species. After germination, MB, TBO and NMBN entered and accumulated in structures present in the cytoplasm of the germlings of all three species. Staining the cells with FM4-64, which has affinity for membranes, indicates that the PS accumulates in vacuoles present in the cytoplasm. Study using Sudan III and FM4-64 showed that NMBN has affinity for lipid bodies and small vesicles present in the cytoplasm of the conidia of Colletotrichum acutatum [31]. The PS entry and accumulation occurred only in the contact with the ungerminated and germinated microconidia and were independent of red light exposure. MB and S137 were not visible inside the ungerminated microconidia in any Fusarium species. Although the accumulation of S137 was not observed in any structure inside the germlings, APDT with S137 at 100 µM caused the formation of large vesicles in the cytoplasm of the F. oxysporum and F. solani (see Fig 4 and 6). It was previously observed that treatment with S137 caused cytoplasmic disorganization and formation of large vesicles in the cytoplasm of conidia of C. acutatum [31]. The disorganization of membranous cytoplasmic vesicles might be the reason for the higher dark toxicity of S137. These structures are particularly important in the early stages of conidia germination, having a role, for example, in the osmotic regulation, protein sorting and lipid storage [44-47]. The internalization of S137 only in germlings could be responsible for the greater effectiveness of APDT in germlings as compared to ungerminated microconidia.

Several studies have shown that the cell membrane is one of the targets of ROS generated during the photodynamic process [41,48-51]. PI is widely used to evaluate the membrane integrity [52,53]. APDT with all PS increased the membrane permeability of microconidia of the three Fusarium species. Only treatment with S137 increased the permeability in the dark, however, the viability of the microconidia of the three species remained higher than 95%. The increase in the membrane permeability without causing cell

death was previously observed in Saccharomyces cerevisiae [54]. S137 is more lipophilic in comparison to the other studied compounds [55-57]. This could explain the increased permeability of the cell membrane observed after treatment with S137 in the dark. [55-57]. Also, the greater affinity for lipids facilitates the interaction of S137 and NMBN with the cell membranes increasing the oxidation of their lipids during the APDT. As expected, APDT with NMBN and S137 increased lipid peroxidation in all Fusarium species. Previous study showed an increase in lipid peroxidation after the photodynamic treatment of conidia of F. poae and F. culmorum using the lipophilic protoporphyrin IX as PS [58].

The use of APDT in the environment will require environmentally safe PS. Phenothiazinium PS present low toxicity to mammalians. MB has been used to treat methemoglobinemia and to oral disinfection [59,60]. Other PS, such as hypericin, furanocoumarins and thiophenes are produced by plants of various genera and have been used in vitro to kill plant-pathogenic fungi, such as Colletotrichum sp. and Fusarium sp. [27,35,58], and foodborne pathogenic bacteria, such as Bacillus, Listeria, and Salmonella [25,61]. The present study showed that in vitro APDT with the four phenothiazinium PS (MB, TBO, NMBN, and S137) were very effective in killing both ungerminated and germinated microconidia of F. oxysporum, F. moniliforme and F. solani. Thus, APDT has great potential to reduce the inoculum of these fungi on seeds, seedling, fruits and vegetables.

5. Conclusion

Photodynamic treatment with phenothiazinium PS efficiently kills both the quiescent ungerminated microconidia and metabolically active germinated microconidia of F. oxysporum, F. moniliforme, and F. solani. However, further studies should be conducted under realistic conditions (in planta) in order to establish the safety of this approach and the best conditions for the antimicrobial photodynamic treatment required for each pathosystem.

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Appendix A. Supplementary material

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Table 1. Minimal inhibitory concentration (MIC - µM) of APDT with phenothiazinium PS MB, TBO, NMBN and S137 to F. oxysporum, F. moniliforme, and F. solani. Results are MIC ranges from three independent experiments.

	PS/Fluence	0	10 J cm^{-2}	15 J cm^{-2}	20 J cm^{-2}
F. oxysporum	MB	>200	50-75	50	12.5-25
	TBO	>200	75	75	25-50
	NMBN	>200	5-12.5	5-10	2.5
	S137	25-30	12.5	10	5 µM
F. moniliforme	MB	>200	75-150	75-100	25
	TBO	>200	75-100	75	10-12.5
	NMBN	>200	5-10	10	10
	S137	25-30	10	10	5
F. solani	MB	>200	75-100	75	25-75
	TBO	>200	75	75	12.5-25
	NMBN	>200	5-25	5-12.5	2.5-5
	S137	12.5-25	5-10	5-10	5

PS, photosensitizer; MB, methylene blue; NMBN, new methylene blue; TBO, toluidine blue O; S137, pentacyclic phenothiazinium derivative; J cm^{-2}, Joules per square centimeter.

Figure legends:

Figure 1. Evaluation of the metabolic activity of ungerminated and germinated microconidia of F. oxysporum, F. moniliforme, and F. solani by using the vital FUN-1 staining. Error bars are standard deviation of two independent experiments.

Figure 2. Effect of APDT on the survival of ungerminated microconidia. Photodynamic inactivation of F.oxysporum, F. moniliforme, and F. solani with MB (A), TBO (B), NMBN (C), and S137 (D). Ungerminated microconidia were incubated with the PS for 30 min and washed or non-washed before red light exposure. Error bars are standard deviation of three independent experiments.

Figure 3. Effect of APDT on the survival of germinated microconidia. Photodynamic inactivation of F. oxysporum, F. moniliforme, and F. solani with MB (A), TBO (B), NMBN (C), and S137 (D). Germinated microconidia were incubated with the PS for 30 min and washed or non-washed before red light exposure. Error bars are standard deviation of three independent experiments.

Figure 4. F. oxysporum ungerminated and germinated microconidia non-stained (A and I), stained with DAPI (B and J), stained with FM®4-64 (C and K), and merged image (D and L) using the Leica Application Suite X software. Ungerminated and germinated microconidia were also treated with MB 100 µM (E and M), NMBN 100 µM (F and N), TBO 100 µM (G and O), and S137 100 µM (H and P). Arrows indicate the large vesicles formed in the cytoplasm of the germinated microconidia after treatment with S137 (P).

Figure 5. Microscopy study with F. moniliforme ungerminated and germinated microconidia non-stained (A and I), stained with DAPI (B and J), stained FM®4-64 (C and K) and merged image (D and L) using the Leica Application Suite X software. Ungerminated and germinated microconidia were also treated with MB 100 µM (E and M), NMBN 100 µM (F and N), TBO 100 µM (G and O), and S137 100 µM (H and P).

Figure 6. F. solani ungerminated and germinated microconidia non-stained (A and I), stained with DAPI (B and J), stained with FM®4-64 (C and K) and merged image (D and L) using the Leica Application Suite X software. Ungerminated and germinated microconidia were also treated with MB 100 µM (E and M), NMBN 100 µM (F and N), TBO 100 µM (G and O), and S137 100 µM (H and P). Arrows indicate the large vesicles formed in the cytoplasm of the germinated microconidia after treatment with S137 (P).

Figure 7. Percentage of cells stained with PI (gray columns) and survival fraction (white squares) of F. oxysporum microconidia after APDT with MB (A), TBO (B), NMBN (C), and S137 (D). Error bars are standard deviation of three independent experiments.

Figure 8. Percentage of cells stained with PI (gray columns) and survival fraction (white squares) of F. moliniforme microconidia after APDT with MB (A), TBO (B), NMBN (C), and S137 (D). Error bars are standard deviation of three independent experiments.

Figure 9. Percentage of cells stained with PI (gray columns) and survival fraction (white squares) of F. solani microconidia after APDT with MB (A), TBO (B), NMBN (C), and S137 (D). Error bars are standard deviation of three independent experiments.

Figure 10. Peroxidation of microconidia lipids measured by the formation of malondialdehyde (gray columns) and survival fraction (white squares) after APDT with MB, TBO, NMBN, and S137 of: (A) Fusarium oxysporum, (B) F. moniliforme, and (C) F. solani. Error bars are standard deviation of three independent experiments.