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Original article

Identification of proteins in *Sporothrix schenckii sensu stricto* in response to oxidative stress induced by hydrogen peroxide

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ABSTRACT

Background: Sporotrichosis is a fungal infection caused by the *Sporothrix schenckii* complex. In order to colonize the host, the pathogen must neutralize the reactive oxygen species produced by the phagocytic cells during the respiratory burst. Little is known about these mechanisms in *S. schenckii*.
Aims: To identify the proteins differentially expressed after the exposure of *S. schenckii sensu stricto* to different concentrations of H_2O_2.
Methods: Yeast cells of *S. schenckii sensu stricto* were exposed to increasing concentrations of H_2O_2. Proteins differentially expressed in response to oxidative stress were analyzed using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and identified by MALDI-MS/MS. RT-PCR assays were performed to evaluate the transcription of genes of the identified proteins.
Results: Concentrations of H_2O_2 as high as 800 mM allowed cell growth, and 200 mM and 400 mM were selected for comparative analysis by 2D-PAGE. This analysis revealed at least five differentially expressed proteins, which were identified as heat shock 70 kDa protein (Hsp70), chaperonin GroEL, elongation factor 1-β (EF1-β), a hypothetical protein, and mitochondrial peroxiredoxin (Prx1). RT-PCR revealed that the transcription of the genes coding for some of these proteins are differentially regulated.
Conclusions: Based on these results, it is proposed that these proteins may be involved in the resistance of *S. schenckii* to oxidative stress, and play an important role in the fungus survival in the host.

Identificación de proteínas en *Sporothrix schenckii sensu stricto* en respuesta al estrés oxidativo inducido por peróxido de hidrógeno

Palabras clave:
Sporothrix schenckii
H_2O_2
Estrés oxidativo
Especies reactivas del oxígeno

RESUMEN

Antecedentes: La esporotricosis es una infección fúngica causada por el complejo *Sporothrix schenckii*. Para colonizar al huésped, los patógenos deben neutralizar las especies reactivas de oxígeno producidas por las células fagocíticas durante el estallido respiratorio. Poco se conoce sobre este mecanismo en *S. schenckii*.
Objetivos: Identificar proteínas diferencialmente expresadas durante la exposición de *S. schenckii sensu stricto* a diferentes concentraciones de H_2O_2.
Métodos: Levaduras de *S. schenckii sensu stricto* fueron expuestas a concentraciones crecientes de H_2O_2. Las proteínas diferencialmente expresadas en respuesta el estrés oxidativo fueron analizadas mediante electroforesis en geles de poliacrilamida en doble dimensión (2D-PAGE) e identificadas por MALDI-MS/MS. Se realizaron ensayos de RT-PCR para evaluar la transcripción de genes de las proteínas identificadas.

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Resultados: Concentraciones altas de H_2O_2 (800 mM) permitieron el crecimiento celular, y se seleccionaron las concentraciones de 200 y 400 mM para el análisis comparativo mediante 2D-PAGE. Este análisis reveló al menos cinco proteínas diferencialmente expresadas, identificadas como proteína de choque térmico de 70 kDa (Hsp70), chaperonina GroEL, factor de alargamiento 1-β (EF1-β), una proteína hipotética y peroxirredoxina mitocondrial (Prx1). La RT-PCR reveló que la transcripción de los genes que codifican para algunas de estas proteínas se regula diferencialmente.
Conclusiones: Con estos resultados pensamos que estas proteínas podrían estar involucradas en la resistencia de *S. schenckii sensu stricto* al estrés oxidativo y jugar un papel importante en la supervivencia del hongo en el huésped.

Sporotrichosis is a subcutaneous mycosis caused by the *Sporothrix schenckii* complex, a thermodimorphic fungus which is endemic in tropical and subtropical areas of Latin America. This disease has been reported as endemic because of the widespread use of immunosuppressive therapy, cancer, alcoholism and the incidence of acquired immunodeficiency syndrome (AIDS).[17] Phenotypic and molecular studies have shown that *S. schenckii* is a complex of different cryptic species, some of which are considered of medical importance, such as *S. schenckii sensu stricto*, *Sporothrix brasiliensis*, *Sporothrix globosa*, *Sporothrix mexicana*, *Sporothrix luriei* and *Sporothrix pallida*.[2,17–19]

As other pathogens, the first barrier that *Sporothrix* must overcome to colonize its host is the immune system itself. Upon interaction with pathogens, phagocytes produce reactive oxygen species (ROS) during the oxidative burst, which are essential components of the immune response against the invading microorganisms.[4,27] ROS are mainly formed in the mitochondrial electron transport chain by the partial reduction of O_2, generating superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$). Pathogens have developed mechanisms involving enzymatic and non-enzymatic systems that enable them to detoxify ROS and evade phagocytic cells.[27] After the phagocytosis of *S. schenckii*, monocytes and macrophages are strongly induced to produce ROS.[25] It has been shown that in this organism, the superoxide radical has fungistatic and fungicidal activity, and its absence is associated with higher mortality in experimental mouse infections.[14] Despite the relevance of this anti-oxidant activity, there are few studies dealing with the responses of *Sporothrix* to oxidative stress (OS).[22] It is well documented that the response to OS (OSR) depends on the phase growth of the pathogen as it has been demonstrated that exponentially growing cells are more susceptible than those in the stationary phase of growth.[6,8,21,31] A recent work in this laboratory has focused on the mechanisms used by *S. schenckii sensu stricto* to respond to OS in the stationary phase of growth. This phase is of special interest as cells remain in quiescent state and very much emulate their lifestyle in nature.[21,32] Quiescent cells represent almost 60% of earth biomass and they can survive for long periods of time, sometimes years, in the absence of nutrients, a feat of astonishing resilience.[11] It has been reported that in pathogenic organisms such as some *Candida* species, the stationary phase favours colonization of the human host where they remain quiescent until optimal nutrient conditions exist to infect and invade it. *S. schenckii* exhibits a similar behaviour. While in some *Candida* species some proteins involved in the OSR have been identified,[7,23] the same is not true for *S. schenckii* complex.

On this background, we considered it relevant to identify proteins potentially involved in the OSR following exposure of stationary phase yeast cells of *S. schenckii sensu strictu* to H_2O_2. We identified at least five proteins that were up- or down-regulated after exposing the pathogen to increasing concentrations of H_2O_2. RT-PCR revealed that transcription of genes coding for some of these proteins are differentially regulated. We propose that these proteins are involved in the mechanisms of resistance against ROS and may contribute to the survival of the fungus in the host.

Material and methods

Strain and culture conditions

Throughout this study, the *S. schenckii* ATCC 58251 strain was used. To obtain the yeast morphotype cells in the stationary-phase, 500-ml Erlenmeyer flasks containing 200 ml of brain heart infusion medium (BHI, Difco) were inoculated with 5×10^5 conidia·ml^{-1} and incubated at 37 °C for 8 days on a rotary shaker at 120 rpm. Yeast cells were harvested by centrifugation at 7000 × *g* for 10 min and used for the assays described below.

Assays of H_2O_2 susceptibility

S. schenckii was grown for 8 days at 37 °C. To assay H_2O_2 susceptibility, yeast cells were diluted in fresh BHI medium to an OD_{600nm} of 0.5 in sterile deionized water. The cultures were divided in equal parts, exposed to different H_2O_2 concentrations (0 to 1000 mM) and shaken (120 rpm) at 37 °C. After 60 min, an aliquot was taken from the cultures treated with the oxidant, adjusted to an OD_{600nm} of 0.5 and used to prepare exponential dilutions in 96-well boxes. Each dilution was spotted onto YPG plates (0.3% yeast extract, 1% peptone, and 2% glucose) and incubated at 37 °C for 48 h. The experiments were carried out thrice. These protocols were adapted from previous studies with other organisms.[6,7]

Extraction of proteins

Non-exposed and H_2O_2-exposed cell cultures were centrifuged at 13,000 × *g* at 4 °C for 15 min and the supernatant was carefully discarded. The cell pellet was washed thrice with washing buffer [200 mM Tris–HCl, pH 8.5, 1 mM phenylmethylsulfonyl fluoride (PMSF)] by centrifugation at 7000 × *g* at 4 °C for 10 min. Washed cells were resuspended in lysis buffer (200 mM Tris–HCl, pH 8.5, 1 mM PMSF, 200 mM NaCl, 0.5% SDS, 25 mM EDTA) and broken with glass beads (0.45–0.5 mm in diameter) by alternate periods of breaking (40 s) and cooling (60 s) until all cells were broken. The cell homogenate was centrifuged at 7000 × *g* at 4 °C for 10 min. The supernatant was carefully aspirated with a Pasteur pipette and the cell pellet was discarded. Proteins in the supernatant were precipitated with 70% (v/v) ethanol at −20 °C for 3 h and stored at −70 °C until further use. Protein concentration was determined by the DC method (Bio-Rad) using bovine serum albumin (BSA) as standard.

2D-PAGE and image analysis

2D-PAGE was performed using 7-cm strips with an immobilized 4–7 pH gradient (Bio-Rad) as described by Ruiz-Baca et al.[26] Briefly, samples containing 80 μg of total protein were cleaned using the cleaning kit from Bio-Rad (2D Clean-Up Kit) as described above and resuspended in 140 μl of rehydration buffer [7 M urea, 2 M thiourea, 4% CHAPS, 50 mM dithiothreitol (DTT), 0.2% ampholytes (Biolyte 3/10) and 0.001% bromophenol blue]. After rehydration for 16 h, the strips were subjected to isoelectric focusing with voltage gradients of 0 to 250 V for 15 min, 250 to 4000 V for 2 h, and 4000 V to completion at 8500 V/h. After isoelectric focusing, the strips were incubated sequentially for 15 min in equilibrium buffer I (0.375 mM Tris/HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS and 0.5% DTT) and equilibrium buffer II (0.375 mM Tris/HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS and 2% iodoacetamide) under constant stirring. For the second dimension, the strips were placed on top of a 10% SDS-PAGE gel and covered with a 0.5% agarose overlay. The proteins were separated at 95 V for 2 h in a Mini-Protean 3 system (Bio-Rad) and then stained with colloidal Coomassie blue. Images were captured with a ChemiDocTM XRS + System (Bio-Rad) and analyzed with a PDQuest™ 2-D (Bio-Rad) software. Comparisons of the gels were done using a synthetic image containing all the protein spots of analyzed gels. The intensity of the spots was normalized and validated in the master gel. A spot was considered relevant when there was a minimum of a two-fold difference in its intensity as compared with the corresponding spot obtained from the oxidant-untreated sample.

Protein identification

Spots of interest were manually excised from 2D electrophoresis gels. The gel pieces were destained and enzymatically digested according to the modified protocol of Shevchenko et al.[30] Resulting tryptic peptides were concentrated to an approximate volume of 10 μl. Nine μl of this sample were loaded onto a ChromXP Trap Column C18-CL precolumn (Eksigent, Redwood City CA); 350 μm × 0.5 mm, 120 A° pore size, 3 μm particle size and desalted with 0.1% trifluoroacetic acid (TFA) in H_2O at a flow rate of 5 μl/min during 10 min. Then, peptides were loaded and separated on a Waters BEH130 C18 column (Waters, Milford, MA); 100 μm × 100 mm, 130 A° pore size, 1.7 μm particle size, using a HPLC Ekspert nanoLC 425 (Eksigent, Redwood City CA) with 0.1% TFA in H_2O and 0.1% TFA in acetonitrile (ACN) as mobile phases A and B, respectively, under the following lineal gradient: 0–3 min 10% B (90% A), 60 min 60% B (40% A), 61–64 min 90% B (10% A), 65 to 90 min 10% B (90% A) at a flow rate of 250 nl/min. Eluted fractions were automatically mixed with a solution of 2 mg/ml of α-cyano-4-hydroxycinnamic acid (CHCA) in 0.1%TFA and 50% ACN as a matrix, spotted in a stainless steel plate of 384 spots using a MALDI Ekspot (Eksigent, Redwood City CA) with a spotting velocity of 20 s per spot at a matrix flow rate of 1.6 μl/min. The spots generated were analyzed by a MALDI-TOF/TOF 4800 Plus mass spectrometer (ABSciex, Framingham MA). Each MS Spectrum was acquired by accumulating 1000 shots in a mass range of 850–4000 Da with a laser intensity of 3700. The 100 more intense ions with a minimum signal-noise of 20 were programmed to fragmenting. The MS/MS spectra were obtained by fragmentation of selected precursor ions using collision induced dissociation and acquired by 3000 shots with a laser intensity of 4400. Generated MS/MS spectra were compared using a Protein Pilot software v. 2.0.1 (ABSciex, Framingham, MA) against *S. schenckii* strain ATCC 58251 and 1099-18 database (downloaded from Uniprot; 8673 and 10292 protein sequences, respectively) using Paragon algorithm. The search parameters were carbamidomethylated cysteine, trypsin as a cut enzyme, all the biological modifications and amino acid substitution set by the algorithm, as well as phosphorylation emphasis and Gel-based ID as special factors. The detection threshold was considered in 1.3 to acquire 95% of confidence. The identified proteins were grouped by ProGroup algorithm in the software to minimize redundancy.

Reverse transcription-PCR (RT-PCR)

Total RNA from *Sporothrix* cells was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's instructions, and DNase I (Invitrogen) was used to eliminate DNA contamination. Synthesis of cDNA and PCR were carried out as described elsewhere using the ImProm-II™ Reverse Transcription System (Promega). The expression levels of *prx1* and *hsp70* genes were normalized by β-tubulin gene (*tub*). The RT primers used for each gene are shown in Table 1. Synthesis of cDNA was carried out at 42 °C for *prx1*, *hsp70* and *tub* genes and PCR was performed at 58 °C for the three analyzed genes. PCR amplification of DNase-treated RNA was performed without the reverse-transcription procedure (No-RT). Lack of amplification of PCR products confirmed the complete elimination of DNA from RNA samples. The PCR products were visualized on agarose gel stained with ethidium bromide. The quantification of PCR products was carried out using an ImageJ1.51j8 software (Wayne Rasband, National Institutes of Health, USA).

Statistical analysis

The quantification of PCR products were analyzed by a single-factor ANOVA in a completely randomized design with three levels of peroxide concentration. Then, a Tukey post hoc multiple comparison of means with a 95% family-wise confidence level was performed. A $p < 0.05$ was considered statistically significant. These analyses were performed using *R*, a programming language for statistical computing.[24]

Table 1
Proteins separated on 2D-PAGE gels and analyzed by MALDI-MS/MS.

Spot	Protein name	Mass (kDa)	pI	Peptide matching	Sequence coverage (%)	Expression	Function
1	Heat shock 70kDa protein	69	4.8	25	37.7	Upregulated/ Downregulated	Interaction with the host, Response to stress
2	Chaperonin GroEL	61	5.4	44	59.6	Upregulated/ Downregulated	Folding, assembly, and translocation of proteins
3	Hypotethical protein	88	5.9	4	2.4	Downregulated	Unknown
4	Elongation factor 1-β	26	4.3	6	30.6	Upregulated/ Downregulated	Protein biosyntesis
5	Mitochondrial peroxiredoxin-1	25	5.6	10	32.9	Upregulated	Response to stress

Mass spectra were analyzed with Protein Pilot software v. 2.0.1 (ABSciex, Framingham MA) against *S. schenckii*. Protein name: protein name as deduced by comparing peptide sequences via the software BLAST. Molecular weight (kDa): theoretical molecular mass predicted from the amino acid sequence of the identified protein. pI: theoretical isoelectric point predicted from the amino acid sequence of the identified protein. Sequence coverage: coverage of the amino acid sequence of the identified protein. Peptide matching: number of matched peptides based on MS/MS data searching, excluding the duplicate matches.

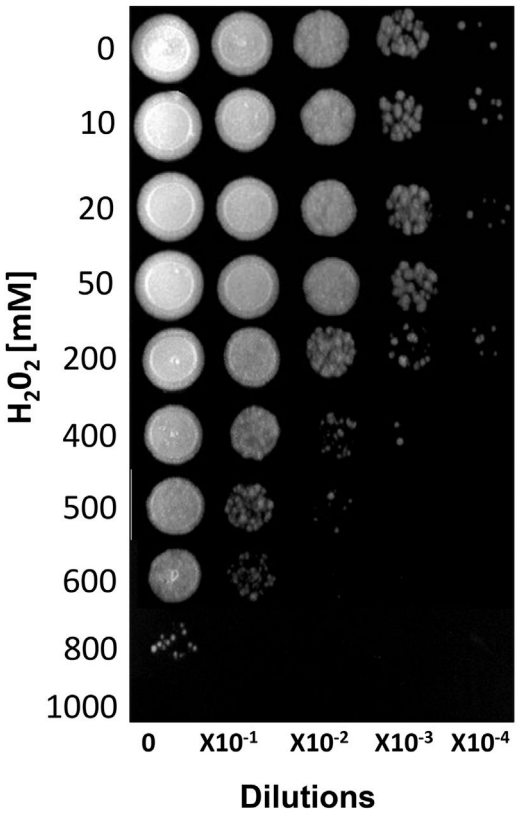

Fig. 1. Susceptibility of *S. schenckii sensu stricto* to H_2O_2. Cultures of stationary-phase yeast cells (OD_{600nm} 0.5) were incubated under constant stirring in the presence of the indicated concentrations of H_2O_2 at 37 °C. Samples of these suspensions were exponentially diluted in 96-well plates and each dilution was spotted onto YPG plates that were incubated at 37 °C. Growth was inspected after 48 h.

Results

Susceptibility to H_2O_2

To increase our knowledge of the resistance mechanisms of *S. schenckii sensu stricto*, we considered it relevant to identify some proteins presumptively involved in OSR. To this purpose, stationary-phase cultures of yeast cells of the fungus were exposed to increasing concentrations of H_2O_2 (Fig. 1). Fungal growth was not affected when cell dilutions were exposed to concentrations between 0 and 50 mM of H_2O_2. At 200 mM, inhibition was observed at a cell dilution of 1×10^{-2}. At 400 mM and at a dilution of 1×10^{-1}, fungal growth decreased and was fully inhibited at all higher cell dilutions with no significant differences at 500 mM. At 600 mM, growth decreased at dilution 0, with poor growth at 800 mM and full inhibition at 1000 mM.

Differential expression of proteins during OSR

Based on their effect on growth, concentrations of 0, 200 and 400 mM H_2O_2 were selected to carry out a comparative analysis of the total protein expression by *S. schenckii sensu stricto*. Analysis by 2D-PAGE at different concentrations of H_2O_2 showed the presence of at least five differentially expressed proteins (Fig. 2) which were identified as a heat shock 70 kDa protein (Hsp70), chaperonin GroEL, elongation factor 1-β (EF1-β), a hypothetical protein and mitochondrial peroxiredoxin (Prx1) (Table 1). Accordingly, an upregulated expression of Hsp70 and Prx1 was observed (Fig. 2; spots 1 and 5, respectively). Other proteins that were either up- or downregulated were chaperonin GroEL and EF-1β (Fig. 2; spots 2 and 4, respectively). Also, a downregulated expression of a hypothetical protein was observed in response to OS (Fig. 2; spot 3).

Analysis of transcriptional expression

To determine whether the regulation of identified proteins occurred at the transcriptional level, we evaluated the expression of genes encoding Prx1 and Hsp70. To this purpose, it was important to assess the quality of the RNA obtained from *S. schenckii sensu stricto* as it has been reported that ROS can damage nucleic acids.[4] It was observed that the ribosomal RNA bands were well defined indicating that it was suitable for analysis (Fig. 3A). Following exposure to H_2O_2, significant differences were found in the expression of the *prx1* and *hsp70* genes at 200 and 400 mM H_2O_2 ($p < 0.05$) as compared with the control. On the other hand, *prx1* showed a significant increase at 400 mM H_2O_2 compared with 200 mM H_2O_2 ($p < 0.05$), while *hsp70* showed a significant decrease at 400 Mm H_2O_2 compared with 200 mM ($p < 0.05$) (Fig. 3B and C).

Discussion

Identification of proteins potentially involved in the resistance of *S. schenckii sensu stricto* to oxidant agents is a useful approach to understand how the fungus can protect itself from OS. Here, stationary-phase yeast cells of the fungus were subjected to OS with different concentrations of H_2O_2. This oxidant started to affect the growth of *S. schenckii* at 200–400 mM, the inhibition increased at 600 mM and no cells remain viable at 1000 mM. In other pathogenic fungi such as *Paracoccidioides brasiliensis* and *Candida* species, the

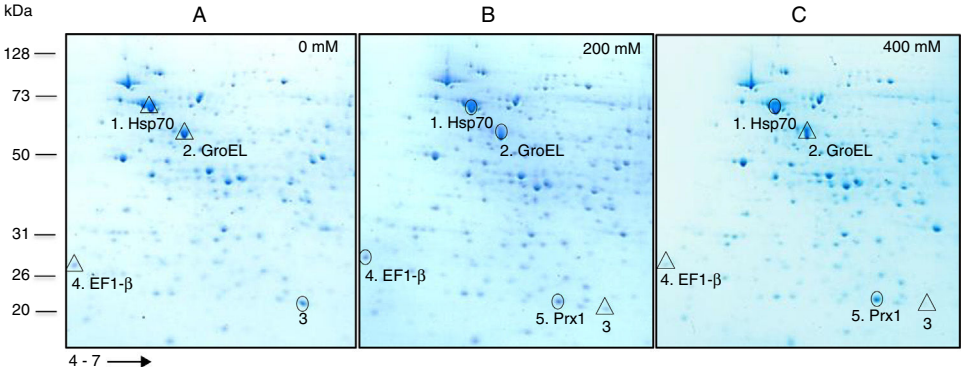

Fig. 2. Analysis of total protein extracts from yeast cells of *S. schenckii sensu stricto* by 2D-PAGE in a pH 4–7 gradient in gels stained with colloidal Coomassie Blue, after exposure to 0 (A), 200 (B) and 400 (C) mM H_2O_2. Spots marked with circles and triangles correspond to up- and down-regulated proteins, respectively, with respect to the reference condition. kDa, kilodalton.

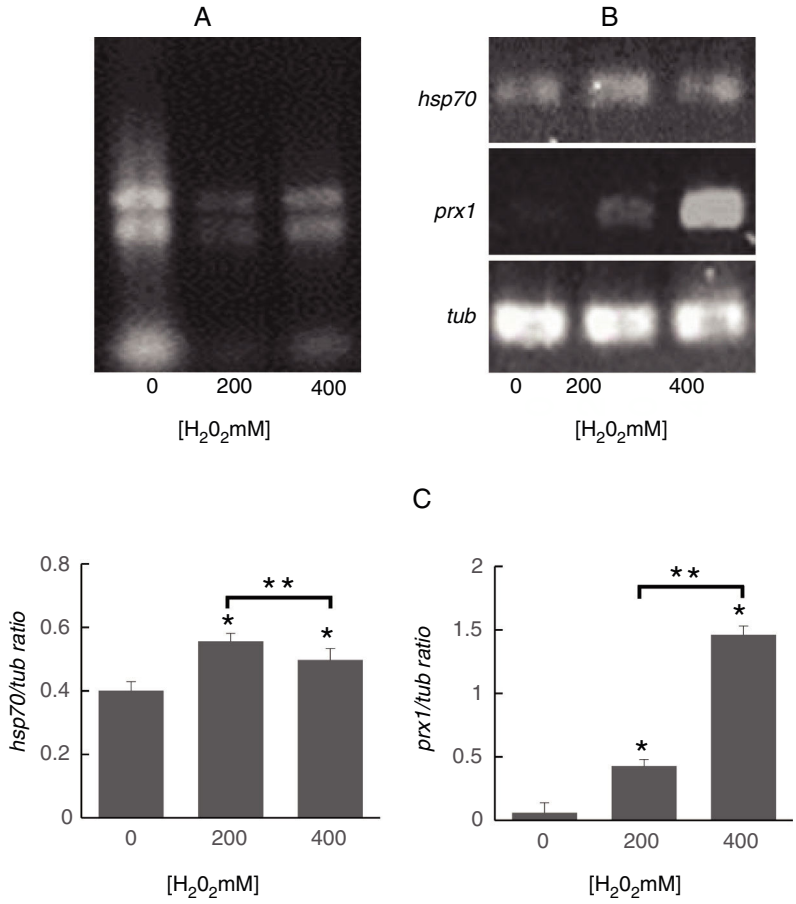

Fig. 3. Analysis of *prx1* and *hsp70* gene expression by RT-PCR. (A) Total RNA of *S. schenckii sensu stricto* exposed to the indicated concentrations of H_2O_2. (B) RT-PCR products from the analysis of *hsp70*, *prx1* and *tub* gene expression in *S. schenckii sensu stricto*. (C) Densitometry of the bands was measured using the ImageJ 1.51j8 software. The quantification of prx1 and hsp70 was perfomed. Data are presented as the mean ± SD, $n = 3$. *Significant differences as compared to control ($p < 0.05$). **Significant differences between concentrations of H_2O_2 ($p < 0.05$).

response to oxidative stress in the growth phase was different.[1,8,23] Yeast cells of *P. brasiliensies* in the stationary phase were more resistant to H_2O_2 than their counterparts in the exponential phase after exposure to 0–100 mM H_2O_2. The survival rates of cells from both phases exceeded 70% with all tested concentrations of H_2O_2.[8] In contrast, species of the *Candida* genus in the stationary phase of growth reveals that *C. albicans* is more susceptible to H_2O_2 as its growth was completely inhibited at 300 mM. Other species such as *C. glabrata* and *C. parapsilosis* survived at 500 mM, while *C. krusei* did so at 1500 mM.[23] Resistance of *Candida* species to H_2O_2 has also been observed by other authors,[1] thus reinforcing the notion that pathogenic organisms have developed mechanisms to detoxify H_2O_2 that enable them to evade the immune system and colonize the host.

2D-PAGE of extracts from *S. schenckii sensu stricto* yeast cells exposed to two concentrations of H_2O_2 showed the presence of at least five differentially expressed proteins. These were identified as Hsp70, chaperonin GroEL, a hypothetical protein, EF-1β and mitochondrial Prx1 (Fig. 2, spots 1–5, respectively. See also Table 1). Fungal Hsp70 has been implicated in various cellular functions, including OS, cell adhesion, macrophage activation, receptor expression and macromolecule internalization.[15,28] Studies in species of *Candida*,[7] *P. brasiliensies*[9] and *Cryptococcus neoformans*[25] suggest an important role of Hsp70 in the interaction with host cells and in the response to OS. On the other hand, the chaperonin GroEL in bacteria is required for the proper folding of many proteins. Studies indicate that these chaperones are overexpressed under conditions of cellular stress, including heat, so they are considered as heat shock proteins[5]. On its part, EF-1β plays an important role in protein synthesis. Accordingly, McGoldrick et al.[20] reported 67 sequences of glutathione transferase-like proteins encoded in 21 fungal species, some related to EF1-β, suggesting their involvement in the ROS. Also, the differentiated expression of EF1-β was noted in *Candida* species exposed to H_2O_2 and menadione, reinforcing its role in the stress response, perhaps as a modulator of transcription of other proteins.[23]

Another protein identified here was a mitochondrial Prx1. Fungal peroxiredoxins are ubiquitous enzymes that protect cells against OS.[12,29] An *in silico* analysis by Ortega et al.[22] demonstrated that both *S. brasiliensis* and *S. schenckii sensu stricto* have four putative peroxiredoxins in their genome, but to date it is unknown which of these genes are modulated by H_2O_2. In accordance with our results, a proteomic analysis in *Aspergillus fumigatus* revealed the differential expression of 28 proteins, including Prx1, which increased their concentration after 45 min of exposure to H_2O_2.[16] In the same line, Kusch et al.[15] exposed *C. albicans* to two oxidizing agents, H_2O_2 and diamide, and observed the differential expression of 52 proteins, including Ipf2431p, which is homologous to Tsa1p in *S. cerevisiae* where it plays the role of a peroxiredoxin. Another study in *Candida* species exposed to H_2O_2 and menadione revealed the differential expression of 15 moonlight-like cell wall proteins including Tsa1p,[23] while in *S. cerevisiae* it was proposed that Prx1 acts as a redox signalling molecule that oxidizes Trx3. At high concentration oxidized Trx3 can produce apoptosis,

indicating that when Prx1 is unable to detoxify reactive mitochondrial oxygen species it induces apoptosis to remove the affected cells.[13] Further studies by proteomics and bioinformatic approaches about the response to OS in exponentially and stationary-phase in *Sporothrix* will be necessary to get a deeper knowledge of the defense mechanisms of this pathogen as they occur in the phagocyte.

To determine whether the regulation of the identified proteins occurred at the transcriptional level, the expression of the genes encoding Hsp70 and Prx1 was evaluated. To this purpose, it was important to assess the quality of the RNA obtained from *S. schenckii sensu stricto* as it has been reported that ROS can damage nucleic acids[4]. The ribosomal RNA bands were well defined indicating that it was suitable for analysis (Fig. 3A). Following the exposure of *S. schenckii sensu stricto* to H_2O_2, significant differences were found ($p < 0.05$) in the expression levels of *prx1* and *hsp70* genes. Thus, while a significant increase in the expression of *prx1* and *hsp70* were observed at 200 and 400 mM H_2O_2 compared with the control, a significant decrease in *hsp70* occurred at 400 mM H_2O_2 compared with 200 mM H_2O_2 (Fig. 3B and C). These results are in agreement with those obtained from the proteomic analysis carried out after the exposure of *S. schenckii sensu stricto* yeast cells to ROS (Fig. 2), therefore indicating that changes observed for the proteins identified in this study are regulated at the transcriptional level.

Comparative studies of the virulence of *S. schenckii* complex demonstrated that *S. brasiliensis* was the most virulent followed by *S. schenckii sensu stricto*, *S. globosa*, *S. mexicana* y *S. pallida*.[3] Fernandes et al.[10] evaluated the secretion profiles of proteins from different isolates of *S. schenckii sensu stricto*, *S. brasiliensis* and *S. globosa*, and reported the expression of different proteins. They also observed that the humoral response of animals infected with these species was different. The most virulent isolates shared two common antigens of 60 kDa and 110 kDa, which are likely to be involved in virulence. In the same line, Ortega et al.[22] observed that *S. brasiliensis* was more resistant to stress by peroxides as compared with *S. schenckii sensu stricto*, suggesting that the *S. schenckii* species exhibit different strategies for adaptation, depending on the route of infection, a phenomenon most likely related with virulence.

Conclusions

Based on the results presented in this study, we propose that the identified proteins are likely to be involved in the resistance of this fungus against OS, helping the pathogen to evade the host phagocytic cells, reach the blood stream and cause an invasive mycosis.

Conflict of interests

The authors declare that there were no conflicts of interest with any organization or entity with a financial interest, or financial conflict with the material discussed in this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.riam.2018.10.004.

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