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Antifungal susceptibility of *Sporothrix schenckii* complex biofilms

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Abstract

Sporotrichosis, caused by species of *Sporothrix schenckii* complex, is the most prevalent subcutaneous mycosis in many areas of Latin America. The aim of this study was to evaluate the ability of *Sporothrix* spp. to form biofilms *in vitro* and to characterize the growth kinetics, morphology, and antifungal susceptibility of biofilms against classical antifungals. We investigated the ability of strains to produce biofilms *in vitro* and determined the effects of exposure to amphotericin B, itraconazole, caspofungin, ketoconazole, voriconazole, and fluconazole at minimum inhibitory concentration (MIC) against planktonic form and at 10× MIC and 50× MIC on the biomass and metabolic activity of these biofilms. Biofilm structure was analyzed by optical microscopy using Congo-red staining, confocal and scanning electron microscopy. Strains were classified for biofilm-forming ability, through the analysis of absorbance of crystal violet retained by biomass of mature biofilms. We found that all *S. brasiliensis* ($n = 10$), *S. schenckii sensu stricto* ($n = 2$), *S. globosa* ($n = 2$), and *S. mexicana* ($n = 4$) strains were strong biofilm-producers. The analyzed biofilms had dense network of hyphae and conidia immersed in extracellular matrix, with presence of water channels. Antifungal drugs at the three tested concentrations showed different effects on biomass and metabolic activity of biofilms. However, the best inhibitory response was observed with 50× MIC of

amphotericin B and caspofungin, which reduced these parameters. Furthermore, high drug concentrations, especially amphotericin B and caspofungin, showed antifungal activity against these biofilms, probably because they damaged the architecture and extracellular matrix, allowing diffusion of the drugs.

Key words: *Sporothrix*, biofilm, antifungal susceptibility, antimicrobial resistance.

Introduction

Sporotrichosis is a cosmopolitan subcutaneous mycosis caused by species of the *Sporothrix schenckii* complex, with several reports in tropical and subtropical areas, including hyperendemic areas. The *Sporothrix* genus is formed by saprophyte, geophilic dimorphic fungi that affect humans and other animals.[1–3] Species associated with sporotrichosis are *S. brasiliensis*, *S. schenckii sensu stricto*, *S. globosa*, and *S. mexicana*.[4]

The etiological agent has a predilection for hot and high humidity areas, the reason why cases have been reported in countries like Australia, China, India, the United States, Mexico, and Panama.[5,6] In Brazil, the main species found in cases of sporotrichosis are *S. brasiliensis* and *S. schenckii sensu stricto*, both considered more virulent than the other species of the genus.[7,8] These species have been implicated in human and feline outbreaks of sporotrichosis in the State of Rio de Janeiro.[1]

Susceptibility studies of species of the *Sporothrix* genus to many currently used antifungals have reported an increase in resistance to amphotericin B, azoles, and echinocandins.[8–10] There is a difference in antifungal susceptibility between species of the *S. schenckii* complex, which can be explained by genetic diversity, revealing the existence of genotypes linked to resistance.[8] This diversity has not only been demonstrated with several traditional antifungal drugs but also with the leishmanicide miltefosine, which has shown different antifungal activity against species of the *S. schenckii* complex, with *S. globosa* as the least susceptible species to this drug.[11]

One of the factors also related to differences in antimicrobial susceptibility is the ability of microorganisms to produce biofilms, which decreases the effectiveness of antimicrobial therapies. Studies on bacterial biofilms are far more abundant in the literature, but there are reports that fungi, including dimorphic fungi, are also capable of forming such structure.[12–13]

Thus, this study aimed to evaluate the ability of species of the *Sporothrix schenckii* complex to form biofilms *in vitro*, as well as to characterize the growth kinetics, morphology and antifungal susceptibility of these biofilms.

Methods

Microorganisms

In this study, 18 strains of the *Sporothrix schenckii* complex in the filamentous phase were used: 10 strains of *S. brasiliensis*, two *S. schenckii sensu stricto*, two *S. globosa*, and four strains of *S. mexicana*. The isolates were obtained from the culture collection of the Specialized Medical Mycology Center, Brazil (CEMM) and were stored in saline solution at 4°C. Before the experiments, all isolates were plated onto potato dextrose agar (PDA; Himedia, India), grown at 25°C, for 5 days, and maintained in the filamentous phase.

Biofilm formation assay

All isolates ($n = 18$) were previously cultured on PDA for 5 days, at 25°C. Inocula with 2×10^4, 2×10^5, and 2×10^6 conidia/ml were transferred to wells of microtiter plates for biofilm formation and were analyzed for 1, 8, and 24 h of adherence. The best conditions to form biofilms were found when using an inoculum of 2×10^5 conidia/ml, with an adherence phase of 24 h. The cells were suspended in 0.9% sterile saline to obtain an inoculum concentration of 2×10^5 conidia/ml (1.0 McFarland). Aliquots of 1000 μl of each inoculum were transferred to flat-bottomed 24-well polystyrene plates containing a Thermanox™ coverslip (Thermo Fisher Scientific, USA) and incubated at 35°C for 24 h under static conditions for the pre-adhesion step. Afterward, the supernatant from each well was carefully removed, and 1000 μl of RPMI 1640 medium (Sigma, Germany), buffered to pH 7.0 with MOPS 0.165 M, were added. The plates were further incubated for 5 days at 35°C, also under static conditions, for biofilm formation and maturation,[14] after which the biofilms were examined by optical, confocal, and scanning-electron microscopic methods.

Biofilm growth kinetics

Following the biofilm formation method described above, the growth kinetics was measured at different incubation periods (24, 48, 72, 96, 120, and 144 h). Fungal cultures were previously grown on potato dextrose agar at 35°C

for 5 days. Then, biofilms were grown in separate 96-well plates in order to be analyzed at different times of incubation (24, 48, 72, 96, 120, and 144 h). For such, inocula of 2×10^5 conidia/ml were prepared for each tested strain, and 200 μl of the inocula were added to each well of the plate and statically incubated at 35°C, for 24 h, for the adhesion step. Afterward, the supernatant of each well was removed, and 200 μl of RPMI medium at pH 7.0 were added to induce biofilm growth and maturation and statically incubated at 35°C, until reaching a total of 144 h of incubation (6 days). At each pre-established time (24, 48, 72, 96, 120, and 144 h), supernatants were discarded and the plates were washed twice with sterile PBS/Tween to remove nonadhered cells. Then, biofilm biomass was evaluated with crystal violet staining and its viability was assessed by the XTT assay.[14,15] Biomass quantification by crystal violet staining is based on the retention of this dye by the fungal biomass, which can be measured in a spectrophotometer. In the crystal violet assay, microplates were incubated at 35°C and at each chosen time the wells were washed three times with 0.9% saline solution to remove non-adhered cells. Subsequently, the fungal structures in the wells were fixated with 100 μl of 100% methanol. Then, the methanol was aspirated and an aliquot of 100 μl of 0.3% crystal violet was added to each well. After 20 min at 35°C, the dye solution was aspirated and the wells were washed twice with 200 μl of sterile distilled water. Finally, 150 μl of 33% acetic acid was added to the stained wells and left for 30 seconds. After this period, the volume was transferred to another plate that was immediately read in a spectrophotometer at 540 nm.[16] Assays were performed in triplicate and fungus-free sterility control wells were included, which were submitted to the same protocol as the other wells and were used to obtains the reference blank of absorbance for the crystal violet strain. These blank values were then used to establish the cutoff optival density (ODc) for biofilm formations, which was defined as the mean optical density obtained for the reference blank, plus 3 times its standard deviation. Then, biofilm biomass of each tested strain was spectrophotometrically quantified through crystal violet staining and the obtained optical densities (OD) were used to classify the biofilm-forming ability of the species of the *Sporothrix schenckii* complex, as follows: non–biofilm-producers ($OD \leq ODc$), weak producers ($ODc < OD \leq 2 \times ODc$), moderate producers ($2 \times ODc < OD \leq 4 \times ODc$) and strong producers ($4 \times ODc < OD$).[17] Finally, the XTT reduction method was used to evaluate the viability of the biofilms at each preestablished time of incubation. This method is based on the ability of mitochondrial dehydrogenases present in viable cells to convert this tetrazol salt into its reduced form, called formazan, a colored product which can be spectrophotometrically evaluated. The XTT assays were performed using stock solutions of XTT (1 mg/ml in phosphate-buffered saline [PBS]) and menadione (1 mM in ethanol). At each pre-established time, 50 μl of XTT solution plus 4 μl of menadione solution were added to all wells containing biofilm and negative control. After 3 h of incubation at 35°C, in the dark, the color change was measured with spectrophotometer at 492 nm.

Sporothrix spp. biofilm structure and morphology

For this step of the research, biofilms were grown on 24 well flat-bottomed plates containing a Thermanox™ in each well, as previously described in this manuscript.

Optical microscopy

Congo red staining was used for microscopic visualization of biofilms, as this staining technique allows the observation of the biofilm polymeric matrix. Therefore, the surface of each coverslip in polystyrene plate wells after formation of mature biofilms was covered with 10 mM of cetylpyridinium chloride and allowed to dry naturally in air for 30 minutes. Then, biofilms were stained with a 2:1 mixture of saturated aqueous solution of Congo red containing 10% (v/v) Tween 80, for 15 minutes. After washing with 1× PBS, the coverslips were stained with a 10% solution (v/v) of Ziehl carbol fuchsin for 6 minutes. Then, the coverslips were washed again with PBS 1× and allowed to dry at 37°C.[18] The coverslips were examined with an Olympus B ×41 microscope and the images were captured with an Olympus DP71 digital camera.

Confocal laser scanning microscopy (CLSM)

The biofilms were also analyzed by confocal microscopy. They were formed as described above on Thermanox™. After incubation, biofilms were washed with PBS and stained using LIVE/DEAD™ fluorescent dye (Invitrogen, USA). The biofilms were analyzed with Nikon C2 microscope at 488 nm for detection of SYTO 9 fluorescent dye, which identifies live cells, and at 561 nm for detection of propidium iodide, which identifies dead or damaged cells. Several sections were obtained in the *x-y* plane with 1 μm intervals along the z-axis. Three-dimensional reconstructions of biofilms were obtained by the resident software.[19] The images were processed with Photoshop software (Adobe Systems, San Jose, California USA).

Scanning electron microscopy (SEM)

To obtain morphological and structural information at the preestablished incubation periods (24, 48, 72, 96, 120, and 144 h) during biofilm formation and maturation, we used scanning electron microscopic images. As before, biofilms were formed on Thermanox™. After incubation, the supernatant was removed and plates were washed with cacodylate buffer (0.15 M) twice. Biofilms were then covered with glutaraldehyde (2.5% in 0.15 M sodium cacodylate buffer with 0.1% Alcian blue) and incubated at 4°C overnight. Subsequently, biofilms were washed twice with cacodylate buffer for 5 minutes and the coverslips were dehydrated in an ascending ethanol concentrations (50, 70, 80, 95, and 100%) twice, for 10 minutes each. Then the biofilms were dried at room temperature and covered with hexamethyldisilazane (HMDS) (Polysciences Europe, Germany) for 30 minutes. Afterward, HMDS was removed and biofilms were dried overnight in a desiccator.[14, 20] Slides were covered with 10 nm of gold (Emitech Q150T) and observed with an FEI Inspect S50 scanning electron microscope in high vacuum mode at 15 kV. Images were processed with Photoshop software (Adobe Systems, San Jose, California).

Antifungal susceptibility of planktonic form

Antifungal susceptibility assays of planktonic *Sporothrix* spp. were performed according to CLSI,[21] with adaptations.[11] Fungal inoculum were prepared from strains in the filamentous phase grown on PDA at 25°C, for 6 to 7 days. A sterile saline solution was added to each fungal

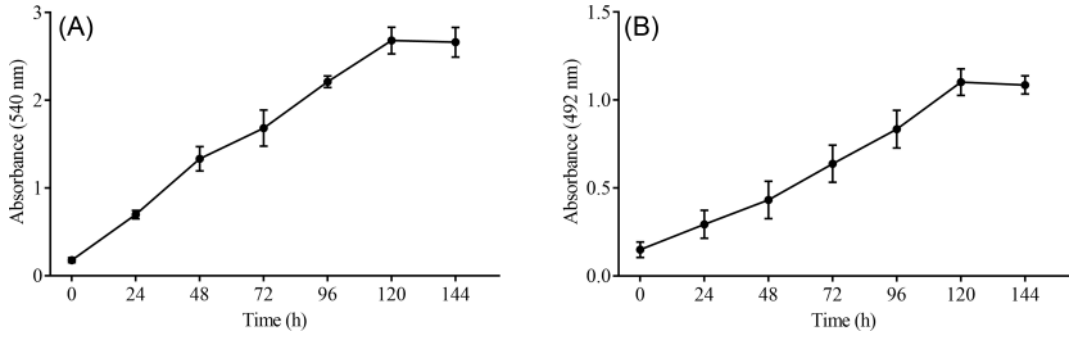

Figure 1. Biomass (A) and metabolic activity (B) of *Sporothrix schenckii* complex ($n = 8$; 2 *S. brasiliensis*; 2 *S. schenckii sensu stricto*; 2 *S. globosa*, and 2 *S. mexicana*) biofilm throughout an incubation period of 144 h. Biomass was quantified through crystal violet staining technique, while metabolic activity was measured by XTT reduction assay.

Table 1. *In vitro* biofilm production by species of the *Sporothrix schenckii* complex.

Strain	Species	OD (mean ± SD)	Biofilm-forming ability
CEMM 05-3-050	*Sporothrix brasiliensis*	2.062 ± 0.002	Strong
CEMM 05-3-052	*S. brasiliensis*	2.232 ± 0.006	Strong
CEMM 05-3-053	*S. brasiliensis*	1.831 ± 0.009	Strong
CEMM 05-3-054	*S. brasiliensis*	1.924 ± 0.035	Strong
CEMM 05-3-055	*S. brasiliensis*	2.029 ± 0.011	Strong
CEMM 05-3-056	*S. brasiliensis*	2.162 ± 0.015	Strong
CEMM 05-3-057	*S. brasiliensis*	2.170 ± 0.001	Strong
CEMM 05-3-058	*S. brasiliensis*	2.577 ± 0.001	Strong
CEMM 05-3-075	*S. brasiliensis*	2.009 ± 0.012	Strong
CEMM 05-3-078	*S. brasiliensis*	2.099 ± 0.004	Strong
CEMM 05-3-090	*S. schenckii sensu stricto*	2.183 ± 0.027	Strong
CEMM 05-3-100	*S. mexicana*	2.017 ± 0.013	Strong
CEMM 05-4-001	*S. mexicana*	2.061 ± 0.036	Strong
CEMM 05-4-002	*S. schenckii sensu stricto*	2.151 ± 0.007	Strong
CEMM 05-3-004	*S. globosa*	2.481 ± 0.004	Strong
CEMM 05-3-005	*S. globosa*	2.353 ± 0.023	Strong
CEMM 05-3-008	*S. mexicana*	2.149 ± 0.019	Strong
CEMM 05-3-009	*S. mexicana*	2.002 ± 0.001	Strong

Biofilm formed in 96-well microplates in RPMI medium with 24 hours of adhesion and 120 hours of maturation.
OD: optical density obtained at 540 nm for the crystal violet staining technique, expressed as mean ± standard deviation.

culture and the mycelial surface was scraped. Afterward, the turbidity of the fungal suspension was adjusted to 2.0 on McFarland scale (approximately 1–5 $\times$ 10^6 conidia/ml for *Sporothrix* spp.). Suspensions were diluted in the proportion of 1:10 with RPMI medium to obtain inocula with final concentrations of 1–5 $\times$ 10^5 conidia/ml.[11] This study evaluated the minimum inhibitory concentration (MIC) of six antifungal agents: amphotericin B (AMB), itraconazole (ITC), voriconazole (VRC), fluconazole (FLC), ketoconazole (KTC), and caspofungin (CAS) (all from Sigma Chemical Corporation, USA). Drug concentrations ranged from 0.03 to 16 μg/ml for AMB, ITC, VRC, KTC, and CAS and from 0.12 to 64 μg/ml for FLC.[21] Microplates were incubated 35°C, for 72 h, the time required for the observation of fungal growth in the drug-free growth control well.[11] All isolates were tested in duplicate. For AMB, ITC, and VRC, the minimum inhibitory concentration (MIC) was defined as the lowest drug concentration that inhibited 100% fungal growth; for KTC and FLC, the MIC was defined as the lowest drug concentration that inhibited fungal growth by 50%, when compared to drug-free growth control; for CAS the minimum effective concentration (MEC) was defined as the lowest concentration of drug that led to the growth of small, round, compact hyphal forms, as compared to the drug-free growth control.[21] *Candida parapsilosis* ATCC 22019 was included as quality control.[21] After the MIC reading, 100 μl aliquots from the wells that did not show visible fungal growth were transferred to tubes containing potato agar and incubated for 7 days at 25 to 28°C. The minimum fungicidal concentration (MFC) was defined as the lowest concentration able to prevent growth of fungal colonies.[11]

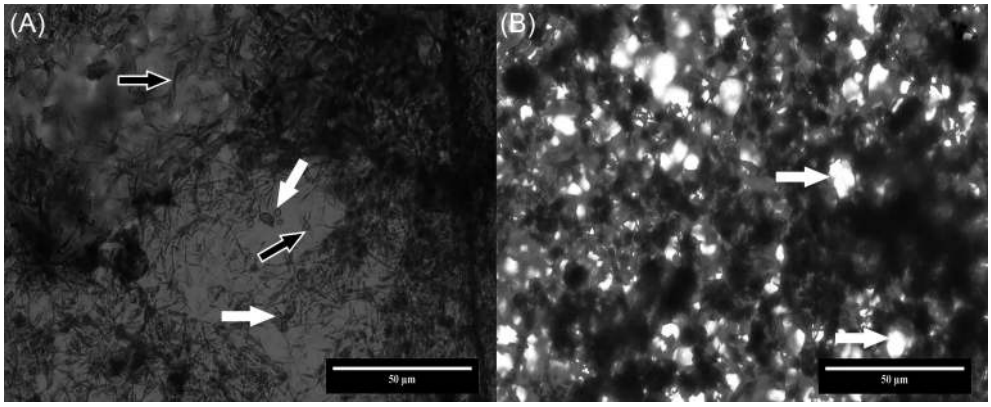

Figure 2. Optical microscopy of mature *Sporothrix globosa* biofilms, stained through Congo Red technique. (A) Dense extracellular matrix (gray background), with hyphae (black arrows with white outline) and conidia (white arrows). (B) Water channels within the hyphal network (arrows). Magnification of 400×.

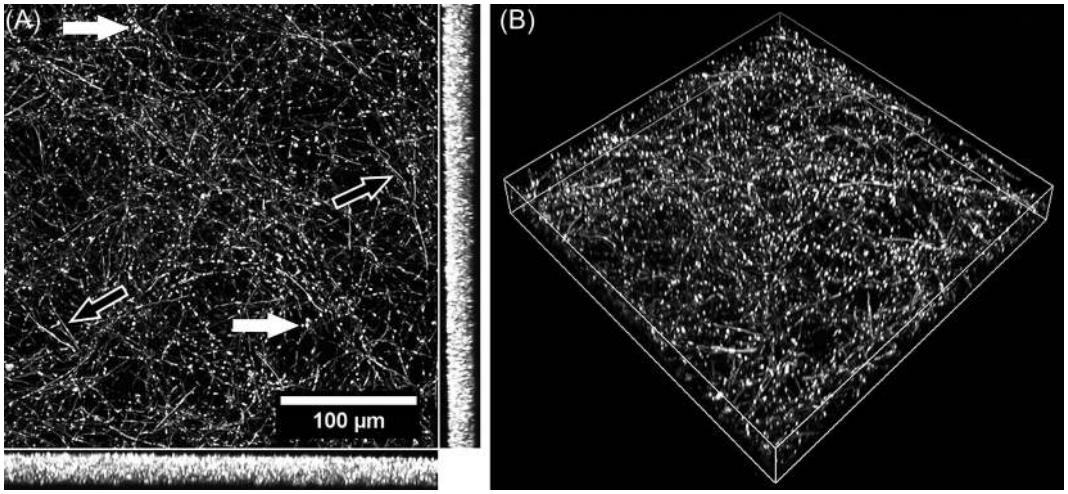

Figure 3. CLSM images of *Sporothrix brasiliensis* mature biofilms. (A) Hyphae (black outlined arrows) and conidia (white arrows) forming a dense biofilm structure. (B) Biofilm three-dimensional view. Magnification of 400×. Images acquired at 488 nm for the detection of SYTO9, which identifies live cells.

Antifungal biofilm susceptibility

To determine the antifungal susceptibility of filamentous biofilms to the tested drugs, biofilms were formed on polystyrene 96-well plates as described above. After 5 days of incubation at 35°C, the supernatant from each well was discarded and, shortly after, 200 μl of three different concentrations of each drug was added (planktonic MIC, 10× MIC and 50× MIC) to three different wells, in duplicate. The plates were incubated at 35°C, for 72 h, and, then, subjected to biomass quantification tests, using the crystal violet staining, and for metabolic activity, using the XTT assay, as previously described.

Statistical analysis

To compare the intraspecific absorbance data (optical density [OD]) for the tested drugs, the data were submitted to ANOVA, followed by the Bonferroni multiple comparison test for *S. brasiliensis* and *S. mexicana*. For *S. schenckii sensu stricto* and *S. globosa*, the nonparametric Fisher's exact test followed by Dunn's post hoc test for comparison between pairs were used. These two tests were also used to analyze the species-specific growth kinetics during the investigated period. In all cases, the maximum significance level adopted for affirmative findings was 5%.

Results

Data analyses related to development and kinetics of biofilms showed that species of *S. schenkii* complex, in filamentous form are capable of forming biofilms *in vitro*. Little biofilm formation was observed with fungal inoculum at a concentration of 2×10^4 conidia/ml, with low adhesion of cells to polystyrene surface. The subsequent concentrations, 2×10^5 and 2×10^6 conidia/ml, led to similar biofilm formation, with robust structure, presenting large amounts of adhered cells, after the adhesion period of 24 h. Thus, the inoculum concentration of the 2×10^5 conidia/ml was chosen for testing the biofilm-forming ability of *Sporothrix* strains. According to the growth curve for biomass quantification and metabolic activity, *Sporothrix* isolates adhered to the polystyrene surfaces, after 24 h of incubation, show-

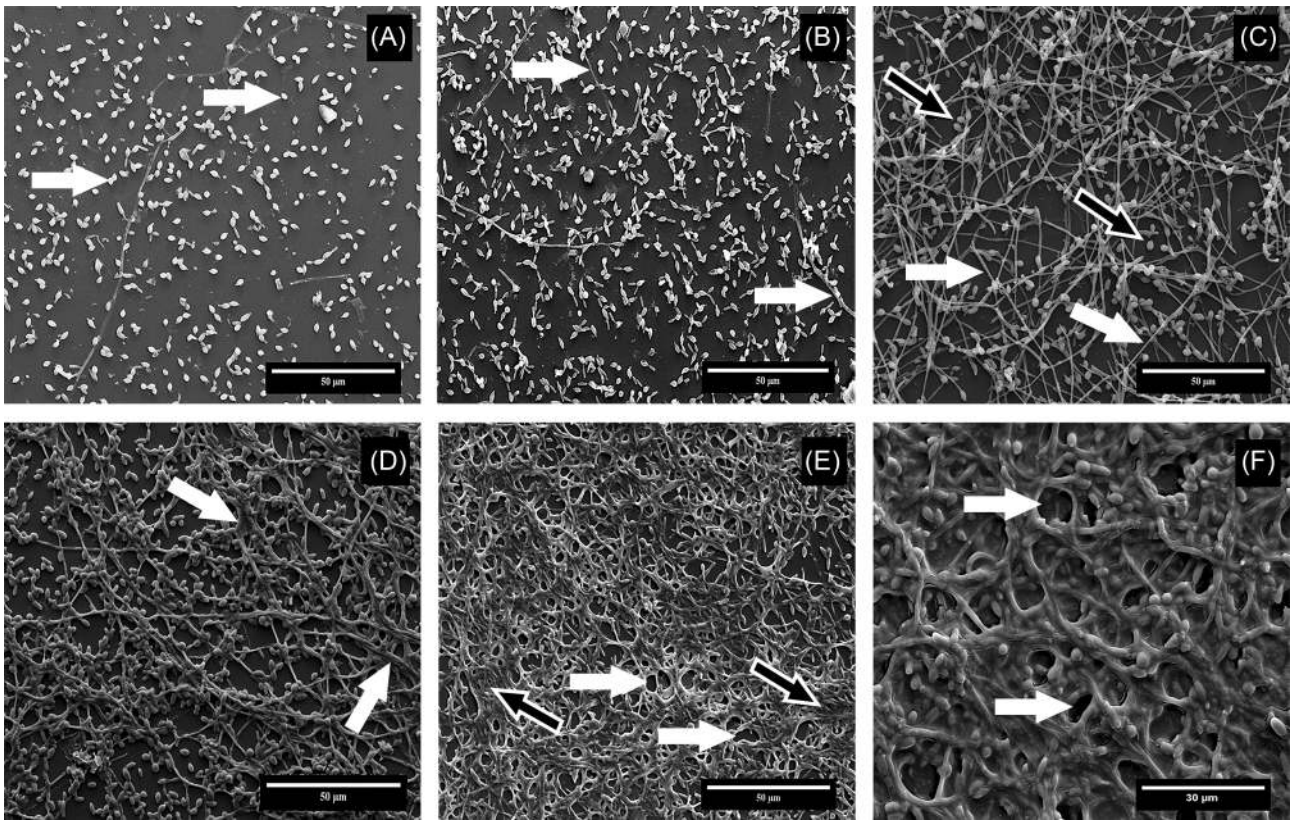

Figure 4. Scanning electron microscopic images of *Sporothrix brasiliensis* biofilms formed on Thermanox™ coverslips after 24, 48, 72, 96, 120, and 144 h of incubation (A–F, respectively). Magnification: 2000× (A–E) and 4032× (F). A. Cells attached to the surface of the coverslip (arrows). (B) Beginning of post-adhesion filamentation (arrows). (C) Formation of hyphal network (white arrows) and conidia (black arrows with white outline). (D) Thickening of the biofilm structure and initial observation of extracellular matrix (arrow). (E) Mature biofilm with presence of hyphae and conidia, matrix (black arrows with white outline) and water channels (white arrows). (F) Water channels (arrows) in 144-h-grown biofilm.

ing an increase in biomass and metabolic activity after the adhesion period. This increase was observed until 120 h of incubation (Fig. 1), reflecting the increase in cell density, during biofilm maturation. The biomass quantification of mature biofilms showed no significant differences between the different species of *Sporothrix* spp., and all of them were classified as strong biofilm-producers, as shown in Table 1.

Through optical microscopy, it was possible to visualize the formation of a dense extracellular matrix with conidia and hyphae embedded in this structure. Water channels for biofilm hydration and distribution of nutrients were also observed. Staining with Congo red revealed the presence of these structures, as shown in Figure 2.

Confocal microscopy allowed differentiation of live and dead cells. Mature biofilms had a larger number of live cells than dead or damaged cells, showing that the viability of biofilms cells remained high after maturation, as demonstrated in Figure 3.

Mature biofilms of *Sporothrix* spp., as revealed by scanning electron microscopy, were formed by a dense and heterogeneous mass of conidia and hyphae. After 24 h of incubation, adhered cells were evident, scattered over the entire surface of the coverslip, but after 48 h the onset of the filamentation process was observed. Hyphal network and conidia were seen after 72 h of incubation, and the maturation process continued at 96 and 120 h, when it was possible to see the formation of water channels. In addition, at 120 h of incubation, extracellular matrix was also observed (Fig. 4). The conidia were oval, with sizes ranging from 2.051 to 2.504 μm in width and 3.613 to 3.636 μm in length.

The *in vitro* antifungal susceptibility of *S. schenckii* species complex are shown in Table 2. Briefly, the MIC values found for these fungi, regardless of species, ranged from 0.25 to 8 μg/ml for AMB, from 0.25 to > 16 μg/ml for ITC, from 8 to > 16 μg/ml for VRC, from 16 to > 64 μg/ml for FLC and from 0.0625 to 2 μg/ml for KTC. For CAS, MEC values were not found, reaching a result of > 16 μg/ml against all strains.

The metabolic activity of *S. brasiliensis* biofilms showed a significant reduction ($P < .05$), when compared to the drug-free growth control, for all concentrations of AMB, ITC, and KTC and at 10× MIC and 50× MIC for VRC and CAS. For *S. schenckii sensu stricto* and *S. globosa*, significant reductions in metabolic activity were observed after exposure of mature biofilms to ITC at all tested concentrations, and AMB, FLC, KTC, and CAS at 50× MIC, while VRC did not affect biofilm metabolic activity of these *Sporothrix* species at any tested concentration. As for *S. mexicana*, significant reductions in metabolic activity ($P <$

Table 2. Minimum inhibitory concentrations (MIC), minimum effective concentrations (MEC) and minimum fungicidal concentrations (MFC) of antifungal drugs against *Sporothrix schenckii* species complex in planktonic growth.

Species	AMB (μg/ml)			ITC (μg/ml)			VRC (μg/ml)			FLC (μg/ml)			KTC (μg/ml)			CAS (μg/ml)		
	MIC		MFC	MIC		MFC	MIC		MFC	MIC		MFC	MIC		MFC	MEC		MFC
	Range	GM		Range	GM		Range	GM		Range	GM		Range	GM		Range	GM	
S. brasiliensis (n = 10)	0.25–1	0.66	0.25–1	0.25–2	0.70	>16	8– > 16	…	>16	64– > 64	>64	>64	0.0625–1	0.233	2 – 8	>16	>16	>16
S. schenckii sensu stricto (n = 2)	0.25	0.25	0.5	0.5– > 16	…	>16	>16	>16	>16	32–64	45.255	>64	0.5–1	0.707	2	>16	>16	>16
S. globosa (n = 2)	0.5	0.5	0.5	1–2	1.41	16	>16	>16	>16	16–32	22.627	>64	0.5	0.5	2–4	>16	>16	>16
S. mexicana (n = 4)	1–8	2.378	8	1–0.5	0.70	16	16– > 16	>16	16– > 16	32– > 64	…	>64	0.5–2	1	4–16	>16	>16	>16

AMB, amphotericin B; CAS, caspofungin; FLC, fluconazole; GM: geometric mean; KTC, ketoconazole; ITC, itraconazole; VRC, voriconazole. MICs for AMB, ITC, and VRC were defined as the minimum antifungal concentration that caused 100% inhibition of fungal growth; MICs for FLC and KTC were defined as the minimum antifungal concentration that caused 50% inhibition of fungal growth, when compared to drug-free growth control.

Table 3. Metabolic activity of *Sporothrix schenckii* complex biofilms, after exposure to antifungal drugs.

Antifungal drug		Species			
		Sporothrix brasiliensis ($n = 10$)	*Sporothrix schenckii sensu stricto* ($n = 2$)	*Sporothrix globosa* ($n = 2$)	*Sporothrix mexicana* ($n = 4$)
Control		0.918 ± 0.195	0.934 ± 0.032	0.930 ± 0.057	0.815 ± 0.055
AMB	MIC	0.870 ± 0.174*	0.915 ± 0.017	0.906 ± 0.064	0.809 ± 0.047
	10× MIC	0.822 ± 0.151*	0.887 ± 0.017	0.823 ± 0.034	0.781 ± 0.037
	50× MIC	0.754 ± 0.147*	0.856 ± 0.022*	0.789 ± 0.037*	0.727 ± 0.029*
ITC	MIC	0.687 ± 0.05*	0.800 ± 0.315*	0.622 ± 0.159*	0.614 ± 0.020*
	10× MIC	0.521 ± 0.070*	0.646 ± 0.235*	0.556 ± 0.128*	0.612 ± 0.050*
	50× MIC	0.404 ± 0.055*	0.579 ± 0.148*	0.402 ± 0.094*	0.455 ± 0.025*
VRC	MIC	0.935 ± 0.183	0.926 ± 0.019	0.988 ± 0.038	0.813 ± 0.054
	10× MIC	0.885 ± 0.182*	0.915 ± 0.013	0.952 ± 0.041	0.791 ± 0.057
	50× MIC	0.851 ± 0.178*	0.902 ± 0.011	0.900 ± 0.045	0.764 ± 0.063*
FLC	MIC	0.921 ± 0.192	0.917 ± 0.035	0.910 ± 0.058	0.813 ± 0.058
	10× MIC	0.909 ± 0.183	0.884 ± 0.008	0.889 ± 0.058	0.797 ± 0.051
	50× MIC	0.889 ± 0.178	0.864 ± 0.012*	0.844 ± 0.031*	0.760 ± 0.048*
KTC	MIC	0.882 ± 0.180*	0.908 ± 0.027	0.890 ± 0.055	0.792 ± 0.037
	10× MIC	0.835 ± 0.176*	0.885 ± 0.028	0.855 ± 0.045	0.783 ± 0.046*
	50× MIC	0.788 ± 0.174*	0.868 ± 0.015*	0.824 ± 0.058*	0.745 ± 0.051*
CAS	MEC	0.915 ± 0.199	0.926 ± 0.015	0.919 ± 0.058	0.806 ± 0.051
	10× MEC	0.891 ± 0.205*	0.911 ± 0.012	0.893 ± 0.053	0.790 ± 0.050*
	50× MEC	0.863 ± 0.209*	0.897 ± 0.011*	0.859 ± 0.040*	0.771 ± 0.046*

Results are shown as means ± standard deviation of the absorbance values obtained through XTT metabolic assay at a wavelength of 492 nm. AMB, amphotericin B; CAS, caspofungin; FLC, fluconazole; KTC, ketoconazole; ITC, itraconazole; MEC, minimum effective concentration; MIC, minimum inhibitory concentration; VRC, voriconazole.

*Significant reduction compared to drug-free growth control ($P < .05$).

The tested drug concentrations varied according to the strain, considering the obtained antifungal MIC values were a strain-specific feature. For those strains against which antifungal MICs were not found, the highest drug concentrations tested in the susceptibility assays were used as the MIC (ITC, VRC, and CAS = 16 μg/ml; FLC = 64 μg/ml).

.05) were observed for all concentrations of ITC, at 10× MIC and 50× MIC for KTC and CAS and only at 50× MIC for AMB, VRC, and FLC. The *in vitro* metabolic activity of *S. schenckii* species complex biofilms, after exposure to AMB, ITC, VRC, FLC, KTC, and CAS are shown in Table 3.

As for the effects of antifungal drugs on biofilm biomass of *S. brasiliensis*, significant reductions ($P < .05$) were observed at all tested concentrations of FLC and CAS, at 10× MIC and 50× MIC of AMB and at 50× MIC of ITC and KTC. For *S. schenckii sensu stricto*, significant biomass reduction ($P < .05$) were only found at 50× MIC for AMB, ITC, and CAS, while for *S. globosa*, all concentrations of at ITC and 50× MIC of the other tested drugs induced significant biomass reductions ($P < .05$). Concerning *S. Mexicana*, significant biomass reductions ($P < .05$) were observed at all tested concentrations of ITC, VRC, and KTC and at 50× MIC for AMB and CAS. The *in vitro* biomass quantification of *S. schenckii* species complex biofilms after exposure to AMB, ITC, FLC, KTC, and CAS are shown in Table 4.

Discussion

Our results show that species of *Sporothrix schenckii* complex have the ability to form biofilms in their filamentous saprophytic phase. Biofilm formation by *Sporothrix* spp. has previously been described[22]; however, it was only evaluated until 48 h of incubation, showing a progressive increase in biofilm biomass. In the present study, a longer period of incubation (144 h) for biofilm formation of *Sporothrix schenckii* species complex was evaluated, demonstrating that complete maturation is achieved at 120 h of incubation, not at 48 h, as described previously.[22] Other researchers have reported that several other fungi have this ability of form biofilm, including yeasts of the genus *Candida* spp.,[12,23] filamentous fungi of the genera *Aspergillus* spp.[24] and *Fusarium* spp.,[25] and dimorphic fungi of the genera *Coccidioides* spp.,[26] *Histoplasma* spp.,[16] and *Paracoccidioides* spp.[27]

The biofilms studied here presented the best growth rate, when using a fungal inoculum of 2×10^5 conidia/ml. Less concentrated inoculum (2×10^4 conidia/ml) led to an inefficient cell adhesion, after 24 h, resulting in a small amount

Table 4. Biomass of *Sporothrix schenckii* complex biofilms, after drug exposure to antifungal drugs.

Antifungal drug		Species			
		Sporothrix brasiliensis ($n = 10$)	*Sporothrix schenckii sensu sensu stricto* ($n = 2$)	*Sporothrix globosa* ($n = 2$)	*Sporothrix mexicana* ($n = 4$)
Control		2.180 ± 0.195	2.252 ± 0.032	2.498 ± 0.057	2.133 ± 0.055
	MIC	2.163 ± 0.175	2.222 ± 0.019	2.500 ± 0.056	2.126 ± 0.044
AMB	10× MIC	2.111 ± 0.162*	2.190 ± 0.027	2.457 ± 0.041	2.113 ± 0.036
	50× MIC	2.059 ± 0.149*	2.159 ± 0.024*	2.418 ± 0.040*	2.086 ± 0.044*
	MIC	2.742 ± 0.450	3.130 ± 0.552	1.071 ± 0.257*	1.369 ± 0.124*
ITC	10× MIC	2.362 ± 0.167	2.796 ± 0.829	0.983 ± 0.202*	0.834 ± 0.121*
	50× MIC	1.190 ± 0.377*	1.740 ± 0.066*	0.847 ± 0.186*	0.636 ± 0.002*
	MIC	2.191 ± 0,167	2.253 ± 0.022	2.607 ± 0,057	2.108 ± 0.052*
VRC	10× MIC	2.189 ± 0,168	2.225 ± 0,025	2.566 ± 0.067	2.083 ± 0.058*
	50× MIC	2.183 ± 0,162	2.193 ± 0,022	2.528 ± 0,060*	2.064 ± 0.051*
	MIC	2.163 ± 0,198*	2.268 ± 0,035	2.511 ± 0,032	2.133 ± 0.056
FLC	10× MIC	2.151 ± 0,197*	2.261 ± 0,030	2.436 ± 0,028	2.125 ± 0.055
	50× MIC	2.131 ± 0,195*	2.230 ± 0,036	2.402 ± 0,015*	2.115 ± 0.052
	MIC	2.173 ± 0,198	2.246 ± 0,035	2.444 ± 0,051	2.107 ± 0.051*
KTC	10× MIC	2.170 ± 0,197	2.194 ± 0,056	2.384 ± 0,022	2.079 ± 0.051*
	50× MIC	2.105 ± 0,197*	2.156 ± 0,055	2.348 ± 0,029*	2.053 ± 0.052*
	MEC	2.164 ± 0,195*	2.247 ± 0,007	2.479 ± 0,068	2.134 ± 0.069
CAS	10× MEC	2.140 ± 0,201*	2.228 ± 0,013	2.469 ± 0,067	2.121 ± 0.069
	50× MEC	2.120 ± 0,203*	2.212 ± 0,008*	2.431 ± 0,054*	2.106 ± 0.067*

Results are shown as means ± standard deviation of the absorbance values obtained through crystal violet assay at a wavelength of 540 nm. AMB, amphotericin B; CAS, caspofungin; FLC, fluconazole; KTC, ketoconazole; ITC, itraconazole; MEC, minimum effective concentration; MIC, minimum inhibitory concentration; VRC, voriconazole.

*Significant reduction compared to drug-free growth control ($P < .05$).

The tested drug concentrations varied according to the strain, considering the obtained antifungal MIC values were a strain-specific feature. For those strains against which antifungal MICs were not found, the highest drug concentrations tested in the susceptibility assays were used as the MIC (ITC, VRC, and CAS = 16 μg/ml; FLC = 64 μg/ml).

of adhered fungal cells; hence, biofilms did not form a characteristic hyphal network, even after several days of incubation. When using the concentration of 2×10^6 conidia/ml, the growth was equivalent to the immediately lower inoculum concentration (2×10^5 conidia/ml), with similar amount of adhered cells and similar biofilm structure, as revealed by scanning electron microscopy.

Like other microbial biofilms, the *in vitro* growth of *S. schenckii* species complex biofilms included a phase of surface adhesion, for 24 h of incubation, followed by cell multiplication and biofilm maturation. *Sporothrix* biofilms presented a slow growth rate, as demonstrated by biomass and biofilm metabolic activity throughout the incubation period, when compared with other microbial biofilms. Growth peaked at 120 h of incubation and then maintained biomass and/or metabolic rated that were similar to those found by other researchers for *Paracoccidioides brasiliensis*[27] and *Histoplasma capsulatum*.[16]

In the present study, *Sporothrix* spp. produced structurally complex biofilms, as shown by their heterogeneity, density, and hyphal network, associated with the presence of extracellular polymeric matrix and water channels, which were similar to the *Sporothrix* biofilms described by Sánchez-Herrera et al.[22] The hyphal network is important for the structural maintenance of the biofilm architecture, while the water channels are essential to warrant nutrient transport to the biofilm cells and the removal of cell metabolites from the biofilm structure.

To investigate the antifungal activity against biofilms of *S. schenckii* species complex, it was initially necessary to determine the planktonic antifungal MICs. Our planktonic antifungal susceptibility findings are consistent with other findings by our group.[28] The MICs vary according to the antifungal agent and species studied. In the present study, for instance, it was observed that AMB, CAS, and KTC have higher MIC and MEC values against planktonic *S. mexicana* than the other species. This finding corroborates other authors, who have reported higher tolerance of this species to antifungals.[8, 11] The MEC values found for caspofungin against planktonic cells (8– > 16 μg/ml) in our study corroborate other studies that demonstrated the susceptibility of *S. brasiliensis* and *S. schenckii sensu stricto* to this drug.[29]

The metabolic activity and biomass of mature biofilms after exposure to antifungal agents indicate differences in antifungal susceptibility between species of the *S. schenckii* complex, similar to what was described for planktonic cells. Antibiofilm activity was observed after exposing biofilms to the planktonic MICs for some drugs, but this activity was more pronounced at 50× MIC. It is worth noting that a similar finding was observed by our group with biofilms of the dimorphic fungus *H. capsulatum* var. *capsulatum*.[16] These findings for biofilms of dimorphic fungi, including *Sporothrix* spp., corroborate researches with *Candida albicans* biofilms, demonstrating that these structures are associated with decreased susceptibility to antifungal drugs.[30]

In conclusion, species of the *Sporothrix schenckii* complex have the ability to form well-structured biofilms *in vitro*, which are less susceptible to antifungal agents than fungal planktonic growth, thus demonstrating protective properties of growing in biofilm arrangements.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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