Tonani, L, Morosini, NS, de Menezes, HD, Bonifácio da Silva, MEN, Wainwright, M, Braga, GÚL and von Zeska Kress, MR

In vitro susceptibilities of Neoscytalidium spp. sequence types to antifungal agents and antimicrobial photodynamic treatment with phenothiazinium photosensitizers

In vitro susceptibilities of *Neoscytalidium* spp. *sequence types* to antifungal agents and antimicrobial photodynamic treatment with phenothiazinium photosensitizers

Ludmilla Tonani[a,b], Natália Silva Morosini[a], Henrique Dantas de Menezes[a], Maria Emília Nadaletto Bonifácio da Silva[a], Mark Wainwright[c], Gilberto Úbida Leite Braga[a], 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]*Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP Brazil.*
[c]*School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool L3 3AF, United Kingdom.*

Running title: Antifungal and photodynamic treatment of *Neoscytalidium* spp.

[*]Corresponding author: Phone: +55 1633150240; Fax: +55 1633154725; E-mail address: kress@fcfrp.usp.br

Keywords: Antimicrobial photodynamic treatment, Phenothiazinium photosensitizers, Antifungal susceptibility testing, *Neoscytalidium dimidiatum, Neoscytalidium dimidiatum* var. *hyalinum*, Multilocus sequence typing, Fungal phylogeny.

ABSTRACT

Neoscytalidium spp. are ascomycetous fungi consisting of pigmented and hyaline varieties both able to cause skin and nail infection. Their color-based identification is inaccurate and may compromise the outcome of the studies with these fungi. The aim of this study was to genotype 32 isolates morphologically identified as *N. dimidiatum* or *N. dimidiatum* var. *hyalinum* by multilocus sequence typing (MLST), differentiate the two varieties by their sequence types, evaluate their susceptibility to six commercial antifungal drugs [amphotericin B (AMB), voriconazole (VOR), terbinafine (TER), 5-flucytosine (5FC), fluconazole (FLU), and caspofungin (CAS)], and also to antimicrobial photodynamic treatment (APDT) with the phenothiazinium photosensitizers (PS) methylene blue (MB), new methylene blue (NMBN), toluidine blue O (TBO) and the pentacyclic derivative S137. The efficacy of each PS was determined, initially, based on its minimal inhibitory concentration (MIC). Additionally, the APDT effects with each PS on the survival of ungerminated and germinated arthroconidia of both varieties were evaluated. Seven *loci* of *Neoscytalidium* spp. were sequenced on MLST revealing eight polymorphic sites and six sequence types (ST). All *N. dimidiatum* var. *hyalinum* isolates were clustered in a single ST. AMB, VOR and TER were the most effective antifungal agents against both varieties. The hyaline variety isolates were much less tolerant to the azoles than the isolates of the pigmented variety. APDT with S137 showed the lowest MIC for all the isolates of both varieties. APDT with all the PS killed both ungerminated and germinated arthroconidia of both varieties reducing the survival by up to 5 logs. Isolates of the hyaline variety were also less tolerant to APDT. APDT with the four PS also increased the plasma membrane

permeability of arthroconidia of both varieties but only NMBN and S137 caused peroxidation of the membrane lipids.

1. Introduction

Neoscytalidium spp. (formerly *Scytalidium*) are ascomycetous, dematiaceous (producer of melanins or melanin-like pigments), keratinophilic and non-dermatophyte fungi [1]. The taxonomy of the genera is still very problematic and has been constantly revised [2-4]. These fungal species are worldwide distributed in tropical and subtropical regions and are principally known as phytopathogens but some species can infect humans via direct contact with colonized plants [1]. *Neoscytalidium dimidiatum* and its albino variant *N. dimidiatum* var. *hyalinum*, which does not produce melanin, are involved in human infections [5]. Traditionally, the fungus has been characterized by producing abundant uni- to tricellular dark arthroconidia [6-9] which are nonsexual propagules produced by hypha fission [10]. In culture, *N. dimidiatum* shows cottony, fast-growing colonies that become pigmented, usually with a brown to gray color [6-7]. There is a great variety in colony color among the so called pigmented isolates and the color-based phenotypic distinction between the two varieties is not always straightforward. Therefore, it is important to establish a more accurate methodology for the identification of the isolates and distinction between the two varieties. The accurate identification of the two varieties is important to guide clinical management, to associate particular genotypes with medically important traits such as virulence and antifungal drug susceptibility and to perform epidemiological studies [2,11]. Unlike *N. dimidiatum*, which is commonly isolated from plants and soil samples, the albino variety has been isolated exclusively from human infections [2, 12-13]. The two varieties have also different geographical distributions. *N. dimidiatum* was isolated from environmental samples and patients in Central

Africa, Asia and the Indian Ocean region, whereas the hyaline variety was exclusively isolated from patients in South America, West Africa and the West Indies [5, 14-15]. The hyaline variety is responsible for chronic infections in toenail, interdigital spaces, soles and palms [1, 2, 5, 16-19] which are clinically similar to *N. dimidiatum* infections and indistinguishable from dermatophytosis [1-2, 16-17]. The dematiaceus variety is responsible not only for superficial infection as *N. dimidiatum* var. *hyalinum*, but also for invasive and deeper infection such as subcutaneous infections [20-21], sinusitis [22-23], endophtalmitis [24], cerebral pheohyphomycosis [25], pneumonia [8,26], and disseminated infections [27-29]. As the number of mycoses caused by both varieties is increasing [7,9,30] there is a renewed interest in the study of these fungi.

Only a few studies have reported the comparison of the antifungal susceptibility of both *N. dimidiatum* and *N. dimidiatum* var. *hyalinum* [2,16-17,31]. Amphotericin B, voriconazole and terbinafine are the only antifungals that have shown *in vitro* activity against both varieties [2,16-17,32-33]. However, unlike infections caused by dermatophytes, the treatment of mycoses caused by *Neoscytalidium* spp. with conventional antifungal drugs results in poor outcomes due to antifungal resistance [1,5,19,34]. Therefore, due to the limitation of current therapeutic options, the development of new approaches to treat mycosis caused by *Neoscytalidium* spp. is necessary.

Antimicrobial photodynamic therapy (APDT) has been described as an effective alternative to conventional fungicides to treat localized mycoses [35]. The photodynamic process results from the combination of three elements: light of an appropriate wavelength, oxygen, and a photosensitizer (PS), which

accumulates preferentially in the target microorganism [36]. As a consequence of the photodynamic process, several reactive oxygen species (ROS) are produced and they subsequently kill the microbial cells without significant damage to the host [36]. More importantly, given the multiple cellular targets of ROS, the selection of tolerant microorganisms is less probable in APDT than in treatments with conventional antifungal drugs [35]. Among the PS examined for antifungal use, the phenothiazinium derivatives methylene blue (MB), new methylene blue (NMBN) and toluidine blue O (TBO) are particularly attractive, mainly due to their low toxicities to mammals and other clinical uses [35,37]. Novel derivatives, such as the pentacyclic S137, and derivatives with basic side chains, more effective than the lead compound MB are still being synthesized [38-39]. Previous studies have reported that APDT with phenothiazinium derivatives kills different fungal structures, such as microconidia of different species including the dermatophyte *Trychophyton* spp. [40, 43] and the non-dermatophyte *Fusarium* spp. [44] and conidia of *Exophiala* spp. [42], *Colletotrichum* spp. [53], *Metarhizium anisoplia*, and *Aspergillus nidulans* [41]. As far as we know there is no data regarding the effect of APDT with phenothiazinium derivatives on arthroconidia of any species.

The aims of this study were: (1) to determine if multilocus sequence typing allows the distinction between the isolates of *N. dimidiatum* and *N. dimidiatum* var. *hyalinum*; (2) to investigate the genotype distribution of Brazilian clinical isolates of the two varieties; (3) to evaluate the *in vitro* susceptibility of the arthroconidia of the different sequence types to conventional antifungal drugs and to APDT with phenothiazinium photosensitizers, and (4) to compare the susceptibilities of the two varieties to antifungals and APDT.

2. Material and Methods

2.1. Fungal clinical isolates

A total of 30 isolates identified by their morphological characteristics as *N. dimidiatum* or *N. dimidiatum* var. *hyalinum* was obtained from the Serviço de Análises Clínicas da Faculdade de Ciências Farmacêuticas de Ribeirão Preto (FCFRP), University of São Paulo (USP). Fungi were isolated from skin and toenail infections of patients residing in Ribeirão Preto (São Paulo, Brazil) and neighboring cities from 2004 to 2014 (Table 1). The American Type Culture Collection (ATCC) strains, ATCC®22190™ (previously identified as *N. dimidiatum*) and ATCC®38907™ (previously identified as *N. dimidiatum* var. *hyalinum*) were also included in the study (Table S1). This study was approved by the Research Ethics Committee of the Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil.

2.2. Fungal isolation and production of arthroconidia and mycelia

Fungal strains were isolated from scrapings of foot and/or toenail placed on both sabouraud dextrose agar (SDA) (Acumedia , Michigan, USA) with chloramphenicol (50 mg L^{-1}) and SDA with chloramphenicol (50 mg L^{-1}) and cycloheximide (400 mg L^{-1}). Plates were incubated at 28ºC for 4 days.

The arthroconidia were produced by culturing the fungi on SDA at 28ºC for 4 days. Arthroconidia were suspended in sterile 0.01% (v/v) Tween 80

solution (Sigma-Aldrich Inc., St. Louis, MO, USA) and filtered through sterile glass wool (Sigma-Aldrich). The filtered arthroconidia were pelleted by centrifugation (3000 *g)* and resuspended in PBS, pH 7.4. The concentrations of arthroconidia were determined with hemocytometer.

Fungal mycelia were obtained by culturing 1×10^7 arthroconidia in 50 mL of potato dextrose broth (PDB) at 28ºC for 24 h and 180 rpm (Ecotron, Infors HT, Switzerland). Subsequently, fungal mycelia were filtered through Miracloth (Calbiochem, La Jolla, CA) and frozen in liquid nitrogen.

2.3. DNA extraction, multilocus sequence typing and phylogenetic analysis

The genomic DNA (gDNA) was extracted from fungal mycelia ground under liquid nitrogen using a mortar and pestle. The gDNA was extracted with extraction buffer (200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% w/v SDS) and phenol-chloroform (1:1). After centrifugation, the gDNA was precipitated with isopropanol and resuspended with RNase-free water (Promega, Madison, WI).

Multilocus sequence typing (MLST) was performed by PCR, DNA sequencing and nucleotide sequence analysis. All PCR reactions were performed with Phusion® High Fidelity DNA Polymerase (Thermo Fisher scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. The following seven *loci* were chosen for MLST: the internal transcribed spacer regions 1 (ITS1) and 2 (ITS2) of the 28S rRNA; the chitin synthase (CHS); the histone H3 (HH3); the glyceraldehyde-3-phosphate dehydrogenase (GPD); the β-tubulin (TUB), and the elongation factor 1α (EF1). The primers for ITS1 and

ITS2 amplification were designed according to White et al. [45], and for the amplifications of the other sequences the following primers were designed based on published sequences in GenBank [46]: CHS (CHS_f 5'-GTGTGCGTTGTCAGCGAC-3', CHS_r 5'-TCCTTCAAACAGAAAAGCATC-3'), TUB (TUB_f 5'-CAGCTGAACTCTGACCTGC- 3', TUB_r 5'-AGCAGTGAACTGGTCACC-3'), HH3 (HH3_f 5'-CGCTGCGTCGCTGTTGC-3', HH3_r 5'-CTACAATACCGTTAGTAATGC-3'), GPD (GPD_f 5'-AGCTGACCCACGCCACCA-3', GPD_r 5'-ACCGTCGACGGTCTTCTG-3'), EF-1 (EF1_f 5'-TCGACAAGCGTACCATCGAG-3', EF1_r 5'-AGCCTTGAGCTTGTCAAGGA-3').

DNA sequences were determined with the ABI3730 DNA Analyzer (Applied Biosystems, Foster City, CA), analyzed with ChromasPro® software (ChromasPro 1.7.6, Technelysium Pty Ltd, Tewantin QLD, Australia) and compared with the DNA sequences database of the Basic Local Alignment Search Tool (BLAST) [47]. The MLST analysis was performed by the multiple DNA alignment method using the 'Molecular Evolutionary Genetics Analysis - MEGA' 6.0 software [48]. The GenBank accession numbers of each sequence are listed in supplementary Table S1. The combination of alleles for the different *loci* defined the sequence type (ST) of each clinical isolate. Phylogenetic analysis was conducted by inferring the evolutionary history using the unweighted-pair group method with arithmetic averages and arithmetic mean (UPGMA) [49]. The robustness of the dendrogram was evaluated from 1000 bootstrap replications. The evolutionary distances were computed using the *p*-distance method [50-51]. The analyses were performed using MEGA 6.0 software [48].

2.4. In vitro antifungal susceptibility test

Data regarding the antifungal susceptibility of the two varieties are scarce, particularly of the Brazilian isolates. As this information is important both for therapeutic and epidemiological purposes, the susceptibilities of all the clinical isolates to seven currently-used antifungal drugs were determined. The susceptibility tests were conducted according to the Clinical Laboratory Standards Institute (CLSI) reference method for broth dilution antifungal susceptibility testing of filamentous fungi (CLSI document M38-A2) [52]. The antifungal drugs amphotericin B (AMB), ketoconazole (KET), fluconazole (FLU), voriconazole (VOR), 5-flucytosine (5FC), and terbinafine (TER) were purchased from Sigma-Aldrich. Caspofungin (CAS) was purchased from Merck Sharp & Dohme Limited (Kenilworth, NJ, USA). The final concentrations were 0.03 to 32 μg mL^{-1} for AMB, 0.03 to 16 μg mL^{-1} for VOR and TER, 0.125 to 64 μg mL^{-1} for CAS, and 0.25 to 128 μg mL^{-1} for 5FC, KET and FLU. The experiments were performed in 96-well, flat-bottomed microtitre plates (TPP, Switzerland) with RPMI 1614 culture medium (Gibco, Invitrogen Corporation, NY, USA) buffered with 0.165 M 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0, antifungal drugs and 2×10^4 arthroconidia mL^{-1}. After 48 h of incubation at 28ºC, minimal inhibitory concentrations (MIC) were determined visually by comparison with the growth in the control well (culture medium and 2×10^4 athroconidia mL^{-1}). The MIC endpoint was defined as the minimal concentration of the drug capable of inhibiting 100% of fungal growth compared with the growth control for amphotericin B and 5-flucytosine and to inhibit 90% for azoles, terbinafine and

caspofungin. Geometric means and MIC ranges were calculated for each species and antifungal drug. The standard quality control strains of the experiment were *Aspergillus flavus* (ATCC®204304™) and *Candida krusei* (ATCC®6258). The tests were repeated three times.

2.5. Phenothiazinium photosensitizers and visible light sources

The phenothiazinium photosensitizers methylene blue (MB), new methylene blue N zinc chloride double salt (NMBN) and toluidine blue O (TBO) were purchased from Sigma-Aldrich. The novel pentacyclic phenothiazinium photosensitizer S137 was synthesized as previously described [38]. The chemical structures of all the PS are show in supplementary Figure S1. Stock solutions of all PS were prepared with phosphate buffered saline (PBS), pH 7.4 and stored as previously described [40].

Light was provided by an array of 96 light-emitting diodes (LED) with emission peak at 631 nm (LED96). The array was made in-house using the Cree®5-mm Round LED (model # LC503UHR1-1 5Q-Q, CREE Inc., Durham, NC, USA). The integrated irradiance in the visible spectrum (400 to 700 nm) was 13.89 mW cm^{-2}. Light measurement was performed by using a cosine-corrected irradiance probe (CC-3-UV, Ocean Optics, USA) screwed onto the end of an optical fiber coupled to an USB4000 spectroradiometer (Ocean Optics, Dunedin, FL, USA). Light was measured inside the well, at the sample level, to reduce interference of the plastic plate. The emission spectra of the LED array and the absorption spectra of the PS are presented in supplementary Figure S2.

2.6. Evaluation of APDT efficacies on arthroconidia based on the PS minimal inhibitory concentration

Minimal inhibitory concentration (MIC)-based experiments were conducted as previously described [39,40,53] to determine the best conditions for APDT of arthroconidia of *N. dimidiatum* and *N. dimidiatum* var. *hyalinum.* Eleven isolates, three of the sequence type 1, one of the sequence type 2, one of the sequence type 3, two of the sequence type 4, three of the sequence type 5 and one of the sequence type 6 were evaluated. Experiments were performed in 96-well microtiter plates (TPP, Switzerland). Fifty µL of the arthroconidia suspension (4×10^4 athroconidia mL^{-1}) and 50 µL of the PS solution were added to each well. Final concentrations of MB, NMBN and TBO were 0, 1, 2.5, 5, 10, 12.5, 25, 50, 75, 100, and 200 µM and the final concentrations of S137 were 0, 0.5, 1, 2.5, 5, 10, 12.5, 20, 25, 30 and 40 µM. Plates were incubated in the dark for 30 min at 28°C and exposed to light fluences of 3, 6, and 11 J cm^{-2} or kept in the dark (dark controls). After the exposures, 100 µL of two-fold concentrate RPMI 1614 culture medium buffered with 0.165 M MOPS pH7.0 were added to each well and plates were incubated at 28°C. Fungal growth in each well was evaluated after 7 days by visual inspection when the MICs were determined. MIC was considered the minimal concentration of the PS for each fluences, in which total growth inhibition was achieved. Three independent experiments were performed in triplicate.

2.7. Effect of APDT with MB, TBO, NMBN or S137 on the survival of ungerminated and germinated arthroconidia

Based on the previous MIC experiments, the best conditions were established and the effect of APDT on the survival of ungerminated arthroconidia was determined as previously described [37,40]. Two selected isolates of *N. dimidiatum* (LMC 302.01 and 301.01) and one of *N. dimidiatum* var. *hyalinum* (LMC 3313.01) were evaluated. Experiments were performed in a 96 microtiter plate (TPP, Switzerland). Fifty µL of the arthroconidia suspension and 50 µL of the PS solution (MB, NMBN, TBO, or S137) were added to each well. The final concentration of the arthroconidia in the mixture was 2×10^7 artroconidia mL^{-1} and the final concentrations of MB, NMBN, TBO and S137 were 200, 200, 200, and 25 µM, respectively, for *N. dimidiatum*; and 25, 25, 10, and 10 µM, respectively, for *N. dimidiatum* var. *hyalinum*. Plates were maintained in the dark for 30 min at 28°C and exposed to light fluences of 3, 6, and 11 J cm^{-2} or kept in the dark. After exposure, arthroconidial suspensions were removed, serially diluted 10-fold in PBS, and 50 µL of each dilution was spread onto potato dextrose agar (Acumedia) supplemented with 0.08 g L^{-1} deoxycholic acid sodium salt (Fluka, Italy). Plates were incubated in the dark at 28°C and colony forming units (CFU) were counted daily at 8× magnification for up to 4 days. Three replicate dishes were prepared for each treatment in each experiment and three independent experiments were performed. The effect of the different treatments was calculated as previously described [53].

The effect of APDT was further evaluated on germinated arthroconidia. For this, the arthroconidia were previously germinated in RPMI 1614 culture

medium buffered with 0.165 M MOPS pH7.0 at 28ºC for 4 h. APDT of germlings was carried out exactly as ungerminated arthroconidia.

2.8. Evaluation of APDT effect on plasma membrane permeability

The permeability of the arthroconidial plasma membrane after APDT was evaluated by measuring the incorporation of propidium iodide (PI) by arthroconidia. PI is a positively-charged fluorescent nucleic acid dye which only penetrates cells with severe membrane damage [44,54]. Experiments were performed in a 24-well flat bottomed microtiter plate (TPP, Switzerland). Ungerminated arthroconidia were exposed to APDT as previously described. After APDT, arthroconidia were transferred from each well to 1.5-mL tubes, washed once with PBS and PI solution was added to a final concentration of 10 μg mL^{-1} immediately before flow cytometric analysis. The single-cell light scattering and fluorescence measurements were performed using a FACSCanto flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). Ten thousand events were analyzed by FACSDiva software (BD, San Jose, CA, USA). The percentage of cells stained with PI after each treatment was determined. Three independent experiments were performed. The results were compared with the 'gold-standard' determination of viable cells (arthroconidia survival fraction) by CFU-based experiments. Arthroconidia not treated with PS neither exposed to light were used as control of conidial viability and arthroconidia treated with 70% ethanol for 15 min were used as positive control (death control).

2.9. Effects of APDT on lipid peroxidation

The evaluation of lipid peroxidation in arthroconidia after APDT was determined by measuring the production of malonildialdehyde (MDA) using the Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich, Inc. - St. Louis, MO, USA) following the manufacturer's protocol with slight modification. MDA is a product of the oxidation of polyunsaturated lipids by reactive oxygen species (ROS). Arthroconidia were exposed to APDT as previously described. After treatments, arthroconidia were recovered from each well, transferred to a 1.5-mL microtube, centrifuged for 5 min at 5000 *g* and lysed with MDA lysis buffer (300 µL) and BHT 100× (3 µL). The insoluble material was removed by centrifugation for 10 min at 13000 *g*. Two hundred microliters of the supernatant from each sample were transferred to a new 1.5-mL microtube and incubated at 95ºC for 1 h with 600 µL of TBA solution. Samples were cooled in ice bath for 10 min and 200 µL of each sample were transferred to a 96-well flat-bottomed microtitre plate. Absorbances at 532 nm were determined using a Spectramax® Paradigm® Multi-Mode Detection Platform (Molecular Device LLC, Sunnyvale, CA). A standard curve was used to calculate MDA concentration in each sample. Three independent experiments were performed in triplicate. The results were also compared with arthroconidial survival determined by CFU counting.

2.10. Statistical analysis

Treatment comparisons were performed using one-way analysis of variance (one-way ANOVA) followed by Tukey's post-test and *P* values of <

0.05 were considered significant. All computations were performed using GraphPad Prism Software (v 5.0 GraphPad Software, La Jolla, CA, USA).

3. Results

3.1 Multilocus sequence typing and phylogenetic analysis

MLST was performed by the sequencing of ITS1 and ITS2 regions of the rDNA and the conserved genes CHS, HH3, GPD, TUB and EF1 in order to determine the genotypes of a collection of 24 *N. dimidiatum* and 6 *N. dimidiatum* var. *hyalinum* strains isolated from skin and toenail infections. Two ATCC strains were also genotyped (Table S1). The nucleotides sequence of ITS2 region and conserved genes CHS and GPD did not show polymorphic sites among the clinical isolates and ATCC strains. However, eight polymorphic sites were found in the sequence of ITS1 region and EF1, TUB and HH3 genes (Table 1). ITS1 has a transition from guanine to adenine at 105 bp of the sequenced region (Table 1). The nucleotide sequence of EF1 has shown four polymorphic sites throughout the intron sequence. The transitions from thymine to cytosine, cytosine to thymine, adenine to guanine and the transversion from adenine to cytosine were found at sites 103, 188, 198 and 214 bp, respectively. Two polymorphic sites were found in the TUB gene. The first site is the transition from thymine to cytosine at position 312 bp. The codon CTT (cytosine thymine thymine) encodes the amino acid leucine while the codon CCT (cytosine cytosine thymine) encodes the amino acid proline. The second polymorphic site has shown a transition from cytosine to thymine at position 319 bp which is a silent mutation since both codons encode serine. The nucleotide

sequence of HH3 has shown a polymorphic site at 265 bp with a transition from adenine to guanine, which represents a silent mutation, in which both codons encode glutamate (Table 1).

The combination of the eight polymorphic sites found in ITS1 region of the rDNA and in the sequences of the EF1, TUB, and HH3 genes determined six sequences types (STs) (Table 1). Most of the *N. dimidiatum* clinical isolates (64%) were clustered in ST1 and 5.2%, 2.6% and 12.8% of the isolates were clustered in ST2, ST3 and ST4, respectively. The *N. dimidiatum* var. *hyalinum* represents 15.4% of the clinical isolates and all of them together with the isolate ATCC®38907™ (*N. dimidiatum* var. *hyalinum*) were clustered in ST5. The only member of ST6 was the isolate ATCC®22190™ (*N. dimidiatum*).

The evolutionary history of *N. dimidiatum* and *N. dimidiatum* var. *hyalinum* isolates using the 2075 bp originated from the concatenation of the seven *loci* dataset (ITS1, ITS2, EF1, TUB, HH3, CHS and GPD), generated the UPGMA dendrogram based on *p*-distance (Fig. 1). The bootstrap values were registered in the nodes of the UPGMA phylogenetic tree. The main set of the isolates could be differentiated into two subgroups (A and B), with strong bootstrap support. Significant association was found between the subgroups, regarding the presence or absence of the dark-brown pigment. All the isolates of subgroup A produce pigments and their arthroconidia and hyphae are pigmented while none of the isolates of the subgroup B produces pigments and their arthroconidia and hyphae are hyaline. The result shows that the isolates of the two varieties can be accurately differentiated by the multilocus sequence typing.

3.2 In vitro antifungal susceptibility test

The *in vitro* activities of seven antifungal drugs were tested against 24 *N. dimidiatum* and 6 *N. dimidiatum* var. *hyalinum* clinical strains isolated from skin and toenail infections, and two ATCC strains. The antifungal drugs which demonstrated lower minimal inhibitory concentrations (MICs) against all isolates (STs 1 to 6) were AMB (MIC range 0.03 - 0.5 μg mL^{-1} and Geometric Mean (GM) MIC 0.126 μg mL^{-1}), TER (0.125 - 4 μg mL^{-1} and 0.75 μg mL^{-1}) and VOR (0.125 - 4 μg mL^{-1} and 1.68 μg mL^{-1}). 5FC (1 - 32 μg mL^{-1} and 12.95 μg mL^{-1}) and CAS (8 - 64 μg mL^{-1} and 27.02 μg mL^{-1}) exhibited lower MICs in comparison with KET (0.5 - >128 μg mL^{-1} and 58.81 μg mL^{-1}) and FLU (8 - >128 μg mL^{-1} and 95.63 μg mL^{-1}) (Table 2). Compared with *N. dimidiatum* isolates (ST1 to 4), *N. dimidiatum* var. *hyalinum* isolates (ST5) were more sensitive to VOR (0.125 - 0.5 μg mL^{-1} and 0.27 μg mL^{-1}), KET (0.5 - 16 μg mL^{-1} and 3.62 μg mL^{-1}) and FLU (8 - 64 μg mL^{-1} and 39 μg mL^{-1} - $P < 0.05$). ST6 showed lower MIC than STs 1 to 4 for FLU (32 - 64 μg mL^{-1} and 45.25 μg mL^{-1}) and KET (1 μg mL^{-1} and 1 μg mL^{-1} – $P < 0.05$) (Table 2).

3.3. Evaluation of APDT efficacies on arthroconidia based on PS minimal inhibitory concentration

Exposure only to red light did not inhibit the growth of any of the isolates. In the dark, treatments with MB, NMBN and TBO in concentrations up to 200 μM did not inhibit the growth of any of the isolates, while S137 at concentrations > 50 μM inhibited the growth of all of them (Table 3). The MICs of each photosensitizer (MB, NMBN, TBO and S137) at each fluence for the 11 selected

isolates of *N. dimidiatum* and *N. dimidiatum* var. *hyalinum* are shown in Table 5. Based on the PS MIC, S137 was the most effective PS followed by NMBN. MB and TBO were less effective. Concentrations of S137 > 25 μM inhibited the growth of all the isolates at all the fluences, and most of the isolates were inhibited by concentrations > 10 μM. MIC-based experiments also showed that arthroconidia of the *N. dimidiatum* var. *hyalinum* isolates (ST 5) are more susceptible to APDT than the pigmented arthroconidia of the *N. dimidiatum* (ST 1, 2, 3 and 4) regardless the photosensitizer used (Table 5).

3.4. Effect of APDT with MB, TBO, NMBN or S137 on the survival of ungerminated and germinated arthroconidia

The criteria used to select the isolates used in this experiment were the MICs for the different PS and colony color. The MICs of the isolates of the ST1, 2, 3 and 4 were similar but they differ from those of the ST5. Regarding the colony color, *N. dimidiatum* isolates have shown different pattern (i.e. brown and gray), while none of the isolates of *N. dimidiatum* var. *hyalinum* produced pigments (ST5) (Fig. 2). Thus, the effects of APDT on the survival of urgeminated arthroconidia was evaluated on two selected isolates of *N. dimidiatum* (LMC 302.01 - brown and 303.01 - gray), which belong to ST1, and one isolate of *N*. *dimidiatum* var. *hyalinum* (LMC 313.01 - hyaline), which belongs to ST5 (Fig. 2). The concentrations of the different PS were selected based on the results of the MIC experiments (Table 5). Thus, PS concentrations used for *N. dimidiatum* were 200 μM for MB, NMBN and TBO, and 25 μM for S137. PS concentrations used for *N. dimidiatum* var. *hyalinum* were 25 μM for

MB and NMBN, and 10 μM for TBO and S137. Exposure only to light and treatments with MB, NMBN or TBO in the dark did not kill the conidia of any isolate. Treatment with S137 in the dark killed 58.3%, 39.8% and 0% of the arthroconidia of *N. dimidiatum* (LMC302.01), *N. dimidiatum* (LMC303.01) and *N. dimidiatum* var. *hyalinum* (LMC313.01), respectively. APDT with MB and TBO at 3 J cm^{-2} reduced the survival of *N. dimidiatum* (both isolates) and *N. dimidiatum* var. *hyalinum* arthroconidia by 1 log and 4 logs, respectively. At 6 J cm^{-2} reductions were 3 logs and 5 logs, and at 11 J cm^{-2} reductions were 4.5 logs and 5 logs, respectively ($P < 0.05$ for all treatment comparisons). APDT with NMBN at 3 J cm^{-2} reduced the survival of *N. dimidiatum* (LMC302.01 and LMC303.01) and *N. dimidiatum* var. *hyalinum* (LMC313.01) arthroconidia by 3 logs, 3 logs and 5 logs, respectively. At 6 and 11 J cm^{-2}, reductions in the survival were 5 logs, 4 logs and 5 logs for the three isolates, respectively ($P < 0.05$ for all treatment comparisons) (Fig. 3). APDT with S137 at 3 J cm^{-2} reduced the survival of *N. dimidiatum* (LMC302.01 and LMC303.01) and *N. dimidiatum* var. *hyalinum* (LMC313.01) by 3.5 logs, 2.5 logs and 2 logs, respectively. At 6 J cm^{-2}, reductions were 5 logs, 2.5 logs and 2.5 logs, and at 11 J cm^{-2} the reductions were 5 logs, 5 logs and 3.5 logs ($P < 0.05$ for all treatment comparisons) (Fig. 3).

In addition, APDT with MB, NMBN, TBO and S137 at a fluence of 6 J cm^{-2} was carried out with 4-h germinated arthroconidia of *N. dimidiatum* (LMC302.01 and LMC303.01) and *N. dimidiatum* var. *hyalinum* (LMC313.01). Results were very similar to those observed with ungerminated arthroconidia (Supplementary Fig. S3). Thus, APDT with MB, NMBN, TBO and S137 are able

to kill both ungerminated and germinated arthroconidia of *N. dimidiatum* and *N. dimidiatum* var. *hyalinum* equally well.

3.5. Effect of APDT on the permeability of the arthroconidia plasma membrane

The effects of APDT with the four PS on the arthroconidial survival and plasma membrane permeability of the three selected isolates are shown in Fig. 4. Ungerminated arthroconidia were treated with MB, NMBN, TBO or S137 and exposed to light or kept in the dark. PS concentrations used for the two *N. dimidiatum* isolates (LMC302.01 and 303.01) were 200 μM for MB, NMBN and TBO, and 25 μM for S137, and concentrations used for the *N. dimidiatum* var. *hyalinum* isolate (LMC313.01) were 25 μM for MB and NMBN, and 10 μM for TBO and S137. After APDT, cells were treated with PI, which only penetrates cells with damage in the plasma membrane. Therefore, the higher the percentage of arthroconidia stained with PI, the greater the damage caused by APDT on the membrane. Exposure only to light did not kill arthroconidia or damage their plasma membrane. In the dark, none of the four PS killed the arthroconidia, but all of them caused some damage to the plasma membrane. The extent of the damage varied depending on the PS and isolate. Despite the lower concentration used, S137 caused more damage to the plasma membrane of the three isolates as assessed by PI incorporation. The results also showed that PI incorporation by arthroconidia does not always mean that the stained arthroconidia are dead. For example, treatment with S137 in the dark resulted in PI incorporation by virtually all the arthroconidia, but they were still viable. APDT with all the PS killed the arthroconidia of the three isolates and also damaged

their plasma membranes. PI incorporation was close to 100% after all treatments (PS and fluences) (Fig. 4).

After APDT, an inverse correlation was observed between the arthroconidia survival and the percentage of conidia stained with PI (Fig. 4).

3.6. Effects of APDT on lipids peroxidation

Lipid peroxidation was evaluated by the cellular quantification of malondialdehyde (MDA). The effects of APDT with MB, NMBN, TBO and S137 on arthroconidial survival and lipid peroxidation are show in Fig. 5. Exposure only to light or treatments only with the PS did not kill the arthroconidia or result in lipid peroxidation. APDT with NMBN or S137 but not with MB or TBO resulted in the oxidation of the lipids in arthroconidia of the three fungal isolates (Fig. 5).

4. Discussion

The accurate identification of the isolates of the two *N. dimidiatum* varieties is important not only to guide the therapy, since the varieties present different profiles of susceptibility to antifungal drugs, but also to allow epidemiological and biogeography studies. To date, few studies regarding the molecular distinction between the two varieties have been reported and their results varied. Chromosomal DNA of both *N. dimidiatum* and *N. dimidiatum* var. *hyalinum* has similar percentages of guanine and cytosine [55]. Amplified ribosomal DNA restriction analysis conducted with isolates from North America, Europe and North Africa showed that *N. dimidiatum* is identical to *N. dimidiatum* var. *hyalinum* [12]. PCR-restriction fragment length polymorphism ribotyping

showed an identical sequence for the 18S ribosomal DNA gene of both *N. dimidiatum* and *N. dimidiatum* var. *hyalinum* [15]. More specific molecular methods, such as the sequencing of the 18S subunit of ribosomal RNA performed with isolates from France, North America and North Africa, identified nucleotide polymorphism between *N. dimidiatum* var. *hyalinum* and *N. dimidiatum* isolates. Additionally, an intronic insertion was observed exclusively in *N. dimidiatum* isolates [13]. The genotyping of *N. dimidiatum* and *N. dimidiatum* var. *hyalinum* by the sequencing of four *loci* (the tubulin and the chitin synthase genes and the internal transcribed spacer region and D1/D2 domain of the 28S rRNA gene) revealed five sequence types (ST) [2]. In our study, the genotyping included the sequence analysis of seven *loci* of 24 *N. dimidiatum* and 6 *N. dimidiatum* var. *hyalinum* clinical isolates. Eight polymorphic sites have been detected and six ST were determined. Interestingly, all the clinical isolates of *N. dimidiatum* var. *hyalinum* evaluated in the present study and the hyaline ATCC®38907™ isolate clustered together in the same sequence type (ST5). The polymorphic sites in ITS1 and TUB allow differentiation of the ST5 isolates from the other ST (ST1 to 4 and ST6). Some of these polymorphic sites were previously described in *N. dimidiatum* and *N. dimidiatum* var. *hyalinum* strains isolated from different sources in Brazil, Egypt, USA, Mali and the United Kingdom [2]. Therefore, our results showed that the two varieties can be accurately differentiated based on the multilocus polymorphism analysis. Also, the genetic difference between the two varieties may be higher than it is generally assumed, involving other *loci* than those responsible for pigments biosynthesis. The extent of this difference will be only known when several isolates of the two varieties have their genomes

sequenced. Thus, for now, differences observed between the two varieties, including their susceptibilities to antifungals and to APDT, should not be attributed only to the difference in pigmentation.

The reference microdilution methodologies for *in vitro* antifungal susceptibility testing have not been standardized for *Neoscytalidium* spp. [4,7,34]. Thus, results of the *in vitro* susceptibility testing with these fungi should be interpreted very carefully. AMB, VOR and TER are ranked as the antifungal agents with the highest *in vitro* antifungal activity against *N. dimidiatum* and the hyaline variety [2,16-17,32-33]. In accordance with these data, we found that the isolates of *N. dimidiatum* (ST 1 to 4) and *N. dimidiatum* var. *hyalinum* (ST 5) were more susceptible to AMB, VOR and TER than to the other antifungal agents tested. Additionally, our results clearly showed that the isolates of the hyaline variety are much less tolerant to the azole antifungal drugs FLU, VOR and KET than the isolates of the pigmented variety. A previous study showed that isolates of *N. dimidiatum* var. *hyalinum* from Brazil and France were less tolerant to VOR than isolates of the pigmented *N. dimidiatum* [2, 17].

Antimicrobial photodynamic therapy with phenothiazinium PS has been shown to be a potential alternative to conventional antifungal agents for the treatment of mycoses. The use of MB and TBO in the photodynamic treatment of onychomycosis was recently reported. Local application of PS (MB and TBO) and illumination of infected nails produced a response in 53 out of 62 patients, without any collateral effect, and 28 patients showed complete clearance [56]. APDT with methyl-aminolevulinate was successfully used to cure a woman with onychomycosis of the toes caused by *N. dimidiatum* [57]. In the present study, we demonstrated the *in vitro* efficacy of APDT with the phenothiazinium PS MB,

NMBN, TBO and S137 both on urgerminated and germinated arthroconidia of the dematiaceous fungus *N. dimidiatum* and the hyaline variety *N. dimidiatum* var. *hyalinum*. The ability to kill ungerminated conidia is a positive aspect of APDT, since conidial tolerance to currently-used antifungal agents is frequently pointed out as one of the causes of therapeutic failure and recurrence of the infections [58-60]. Arthroconidia of *N. dimidiatum* isolates were more tolerant to APDT with all the four phenothiazinium PS than the hyaline isolates (see Table 5). The protective effect of melanins and melanin-like pigments against conventional antifungal drugs [61-63] and also to APDT [64-67] was observed previously both in conidia and in vegetative yeast cells. Pigmented conidia of *Penicillium* and *Metarhizium* are much more resistant to APDT with different PS than the colorless mutants [65-66]. In yeasts, *Cryptococcus neoformans* melanized cells are more resistant to APDT than the nonmelanized cells [64,67]. Unfortunately the mechanisms responsible for increasing resistance due the presence of pigments appear to be diverse and are not yet fully understood. Interestingly, arthroconidia of *N. dimidiatum* are intrinsically much more resistant to APDT with the phenothiazinium PS than conidia of other previously studied species, such as *Colletotrichum acutatum*, *C. gloesporioides* [53], *Aspergillus nidulans* [41], *Fusarium oxysporium*, *F. moniliforme* and *F. solani* [44]. This is not surprising, since the different types of fungal asexual propagules (i.e. conidia, microconidia and arthroconidia) are very different in relation to their ontogeny, dimensions, morphology, physiology, and physical properties and chemical composition [10]. Among the tested photosensitizers, the pentacyclic derivative S137 was the most effective. The MICs of S137 were lower than the MIC of other PS for the isolates of both the varieties *N.*

dimidiatum and *N. dimidiatum* var. *hyalinum* (see Table 5). Higher concentrations of MB, TBO or NMBN than S137 were necessary to kill ungerminated and germinated arthroconidia of the dematiaceous isolates (see Figs. 2 and S3). Additionally, it should be taken into account that, despite the higher activity of S137 compared with the other PS, the light source employed was optimal (i.e. coinciding with the longer-wavelength monomer peak) for TBO and NMBN but not for S137 or MB in terms of output wavelength (supplementary Fig. S2) [37]. The higher efficacy of APDT with S137 compared with the other phenothiazinium PS, including MB, was previously observed against microconidia of the dermatophytes *T. rubrum* and *T. mentagrophytes* [40]; the plant-pathogenic fungi *Colletotrichum acutatum*, *C. gloeosporioides* [53], *Fusarium oxysporum*, *F. moniliforme* and *F. solani* [44]; the saprophyte *Aspegillus nidulans* [41], and also against planktonic cells of several species of *Candida* [37]. The ability of the PS to bind to microbial cells and to produce singlet oxygen is important to the efficiency of the APDT. Although MB, NMBN, TBO and S137 do not differ much in the production of singlet oxygen, they differ markedly in their ability to bind to plasma membranes [68]. Both MB and TBO display log $P_{oil/water} < 0$ which indicate a low lipophilicity. While NMBN and S137 exhibit a higher lipophilic profile (log $P_{oil/water} > 0$) [68-70]. The more lipophilic nature of these PS molecules determines their capacity to bind to cell membranes and is critical to define the extent of photoinduced membrane damage and consequently the efficiency of cell killing [70,44]. We evaluated the effect of the APDT with the four phenothiazinium PS on arthroconidia plasma membranes by evaluating changes in membrane permeability and peroxidation of the lipids. The propidium iodide (PI) staining-based assay is widely used to

evaluate the membrane integrity [54] and also has been considered a good marker for cell death associated with membrane alterations in fungi [71]. PI is a membrane-impermeable fluorescent dye that only penetrates cells with damaged plasma membranes. Treatments with MB, NMBN and TBO at 200 µM in the dark increased the membrane permeability of *N. dimidiatum* arthroconidia, and treatment with S137 at 10 µM had an even stronger effect, causing the staining of 100% of the arthroconidia of the three isolates (see Fig. 4). Interestingly, despite the disturbance of the membrane that increased cell permeability to PI, most of the arthroconidia were still viable (see Fig. 4), which clearly indicates that the permeability to PI is not always a good marker for cell death in fungi. APDT with MB, NMBN, TBO and S137 increased the plasma membrane permeability of arthroconidia of *N. dimidiatum* and *N. dimiditum* var. *hyalinum* isolates, causing the PI staining of approximately 100% of the arthroconidia.

Polyunsaturated lipids are easily oxidized by reactive oxygen species resulting in a chain of reactions that has malondialdehyde as a major product [72]. APDT with S137 or NMBN but not with MB or TBO caused the peroxidation of arthroconidial lipids and consequently malondialdehyde production (see Fig. 5). Membrane damage efficiency of photodynamic treatment with phenothiazinium PS such as MB, TBO, 1,9-dimethyl methylene blue (DMMB) and S137 was previously studied using artificial liposomes [68]. The membrane damage efficiency followed the order S137 > DMMB > MB > TBO. Only the PS that had higher membrane/solution partition (S137 and NMMB) could permeabilize the lipid bilayer and cause membrane leakage and lipid oxidation. Moreover, only S137 altered the membrane structure. To some

extent, the present study confirmed these findings in living cells. In a recent study performed with microconidia of three *Fusarium* species we reported that APDT with the same phenothiazinium PS used in the present study damages the microconidial membrane but only with NMBN and S137 caused the peroxidation of the membrane lipids. Thus, despite the great difference between the two types of propagules (arthroconidia and microconidia), the effects of APDT on the membranes were similar and seem to be common to the different types of conidia.

5. Conclusion

The pigmented variety *N. dimidiatum* differs from the hyaline variety in other *loci* than the ones involved in the pigment biosynthesis, and the two varieties can be differentiated by multilocus sequence typing. The arthroconidia of the hyaline variety are more susceptible to antifungal drugs as well to APDT with phenothiazinium photosensitizers. APDT with the four PS damages the plasma membrane of arthroconidia of both varieties increasing its permeability to PI but only NMBN and S137 caused peroxidation of the membrane lipids.

Acknowledgment

The authors are thankful to the ‘Serviço de Analises Clínicas da Faculdade de Ciências Farmacêuticas de Ribeirão Preto (FCFRP) - Universidade de São Paulo’ which has provided the clinical isolates studied in this work. We also thank John Carpenter, Ribeirão Preto, Brazil, for the English revision.

This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo - FAPESP grants (2012/17028-2 and 2012/23920-5).

References

[1] R.J. Hay, *Scytalidium* infections, Curr. Op. Infect. Dis. 15 (2002) 99-100..

[2] H. Madrid, M. Ruiz-Cendoya, J. Cano, A. Stchigel, R. Orofino, J. Guarro, Genotyping and *in vitro* antifungal susceptibility of *Neoscytalidium dimidiatum* isolates from different origins, Int. J. Antimicrob. Agents 34 (2009) 351-354.

[3] A.J.L. Phillips, A. Alves, J. Abdollahzadeh, B. Slippers, M.J. Wingfield, J.Z. Groenewald, P.W. Crous, The Botryosphaeriaceae: genera and species known from culture, Stud. Mycol. 76 (2013) 51-167.

[4] N. Valenzuela-Lopez, D.A. Sutton, J.F. Cano-Lira, K.Paredes, N. Wiederhold, J. Guarro, A.M. Stchigel, Coelomycetous fungi in the clinical setting: morphological convergence and crypyic diversity, J. Clin. Microbiol. 55(2) (2017) 552-567.

[5] M. Machouart, P. Menir, R. Helenon, D. Quist, N. Desbois, *Scytalidium* and scytalidiosis: what's new in 2012? J. Mycol. Med. 23 (2013) 40-46.

[6] P.W. Crous, B. Slippers, M.J. Wingfield, J. Rheeder,W.F.O. Marasas, A.J.L. Philips, A. Alves, T. Brugess, P. Barber, J.Z. Groenewald, Phylogenetic lineages in the *Botryosphaeriaceae*, Stud. Mycol. 55 (2006) 235-253.

[7] A. Chowdhary, J.F. Meis, J. Guarro *et al.*, ESCMID and ECMM joint clinical guidelines for the diagnosis and management of systemic phaeohyphomycosis; diseases caused by black fungi, Clin. Microbiol. Infec. 20 (2014) suppl. 3 47-75.

[8] B. Dionne, L. Neff, S.A. Lee, D.A. Sutton, N.P. Wiederhold, J. Lindner, H. Fan, B. Jakeman, Pulmonary Fungal Infection Caused by *Neoscytalidium dimidiatum*. J. Clin. Microbiol. 53 (2015) 2381-2384.

[9] A. Miqueleiz-Zapatero, C.S. Olalla, B. Buendía, J. Barba, Dermatomycosis due Neoscytalidium spp., Enferm Infecc. Microbiol. Clin. 35 (2017) 130-131.

[10] G. Turian, Spores in ascomycetes, their controlled differentiation, in: D. Weber and W.M. Hess (Eds.), The fungal spore, John Wiley & Sons, Inc., Brigham, Provo, Utah, 1976, pp.715-788.

[11] K. Alshawa, J.L. Beretti, C. Lacroix, M. Feuilhade, B. Dauphin, G. Quesne, N. assouni, X. Nassif, M.-E. Bougnoux, Successful identification of clinical dermatophyte and Neoscytalidium species by matrix-assisted laser desorption ionization-time of flight mass spectrometry, J. Clin. Microbiol. 50 (2012) 2277-2281.

[12] H.J. Roeijmans, G.S. De Hoog, C.S. Tan, M.J. Figge, Molecular taxonomy and GC/MS of metabolites of *Scytalidium hyalinum* and *Nattrassia mangiferae* (*Hendersonula toruloidea*), J. Med. Vet. Mycol. 35 (1997) 181-188.

[13] M. Machouart, C. Lacroix, H. Bui, M. Feuilhade de Chauvin, F. Derouin, F. Lorenzo, Polymorphisms and intronic structures in the 18S subunit ribosomal RNA gene of the fungi *Scytalidium dimidiatum* and *Scytalidium hyalinum*. Evidence of an IC1 intron with an His-Cys endonuclease gene, FEMS Microbiol. Lett. 238 (2004) 455-467.

[14] R. Morris-Jones, S. Youngchim, J.M. Hextall, B. L. Gomez, S. D. Morris-Jones, R. J. Hay, A. Casadevall, J. D. Nosanchuk, and A. J. Hamilton, *Scytalidium dimidiatum* causing recalcitrant subcutaneous lesions produces melanin, J. Clin. Microbiol. 42 (2004) 3789-3794.

[15] M. Machouart-Dubach, C. Lacroix, C. Vaury, M. Feuilhade de Chauvin, C. Bellanné, F. Derouin, F. Lorenzo, Nucleotide structure of the *Scytalidium hyalinum* and *Scytalidium dimidiatum* 18S subunit ribosomal RNA gene: evidence for the insertion of a group IE intron in the rDNA gene of *S. dimidiatum*, FEMS Microbiol. Lett. 208 (2002) 187-196.

[16] J. Dunand, A. Paugam, *In vitro* efficacy of voriconazole against clinical isolates of *Scytalidium* spp. from clinical lesions, Int. J. Antimicrob. Agents 31 (2008) 176-177.

[17] C. Lacroix, M.F. de Chauvin, *In vitro* activity of amphotericin B, itraconazole, voriconazole, posaconazole, caspofungin and terbinafine against *Scytalidium dimidiatum* and *Scytalidium hyalinum* clinical isolates, J. Antimicrob. Chemother. 61 (2008) 835-7.

[18] Z.U. Khan, S. Ahmad, L. Joseph, R. Chand, Cutaneous phaeohyphomycosis due to *Neoscytalidium dimidiatum*: First case report from Kuwait, J. de Mycologie Médicale 19 (2009) 138-142.

[19] B.E. Elewski, Onychomycosis caused by *Scytalidium dimidiatum*. J. Am. Acad. Dermatol. 35 (1996) 336-338.

[20] L. Sigler, R.C. Summerbell, L. Poole, M. Wieden, D.A. Sutton, M.G. Rinaldi, M. Aguirre, G.W. Estes, J N. Galgiani, Invasive *Nattrassia mangiferae* infections: case report, literature review, and therapeutic and taxonomic appraisal, J. Clin. Microbiol. 35 (1997) 433-440.

[21] M.K. Dhindsa, J. Naidu, A. Singh, A case of subcutaneous infection in a patient with discoid lupus erythematosus caused by a *Scytalidium* synanamorph of *Natrassia mangiferae,* and its treatment. Med. Mycol. 36 (1998) 425-427.

[22] J.J. Dunn, M.J. Wolfe, J. Trachtenberg, J.D. Kriesel, R.R. Orlandi, K.C. Carroll, Invasive fungal sinusitis caused by Scytal*idium dimidiatum* in a lung transplant recipient. J. Clin. Microbiol. 41 (2003) 5817-5819.

[23] M. Bakhshizadeh, H.R. Hashemian, M.J. Najafzadeh, S. Dolatabadi and H. Zarrinfar, First report of rhinosinusitis caused by *Neoscytalidium dimidiatum* in Iran. J. Med. Microbiol. 63 (2014) 1017–1019.

[24] A.A. Al-Rajhi, A.H. Awad, S.S.A. Al-Hedaithy, R.K. Forster, K.C. Caldwell, *Scytalidium dimidiatum* fungal endophthalmitis. British J. Ophthalmol. 77 (1993) 388-390.

[25] R.S. Mani, Y.T. Chickabasaviah, S. Nagarathna A. Chandramuki, M.R. Shivprakash, J. Vijayan, D.K. Prashantha, P.S. Vasudevan, A. Natarajan, J. Kovoor, Cerebral phaeohyphomycosis caused by *Scytalidium dimidiatum*: a case report from India. Med. Mycol. 46 (2008) 705-11.

[26] H. Elinav, U. Izhar, S. Benenson, D. Admon, C. Hidalgo-Grass, I. Polacheck, M. Korem, Invasive *Scytalidium dimidiatum* infection in an immunocompetent adult, J. Clin. Microbiol. 47 (2009) 1259-1263.

[27] B. Willinger, G. Kopetzky, F. Harm, P. Apfalter, A. Makristathis, A. Berer, A. Bankier, S. Winkler, Disseminated infection with *Nattrassia mangiferae* in an immunosuppressed patient, J. Clin. Microbiol. 42 (2004) 478-480.

[28] D.H.S. Tan, L. Sigler, C.F.C. Gibas and I.W. Fong, Disseminated fungal infection in a renal transplant recipient involving *Macrophomina phaseolina*

and *Scytalidium dimidiatum*: case report and review of taxonomic changes among medically important members of the *Botryosphaeriaceae*, Med. Mycol. 46 (2008) 285-292.

[29] A. Ikram, W. Hussain, M.L. Satti, M.A. Wiqar. Invasive infection in a young immunocompetent soldier caused by *Scytalidium dimidiatum*, J. Coll. Physicians. Surg. Pak. 19 (2009) 64-66.

[30] S. Garinet, J. Tourret, S. Barete, N. Arzouk, I. Meyer, C. Frances, A. Datry, D. Mazier, B. Barrou, A. Fekkar, Invasive cutaneous *Neoscytalidium* infections in renal transplant recipients: a series of five cases, BMC Infect Disease 15 (2015) 535.

[31] C.A. Oyeka, H.C. Gugnani, *In vitro* activity of seven azole compounds against some clinical isolates of non-dermatophytic filamentous fungi and some dermatophytes, Mycopathologia, 110 (1990) 157-161.

[32] A.J. Carrillo-Munoz, G. Giusiano, J. Guarro, G. Quindós, C. Guardia, O. del Valle, V. Rodríguez, D. Estivill, C.D. Cárdenes, *In vitro* activity of voriconazole against dermatophytes, *Scopulariopsis brevicaulis* and other opportunistic fungi as agents of onychomycosis, Int. J. Antimicrob. Agents 30 (2007) 157-161.

[33] J.G. Bueno, C. Martinez, B. Zapata, G. Sanclemente, M. Gallego, A.C. Mesa, *In vitro* activity of fluconazole, itraconazole, voriconazole and terbinafine against fungi causing onychomycosis, Clin. Exp. Dermatol. 35 (2010) 658-663.

[34] J.E. James, J. Santhanam, M.C. Lee, C.X. Wong, P. Sabaratnam, H. Yusoff, M.N. Tzar, M.F. Razak, *In vitro* antifungal susceptibility of *Neoscytalidium dimidiatum* clinical isolates from Malaysia, Mycopathologia, 182 (2017) 305-313.

[35] T. Dai T, Y.Y Haung, M.R. Hamblin, Photodynamic therapy for localized infections – state of the art, Photodiag. Photodyn. Ther. 6 (2009) 170-188.

[36] R.F. Donnelly, P.A. McCarron, M.M. Tunney, Antifungal photodynamic therapy, Microbiol. Res. 163 (2008) 1–12.

[37] G.B. Rodrigues, M. Dias-Baruffi, N. Holman, M. Wainwright, G.U.L. Braga, *In vitro* photodynamic inactivation of *Candida* species and mouse fibroblasts with phenothiazinium photosensitisers and red light, Photodiagnosis Photodyn.Ther. 10 (2013) 141-149.

[38] M. Wainwright, K. Meegan, C. Loughran, Phenothiazine Photosensitisers IX. Tetra- and pentacyclic derivatives as photoantimicrobial agents, Dyes Pigm. 91 (2011) 1-5.

[39] M. Wainwright, C. O`Kane, S. Rawthore, Phenothiazinium photosensitisers XI. Improved toluidine blue photoantimicrobials, J. Photochem. Photobiol. Biol. B, 160 (2016) 68-71.

[40] G.B. Rodrigues, L.K. Ferreira, M. Wainwright, G.U.L. Braga, Susceptibilities of the dermatophytes *Trichophyton mentagrophytes* and *T. rubrum* microconidia to photodynamic antimicrobial chemotherapy with novel phenothiazinium photosensitizers and red light, J. Photochem. Photobiol. B 116 (2012) 89-94.

[41] F.P. Gonzales, S.H. Silva, D.W. Roberts, G.U.L. Braga, Photodynamic Inactivation of Conidia of the Fungi *Metarhizium anisopliae* and *Aspergillus nidulans* with Methylene Blue and Toluidine Blue, Photochemistry and Photobiology, 86 (2010) 653-661.

[42] L. Gao, S. Juang, Y. Sun, M. Deng, Q. Wu, M. Li, T. Zeng, Evaluation of the effects of photodynamic therapy alone and combined with standard antifungal therapy on planktonic cells and biofilms of *Fusarium* spp. and *Exophiala* spp., Front. Microbiol. 7 (2016) 617.

[43] D. Watanabe, C. Kawamura, Y. Masuda, Y. Akita, Y. Tamada, Y. Matsumoto, Successful treatment of toenail onychomycosis with photodynamic therapy, Arch. Dermatol. 144 (2008) 19–21.

[44] H.D. de Menezes, L. Tonani, L. Bachmann, M. Wainwright, G.U.L Braga, M.R. von Zeska Kress, Photodynamic treatment with phenothiazinium photosensitizers kills both ungerminated and germinated microconidia of the pathogenic fungi *Fusarium oxysporum*, *Fusarium moniliforme* and *Fusarium solani*, J. Photochem. Photobiol. Biol. B, 164 (2016) 1-12.

[45] T.J.B. White, S. Lee, T.W. Taylor, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Michael A. Innis DHG, John J. Sninsky, Thomas J. White (ed) *PCR protocols: a guide to methods and applications*. New York, N.Y.: Academic Press, INC Harcourt Brace Jovanovich, 1990: 482.

[46] D.A. Benson, M. Cavanaugh, K. Clark, I. Karsch-Mizrachi, D.J. Lipman, J. Ostell, E.W. Sayers, GenBank, Nucleic Acids Res. 41 (2013) D36-42.

[47] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403-410.

[48] K. Tamura. G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: Molecular Evolutionary Genetics Analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725-2729.

[49] P.H.A. Sneath and R.R. Sokal, Numerical taxonomy, in: The principles and practice of numerical classification, San Francisco, W.H. Freeman and Company (1973) 1-573.

[50] J.M. Bain, A. Tavanti, A.D. Davidson, M.D. Jacobsen, D. Shaw, N.A.R. Gow, F.C. Odds, Multilocus sequence typing of the pathogenic fungus *Aspergillus fumigatus*, J. Clin. Microbiol. 45 (2007) 1469-1477.

[51] N. Masatoshi and S. Kumar, Molecular Evolution and Phylogenetics, Oxford University Press (2000) 1-352.

[52] P.A. Wayne, CLSI. Reference Methods for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi, approved standard M38-A. 3rd edition: Clinical Laboratory Standard Institute (2008).

[53] H.D. de Menezes, G.B. Rodrigues, S.P. Teixeira, N.S. Massola Júnior, L. Bachmann, M. Wainwright, J.K. Bastos, G.U.L Braga, *In vitro* photodynamic inactivation of plant-pathogenic fungi *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* with novel phenothiazinium photosensitizers, Appl. Environ. Microbiol. 80 (2014) 1623-1632.

[54] H.M. Davey, P. Hexley, Red but not dead? Membranes of stressed *Saccharomyces cerevisiae* are permeable to propidium iodide. Environ Microbiol 13 (2011) 163-171.

[55] F.D. Davison, D.W.R. Mackenzie, R.J. Owen, Deoxyribonucleic Acid Base Compositions of Dermatophytes, J. Gen.Microbiol. 118 (1980) 465-470.

[56] J.P. Tardivo, M. Wainwright, M. Baptista, Small scale trial of photodynamic treatment of onychomycosis in São Paulo, J. Photochem. Photobiol. B Biol. 150 (2015) 66-68.

[57] C. Aspiroz, B. Fortuño-Cebamanos, A. Rezusta, Y. Gilaberte, Photodynamic therapy with methyl-aminolevulinate can be useful in the

management of *Scytalidium* infections, Actas Dermosfiliogr. 104(8) (2013) 725-727.

[58] L.M. Coelho, R. Aquino-Ferreira, C.M.L. Maffei, N.M. Martinez-Rossi, *In vitro* antifungal drug susceptibilities of dermathophytes microconidia and arthroconidia, J. Antimicrob. Chemother. 62 (2008) 758–761.

[59] N.M. Martinez-Rossi, N.T.A. Peres, A. Rossi, Antifungal resistance mechanisms in dermatophytes, Mycopathologia 166 (2008) 369–383.

[60] T. Hashimoto, H.J. Blumenthal, Survival and resistance of *Trichophyton mentagrophytes* arthrospores, Appl. Environ. Microbiol. 35 (1978) 274-277.

[61] D. van Duin, A. Casadevall, J.D. Nosanchuk, Melanization of *Cryptococcus neoformans* and *Histoplasma capsulatum* reduces their susceptibilities to amphotericin B and caspofungin, Antimicrob. Ag. Chemoth 46 (2008) 3394-3400.

[62] J.D. Nosanchuck and A. Casadevall, Impact of Melanin on microbial virulence and clinical resistance to antimicrobial compounds, Antimicrob. Agents Chemothe. 50 (2006) 3519-3528.

[63] L.M. Baltazar, S.M.C. Werneck, B.M. Soares, M.V.L. Ferreira, D.G. Souza, M. Pinotti, D.A. Santos, P.S. Cisalpino, Melanin protects *Paracoccicioides brasiliensis* from the effects of antimicrobial photodynamic inhibition and antifungal drugs, Antimicrob. Ag. Chemoth. 59 (2015) 4003-4011.

[64] G.B. Rodrigues, F.L. Primo, A.C. Tedesco, G.U.L. Braga, *In vitro* photodynamic inactivation of *Cryptococcus neoformans* melanized cells with chloroaluminium phthalocyanine nanoemulsion, Photochem. Photobiol. 88 (2012) 440-447.

[65] A. Asthana, R.W.Tuvson, Effects of UV and phototoxions on selected fungal pathogens of citrus, Int. J. Plant Sci. 153 (1992) 442-452. et al 1992

[66] F.P. Gonzales, S.H. da Silva, D.W. Roberts, G.U.L.B., Photodynamic inactivation of conidia of the fungi *Metarhizium anisopliae* and *Aspergillus nidulans* with methylene blue e toluidina blue, Photochem. Photobiol. 86(3) (2010) 653-661.

[67] R.A. Prates, B.B. Fuchs, K. Mizuno, Q. Naqvi, I.T. Kato, M.S. Ribeiro, E. Mylonakis, G.P. Tegos, M.R. Hamblin, Effect of virulence factors on the Photodynamic Inactivation of *Cryptococcus neoformans*, PLOS One 8 (2013) e54387.

[68] I.O.L. Bacellar, C. Pavani, E.M. Sales, R. Itri, M. Wainwright, M. Baptista, Membrane damage efficiency of phenothiazinium photosensitizers, Photochem. Photobiol. 90 (2014) 801-813.

[69] M. Wainwright, H. Smalley, O. Scully, E. Lotfipour, Comparative photodynamic evaluation of new phenothiazinium derivatives against *Propionibacterium acnes*, Photochem. Photobiol. 88 (2012) 523-526.

[70] M. Wainwright, D.A. Phoenix, S.L. Laycock, D.R. Wareing, P.A. Wright, Photobactericidal activity of phenothiazinium dyes against methicillin-resistant strains of *Staphylococcus aureus*, FEMS Microbiol. Lett. 160 (1998) 177-181.

[71] C. Pina-Vaz, S. Costa-de-Oliveira, A.G. Rodrigues, A. Espinel-Ingroff, Comparison of two probes for testing susceptibilities of pathogenic yeast to voriconazole, itraconazole and caspofungin by flow cytometry, J. Clin. Microbiol. (2005) 4674-4679.

[72] V. Nair, G.A. Turner, The thiobarbituric acid test for lipid peroxidation: structure of the adduct with malondialdehyde, Lipids 19 (1984) 804–805.

Figure legends:

Fig. 1. UPGMA dendrogram showing the genetic relationship among the 24 *N. dimidiatum*,6 *N. dimidiatum* var. *hyalinum* isolates and ATCC strains. The percentages of replicate dendrograms in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. LMC, laboratório de micologia clínica; ATCC 38907, *N. dimidiatum* var. *hyalinum*; ATCC 22190, *N. dimidiatum*.

Fig. 2. *N. dimidiatum* and *N. dimidiatum* var. *hyalinum* colony color. A, C and E are the front of the colony; B, D and F are the back of the colony. The isolates were grown on potato desxtrose agar (PDA) media culture at 28ºC for 6 days.

Fig. 3. Arthroconidia survival fraction of *N. dimidiatum* and *N. dimidiatum* var. *hyalinum* after APDT with MB (A), TBO (B), NMBN (C) and S137 (D). Arthroconidia were incubated with the PS for 30 min in the dark and exposed to fluences of 0 (dark controls), 5, 10 and 20 J cm^{-2}. Error bars are standard deviation of three independent experiments.

Fig. 4. Plasma membrane permeability of *N. dimidiatum* and *N. dimidiatum* var. *hyalinum* after APDT with MB, TBO, NMBN and S137. Arthroconidia stained

with propidium iodide (PI) (gray columns) and survival fraction (white squares). Error bars are standard deviation of three independent experiments.

Fig. 5: Peroxidation of arthroconidia lipids of *Neoscytalidium dimidiatum* and *N. dimidiatum* var. *hyalinum* after APDT with MB, TBO, NMBN, and S137. Gray columns show the *in situ* generation of malondialdehyde (a marker for lipid peroxidation) and white squares show the arthroconidia survival fraction. Error bars are standard deviation of three independent experiments.

Fig. S1. Chemical structures of the photosensitisers used in the study. Methylene blue (MB), new methylene blue (NMBN), toluidine blue O (TBO), and the new pentacyclic phenothiazinium photosensitizer S137.

Fig. S2. Visible absorption spectra for the photosensitisers (10 μM) employed in the study and LED96 output. MB, methylene blue; NMBN, new methylene blue; TBO, toluidine blue O and S137, new pentacyclic phenothiazinium photosensitizer.

Figure S3: Four-hour germinated arthroconidia survival fraction of *N. dimidiatum* and *N. dimidiatum* var. *hyalinum* after APDT with MB (A), TBO (B), NMBN (C) and S137 (D). Arthroconidia were germinated in potato dextrose broth at 28ºC for 4 h. After, they were incubated with the PS for 30 min in the dark and exposed to fluences of 0 (dark controls) and 10 J cm^{-2}. Error bars are standard deviation of three independent experiments.