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HUGO FELIX PERINI

SWITCHING FENOTÍPICO EM *Candida tropicalis*: ALTERAÇÕES NA RESPOSTA CELULAR E FAGOCITOSE FRENTE A ESTRESSES OXIDATIVO E OSMÓTICO

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Tese apresentada à Universidade Estadual de Londrina - UEL, como requisito final à obtenção do título de Doutor em Microbiologia.

Orientadora: Profa. Dra. Márcia Cristina Furlaneto

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- Paulo Freire

PERINI, Hugo Felix. ***Switching* fenotípico em *Candida tropicalis*:** Alterações na resposta celular e fagocitose frente a estresses oxidativo e osmótico. 2021. 127 f. Tese de Doutorado (Programa de Pós-Graduação em Microbiologia) – Universidade Estadual de Londrina, Londrina, 2021.

RESUMO

Switching fenotípico em *Candida tropicalis* promove alterações na manifestação de virulência e perfil de sensibilidade a antimicrobianos. Uma vez que o evento pode estar relacionado com alterações na resposta adaptativa, o objetivo do presente trabalho foi availiar o papel do *Switching* fenotípico na resposta ao estresse osmótico e oxidativo. Para tal, dois sistemas de *Switching* fenotípico de *C. tropicalis* (49.07 e 100.10) compostos de cinco morfotipos cada (Parental, Crepe, Rugoso, Revertente de Rugoso e Revertente de Crepe) foram expostos à 1 M de NaCl e 5 mM de H_2O_2 por 10 e 60 min. Análises de resitência ao estresse foram realizadas por contagem de *UFC* após exposição e o dano em membrana com ensaio de iodeto de propídio. Análises de expressão dos genes *HOG1*, *EFG1* e *WOR1* foram realizadas por qPCR e a quantidade de componentes de parede por ensaio fluorimétrico. Análizou-se também, o efeito do *Switching* sobre a fagocitose e perfil de filamentação no contato com hemócitos e macrófagos e o efeito da pré-exposição ao H_2O_2 nessas variáveis. Respostas de alterações na parede celular, expressão dos genes testados e resistência ao estresse foram sistemas-dependente, não apresentando relação direta com o padrão morfológico do morfotipo. Variantes morfológicos (Crepe e Rugoso - 100.10) apresentaram menor e complexidade celular que o Parental, assim como alterações na quantidade de manana e na porosidade da parede. Revertentes morfológicos também apresentam maior quantidade de quitina, β-glucana e porosidade que o Parental. Em resposta ao estresse, 62,5% dos morfotipos derivados de *Switching* foram mais resistentes ao choque osmótico (10 min) e 25% à osmoadaptação (60 min). Sob estresse oxidativo 37,5% apresentaram maior viabilidade que o parental após 10 min, e 25% sob estresse prolongado (60 min). O morfotipo Crepe destacou-se como morfotipo com maior capacidade de resposta aos estresses testados. Esse morfotipo apresentou menor decréscimo no volume celular sob estresse osmótico e aumento de quitina e manana na parede celular. Sob estresse prolongado *HOG1* manteve alta expressão nesse morfotipo (49.07). Sob estresse oxidativo Crepe teve alta expressão de todos os genes testados. Hemócitos e macrófagos apresentaram mesmo perfil de fagocitose. Crepe foi mais fagocitado que Parental, no entanto, houve redução no número de células do morfotipo fagocitadas após pré-exposição ao H_2O_2. Crepe apresentou alta capacidade de formação de hifas verdadeiras em co-cultivo com os fagócitos. A pré-exposição ao H_2O_2 promoveu regulação positiva de *WOR1* e *HOG1* em Crepe (49.07). Células de Crepe pré-expostas apresentaram maior quantidade de quitina (100.10) e maior porosidade (ambos os sistemas) que o parental. O presente trabalho pode concluir que *switching* fenotípico promove alteração do *fitness* celular e consequentemente resposta alterada aos estresses osmótico e oxidativo em *C. tropicalis*, refletidas na regulação gênica, alterações da parede celular e na capacidade de morfogênese.

Palavras-chave: *C. tropicalis*; *switching* fenotípico; expressão gênica; parede celular; morfogênese.

PERINI, Hugo Felix. **Phenotypic s*witching* in *Candida tropicalis:*** Changes in cell response and phagocytosis facing oxidative and osmotic stress. 2021. 127 p. PhD thesis (Postgraduate Program in Microbiology) – State University of Londrina, Londrina, 2011.

ABSTRACT

Phenotypic switching in *Candida tropicalis* promotes changes in the manifestation of virulence and antimicrobial sensitivity profile. The event may be related to changes in the adaptive response. The aim of the present work was to assess the role of phenotypic *Switching* in the response to osmotic and oxidative stress. For this purpose, two *C. tropicalis* phenotypic *Switching* systems (49.07 and 100.10) composed of five morphotypes each (Parental, Crepe, Rough, Crepe revertant and Rough revertant) were exposed to 1 M NaCl and 5 mM H_2O_2 for 10 and 60 min. Stress resistance analyzes were performed by counting CFU after exposure and membrane damage with propidium iodide assay. Expression analyzes of *HOG1, EFG1* and *WOR1* genes were performed by qPCR and the amount of cell wall components by fluorimetric assay. We also analyzed the effect of *Switching* on phagocytosis and filamentation profile in contact with hemocytes and macrophages and the effect of pre-exposure to H_2O_2 on these variables. Responses to cell wall changes, expression of tested genes and resistance to stress were system-dependent, not directly related to the morphological pattern of the morphotype. Morphological variants (Crepe and Rough - 100.10) showed lower cellular complexity than Parental, as well as changes in the amount of mannan and cell wall porosity. Morphological revertants also have greater amounts of chitin, β-glucan and porosity than Parental. In response to stress, 62.5% of the morphotypes derived from *Switching* were more resistant to osmotic shock (10 min) and 25% to osmoadaptation (60 min). Under oxidative stress 37.5% showed greater viability than the parental after 10 min, and 25% under prolonged stress (60 min). The Crepe morphotype stood out as the morphotype with the greatest capacity to respond to the stresses tested. This morphotype showed less decrease in cell volume under osmotic stress and an increase in chitin and mannan in the cell wall. Under prolonged stress HOG1 maintained high expression in this morphotype (49.07). Under oxidative stress Crepe had high expression of all tested genes. Hemocytes and macrophages showed the same phagocytosis profile. Crepe was more phagocytosed than Parental, however, there was a reduction in the number of cells of the morphotype phagocytosed after pre-exposure to H_2O_2. Crepe showed a high capacity to form true hyphae in co-culture with phagocytes. Pre-exposure to H_2O_2 promoted upregulation of *WOR1* and *HOG1* in Crepe (49.07). Pre-exposed Crepe cells had a greater amount of chitin (100.10) and greater porosity (both systems) than the Parental. The present work can conclude that phenotypic *Switching* promotes changes in cell fitness and, consequently, an altered response to osmotic and oxidative stresses in *C. tropicalis*, reflected in gene regulation, cell wall changes and in the capacity of morphogenesis.

Keywords: *Candida tropicalis*; phenotypic switching; Gene expression; Cell wall; Morphogenesis.

LISTA DE ILUSTRAÇÕES

ARTIGO 1

LISTA DE ABREVIATURAS E SIGLAS

UFC	Unidade formadora de colônia
WOR1	*White opaque regulator*
HOG1	*Mitogen-activated protein kinase*
EFG1	*Enhanced filamentous growth protein 1*
ROS	*Reactive oxygen species*
CFW	*Calcofluor white*
FITC-CONA	*FITC-Concanavaline A conjugated*
YPD	*Yeast extract – Peptone – Dextrose medium*
PI	*Propidium iodide*
RC	Revertente de Crepe
RR	Revertente de Rugoso
qPCR	*Quantitative PCR*

SUMÁRIO

1 INTRODUÇÃO

Candida tropicalis está entre as espécies mais isoladas em casos de candidemia levando a taxas de mortalidade superiores a 50 % (KONTOYIANNIS et al., 2001; COLOMBO et al., 2007). Dentre os fatores de virulência que favorecem a patogenicidade de *C. tropicalis*, *switching* fenotípico destaca-se por promover variabilidade em populações isogênicas e consequentemente vantagem adaptativa (SOLL, 2014).

Dentre os sistemas descritos de *switching* fenotípico, "*white-opaque*" é caracterizado pela alteração da morfologia de colônia de um estado branco, para um opaco, impactando na capacidade de morfogênese, alterações na virulência e na interação com células imunes (SLUTSKY et al., 1987; PUJOL et al., 2004; PORMAN et al., 2011). No entanto outros sistemas com variações na complexidade de colônias foram descritos para a espécie (MORALEZ et al., 2014).

Nesses sistemas, morfotipos derivados de *switching* apresentaram alterações na capacidade de adesão e formação de biofilme (MORALEZ et al., 2020; SOUZA et al., 2020); capacidade hemolítica (FRANÇA et al., 2011); morfogênese (MORALEZ et al., 2014); citoxicidade em células FaDu (MORALEZ et al., 2016); virulência em larvas de *Galleria mellonella* (MORALEZ et al., 2016); alteração no perfil de sensibilidade a antimicrobianos (MORALEZ et al., 2014); além de apresentar maior sobrevivência após fagocitose e indução de resposta imune alterados em *G. mellonella* (PERINI et al., 2019).

Durante o curso da infecção, além da manifestação de fatores de virulência, a adaptação a estresses impostos pelo sistema imune e a variações de nichos no corpo do hospedeiro é crucial para a sobrevivência de *C. tropicalis* (LEACH et al., 2012; BROWN et al., 2014; ENE; BROWN, 2014). Dentre as respostas desencadeadas pelo estresse oxidativo causado pela fagocitose, estão a regulação de fatores relacionados com a capacidade de formação de hifas verdadeiras e alterações na organização e biossíntese da parede celular ENJALBERT et al., 2006; DANTAS et al., 2010; PIERCE et al., 2013). Em resposta ao estresse osmótico presente em alguns micronichos, vias de sinalização de resposta ao estresse são ativadas, culminando no acúmulo

intracelular de osmólitos, que restabelecem a tensão osmótica da célula e permitem a retomada do crescimento, além do espessamento de parede celular (KUHN; KLIPP 2012; BROWN et al., 2014; GIL-BONA et al., 2015).

Uma vez que s*witching* fenotípico promove alteração da relação parasito/hospedeiro e alterações na manifestação de virulência em *C. tropicalis*. A compreensão da resposta de variantes morfológicos derivados de s*witching* à estressores pode auxiliar na melhor compreensão da patogênese causada pela espécie.

2 OBJETIVOS

2.1 GERAIS

Analisar o efeito do *switching* fenotípico sobre a resposta ao estresse osmótico e oxidativo dos sistemas 49.07 e 100.10 de *Candida tropicalis*.

2.2 ESPECÍFICOS

- Avaliar o efeito do *switching* fenotípico sobre a sensibilidade ao estresse osmótico e oxidativo bem como alterações celulares e na parede celular em resposta a essas variáveis.

- Avaliar o efeito da pré-exposição ao estresse oxidativo na interação com hemócitos e macrófagos.

- Avaliar a indução da formação de formas filamentosas mediadas por *switching* fenotípico em co-cultivo com hemócitos e macrófagos e sob pré-exposição ao estresse oxidativo.

- Avaliar a expressão mediada por *switching* de genes centrais na resposta ao estresse e transição morfológica, assim como a expressão desses reguladores em resposta ao estresse osmótico, oxidativo e em presença de hemócitos e macrófagos.

3 REVISÃO BIBLIOGRÁFICA

3.1 GÊNERO *Candida*

Infecções fúngicas são relacionadas à altas taxas de mortalidade, sendo comparável às de tuberculose e malária. Uma estimativa feita por Bongomin et al. (2017) sugere que cerca de 1,6 milhão de mortes estão relacionadas a infecções fúngicas crônicas ou agudas anualmente. Além das altas taxas de mortalidade, a carga global associada a doenças fúngicas tem um grande impacto na economia e na saúde dos países, especialmente no mundo em desenvolvimento (KAUR; CHAKRABARTI, 2017; LAMOTH et al., 2018).

Dentre os fungos considerados patógenos humanos, os membros do gênero *Candida* são os mais frequentemente isolados a partir de infecções fúngicas (CALDERONE, 2002). O gênero contempla mais de 150 espécies, e dessas, aproximadamente 65% são incapazes de crescer a uma temperatura de 37 ºC, o que impede a colonização e invasão do corpo humano. São clinicamente relevantes cerca de 20 espécies descritas de *Candida* (CALDERONE, 2002), tendo destaque: *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* e *C. krusei* (DIEKEMA et al., 2012; BASSETTI et al., 2013; MAUBON et al., 2014). A distribuição dessas espécies depende de vários fatores. Isso inclui a localização geográfica, idade da população afetada, condições socioeconômicas, níveis de urbanização e aplicação de antifúngicos (BONGOMIN et al., 2017; BOHNER et al., 2021).

As espécies de *Candida* podem se encontrar como comensais da pele humana, do trato geniturinário e gastrointestinal (KABIR; AHMAD, 2013). No entanto, a mudança comensal para patogênica pode ocorrer sob certas condições predisponentes, levando a um grande número de infecções. Isso pode variar de infecções locais da mucosa e superficiais a manifestações mais graves, como candidíase profunda e infecções da corrente sanguínea (candidemia) (KULLBERG; ARENDRUP, 2015).

O surgimento de casos de candidíase invasiva pode ser associado a intervenções médicas que incluem o uso em longo prazo de cateteres venosos centrais, diálise, nutrição parenteral, antibioticoterapia de amplo espectro, hospitalização em unidades de terapia intensiva (UTIs) e imunossupressão

terapêutica. Todos esses fatores associados aos cuidados de saúde aumentam o risco de infecção (PAPPAS et al., 2018). Significativamente, várias doenças também predispõem direta ou indiretamente os pacientes a um risco aumentado de infecções fúngicas. Isso inclui AIDS, diabetes, neutropenia, parto prematuro e imunodeficiências primárias (KULLBERG; ARENDRUP, 2015; BHATTACHARY et al., 2020). Candidíase sistêmica está associada a taxas de mortalidade de até 50% (PERLROTH et al., 2007).

Fatores de risco comuns para o desenvolvimento de candidemia incluem tratamento prolongado em unidades de terapia intensiva, cateteres venosos centrais, cirurgia gastrointestinal, politrauma, idade extrema, imunossupressão grave, neutropenia, tumores sólidos e malignidades hematológicas (KOH et al., 2008; BROWN; DENNING, et al., 2012; SPELLBERG et al., 2012; YAPAR, 2014).

C. albicans se destaca como a espécie mais prevalente como comensal e patogênica, representando até 80 % dos isolados de cadidemias humanas (CALDERONE, 2002). Embora essa espécie apresente maior prevalência, o número de infecções causadas por espécies de *Candida*, não-*C. albicans* têm aumentado significativamente nas últimas décadas (KAUFFMAN et al., 2000; MANZANO-GAYOSSO et al., 2008; RUAN; HSUEH, 2009), indicando que essas leveduras possuem mecanismos de resposta frente ao hospedeiro e estratégias que permitem o seu prevalecimento no ambiente clínico (MAUBON et al., 2014; YAPAR, 2014). Além disso várias das espécies não-*C. albicans* apresentam maior resistência às terapias antifúngicas comumente usadas (PATEL et al., 2012).

Estudos em modelos experimentais apontam diferenças na virulência entre as espécies de *Candida* (ARENDRUP et al., 2002; PRIEST; LORENZ, 2013; DABIRI et al., 2018) indicando que *C. albicans* e *C. tropicalis* são mais virulentas do que outras espécies de *Candida*. Além disso, pacientes infectados por *C. albicans* e *C. tropicalis* apresentam pior prognóstico do que aqueles infectados por outras espécies (PUIG-ASENSIO et al., 2014; FERNANDEZ-RUIZ et al., 2015)

3.2 EPIDEMIOLOGIA DE *C. tropicalis*

Dentre as espécies não-*C. albicans*, *C tropicalis* destaca-se como importante agente etiológico de doenças humanas. Essa espécie é considerada uma das espécies não-*C. albicans* mais frequentemente isoladas em culturas de sangue e urina (KAUFFMAN et al., 2000). No Brasil, de acordo com dados epidemiológicos recentes, *C. tropicalis* tem a maior prevalência do grupo de não-*C. albicans* (BOHNER et al., 2021). A espécie também é comumente isolada de pacientes com neoplasias e neutropenia (YAPAR, 2014).

A epidemiologia da espécie aponta que isolados de *C. tropicalis* estão amplamente associados a surtos hospitalares (SHIN et al., 2002; COLOMBO et al., 2006; NUCCI e COLOMBO, 2007). Nacionalmente, dados apontam maior isolamento de *C. tropicalis* em casos de candidemia que os apresentados pela América do Norte e Europa (NUCCI e COLOMBO, 2007). Em alguns estudos, candidemia causada por *C. tropicalis* tem sido associada a mortalidade superior a observada por outras espécies de *Candida* (KONTOYIANNIS et al., 2001; COLOMBO et al., 2007).

C. tropicalis também é frequentemente encontrada em pacientes internados em unidades de terapia intensiva, especialmente em pacientes que requerem cateterismo prolongado, recebendo antibióticos de amplo espectro (KAUFFMAN et al., 2000; COLOMBO et al., 2007; NUCCI; COLOMBO, 2007). As infecções por *C. tropicalis* são particularmente problemáticas em pacientes neutropênicos e com câncer, especialmente em ambientes nosocomiais (SILVA et al.,2012; TURNER; BUTLER, 2014). A propensão de *C. tropicalis* à disseminação e a alta mortalidade associada podem estar relacionadas aos fatores de virulência exibidos por esta espécie, como formação de biofilme, secreção de proteinases e dimorfismo (KRCMERY, 1999; NEGRI et al., 2010).

Após o evento de colonização, *C. tropicalis* tem capacidade de se disseminar rapidamente em hospedeiros imunocomprometidos e causar alta mortalidade (CHAI et al., 2010). Kontoyiannis et al. (2001) descrevem que infecções sistêmicas causadas por *C. tropicalis* apresentam maior persistência que às causadas por *C. albicans*, levando a uma permanência mais longa no ambiente hospitalar (KRCMERY, 1999; KONTOYIANNIS et al., 2001; EGGIMANN et al., 2003). A maior persistência de *C. tropicalis* e consequente

aumento no tempo de internação pode implicar em uma maior virulência e maior resistência aos antifúngicos comumente usados na prática clínica (EGGIMANN et al., 2003; COLOMBO et al., 2007).

Além de doenças sistêmicas, *C. tropicalis* também está relacionada com a manifestação de endocardite fúngica. Em um estudo de coorte retrospectivo em cinco hospitais realizado entre 1980 e 2015, 85% dos pacientes que apresentavam a doença estavam infectados com *Candida* spp. Em relação à distribuição das espécies, isolados de *C. albicans* foram identificados em apenas 5% dos casos, enquanto *C. tropicalis* em 21% (SICILIANO et al., 2018). Em infecções ungueais, um estudo no Brasil apontou a prevalência de 26 % de *C. tropicalis*. Esses isolados apresentaram alta resistência à antifúngicos (FIGUEIREDO et al., 2007).

3.3 ASPECTOS BIOLÓGICOS DE *CANDIDA TROPICALIS*

C. tropicalis foi originalmente isolada de um paciente com bronquite fúngica em 1910 e então nomeada como *Oidium tropicale* (CASTELLANI, 1912). É uma levedura pertencente ao filo Ascomycota, da classe Hemiascomycetes (BLANDIN et al., 2000). O filo possui uma única Ordem criada em 1960 por Kudrjavzev, denominada Saccharomycetales (KIRK et al., 2001). Esta linhagem monofilética compreende cerca de 1.000 espécies conhecidas, incluindo várias leveduras de importância médica (DIEZMANN et al., 2004).

Quando cultivadas em meio Sabouraud dextrose ágar (SDA) *C. tropicalis* apresenta colônias de coloração esbranquiçada, textura cremosa e aparência lisa. Bordas ligeiramente enrugadas também podem ser observadas a partir desse crescimento. As formas celulares são majoritariamente blastoconidiais (esféricos ou ovóides) medindo cerca de 4-8 x 5-11 µm e se encontram individualizadas, em pares ou em grupamentos (KURTZMAN et al., 2011). A espécie também apresenta capacidade de morfogênese, apresentando pseudohifas em cadeias ramificadas e hifas verdadeiras (SILVA et al., 2012).

O tamanho genômico de *C. tropicalis* é de cerca de 14,5 Mb com conteúdo de guanina-citosina de 33,1 % e cerca de 6.258 genes dispostos em 5 a 6 cromossomos (BUTLER et al., 2009; CIUREA et al., 2020). Embora a levedura se reproduza majoritariamente de forma assexuada alguns estudos

realizados apontam a capacidade de desenvolvimento de ciclo parassexual na espécie (PORMAN et al., 2011; XIE et al., 2012; SEERVAI et al., 2013).

C. tropicalis apresenta alta proximidade filogenética com *C. albicans,* estando ambas posicionadas no clado CTG (BUTLER et al., 2009). Essa relação evolutiva íntima é evidente no compartilhamento de características fenotípicas e bioquímicas entre as espécies (ZUZA-ALVES et al., 2017). O clado leva esse nome pelo códon CTG ser amplamente traduzido em serina em detrimento de leucina, como seria na maioria dos outros organismos (GOMES et al., 2007; SANTOS et al., 2011). No entanto, a tradução do CUG é ambígua e a reversão parcial da identidade serina para leucina é possível (GOMES et al., 2007), levando a uma expansão exponencial do proteoma das espécies que compartilham essa característica. Isso, por sua vez, aumenta a variação fenotípica realizada com as habilidades de sobrevivência mais importantes das espécies (GOMES et al., 2007; MIRANDA et al., 2013). Um exemplo importante da geração de variabilidade pós-genômica é a pluralidade de moléculas da superfície celular, que é criada pela tradução incorreta do CUG e que pode ter um grande impacto na interação fungo-hospedeiro (MIRANDA et al., 2013).

3.4 *SWITCHING* FENOTÍPICO EM *Candida tropicalis*

Transições epigenéticas são responsáveis pela capacidade das células de sofrer alterações de caráter hereditário sem uma alteração subjacente na sequência primária de DNA (ANDERSON et al., 2016). A regulação genética celular é determinada por fatores de transcrição que atuam em circuitos interconectados para conduzir a expressão do gene específico da linhagem (KANG et al., 2006; NOVERSHTERN et al., 2011). Alterações na estrutura da cromatina também desempenham papéis importantes na herança epigenética, incluindo modificações pós-traducionais de proteínas histonas e remodelamento da estrutura da cromatina (ORKIN; HOCHEDLINGER, 2011; POLO et al., 2012).

Em *C. tropicalis* s*witching* fenotípico ocorre naturalmente, sendo caracterizado como um evento epigenético reversível que promove a alteração da morfologia de colônias, ocorrendo em frequências maiores que mutações somáticas (SOLL, 1992). Sinais ambientais importantes como elevada concentração de CO_2, alteração de fontes de carboidratos, temperatura, estresse

genotóxico e oxidativo e interação com células imunes podem induzir o *switching* fenotípico (SOLL, 2014). Muitos desses sinais são considerados desfavoráveis para o crescimento microbiano, apoiando a ideia de que o aumento da heterogeneidade genética promove o surgimento de subpopulações de *Candida* que podem ser mais bem adaptadas a condições desfavoráveis, favorecendo assim a sobrevivência no hospedeiro (SOLL, 2014).

O sistema mais bem caracterizado de *switching* fenotípico é o "*white-opaque*" em *C. albicans*, também sendo observado em *C. tropicalis* (SLUTSKY et al., 1987; ZHENG et al., 2017). A transição entre o estado "branco" e "opaco" é marcado por diferenças fenotípicas celulares, competência de *mating* sexual, capacidade de morfogênese, alterações na virulência e na interação com células imunes (SLUTSKY et al., 1987; PUJOL et al., 2004; PORMAN et al., 2011) A troca entre os dois tipos de células ocorre espontaneamente e também pode ser induzida por sinais ambientais (SOLL, 2009). A biestabilidade entre as duas formas é alcançada por *loops* de *feedback* entrelaçados entre vários fatores de transcrição (SRIKANTHA et al.,2000; ZORDAN et al., 2006; HERNDAY et al., 2013; IWATA-OTSUBO et al., 2016). Este sistema foi modelado por uma rede centrada em dois fatores de transcrição, Wor1 e Efg1, que são mutuamente antagônicos (SRIRAM et al., 2009). Além disso, ambos os fatores de transcrição se autorregulam positivamente, caracterizando estabilidade ao sistema (ANDERSON et al., 2016). Esse sistema regulatório é altamente conservado nas espécies de *Candida* (BUTLER et al., 2009).

Além da transição "*White-opaque*" há a emergência de um fenótipo intermediário (híbrido) de s*witching* (ANFERSON et al., 2016). O fenótipo híbrido apresenta regulação intermediária entre *WOR1* e *EFG1*. O estado "branco" é definido pela expressão alta de Efg1 e baixa de Wor1, e o estado "opaco" pela expressão baixa de Efg1 e alta de Wor1. De acordo com este modelo, os níveis de expressão de *WOR1* e *EFG1* são inversamente correlacionados; *WOR1* aumenta de "branco" para "híbrido" e então para estados "opacos", enquanto a expressão de *EFG1* diminui entre esses mesmos três estados (ANDERSON et al., 2016). No sistema "*White-grey-opaque*" também há a emergência de um fenótipo intermediário entre "branco" e "opaco", no entanto a regulação desse

fenótipo é distinta da observado para o fenótipo híbrido (ANDERSON et al., 2016; ZHANG et al., 2016).

Além das transições fenotípicas “branco-opaco” e “branco-cinza-opaco”, *C. tropicalis* pode sofrer outras formas de troca fenotípica, apresentando uma variedade de morfologias derivadas de *switching* fenotípico (SOLL et al., 1988; MORALEZ et al., 2014). Embora o evento ocorra naturalmente, altas frequências de variações fenotípicas foram observadas em infecção prolongada por *C. tropicalis* em um hospedeiro imunocomprometido (SOLL et al., 1988).

Em isolados clínicos de *C. tropicalis*, *switching* fenotípico promoveu a emergência de morfotipos com características alteradas de virulência *in vitro*, incluindo morfogênese (MORALEZ et al., 2014; MORALEZ et al., 2016), capacidade de adesão a superfícies bióticas e não-bióticas (SOUZA et al., 2020), formação de biofilme (MORALEZ et al., 2020), capacidade de hemólise e citoxicidade em células *FaDu* (MORALEZ et al., 2016). Além disso, em modelo de infecção de *Galleria mellonella*, morfotipos advindos de *switching* apresentaram potencial de virulência aumentado e alteração na relação com células imunes do hospedeiro (PERINI et al., 2019).

A regulação epigenética dos estados celulares pode promover variação fenotípica em uma população unicelular, o que por sua vez culmina na variabilidade e permite uma adaptação mais rápida a ambientes flutuantes (ACAR et al., 2008; HALFMANN et al, 2012; BEN-JACOB et al., 2014). *Switching* fenotípico por promover alteração de virulência e alterações na relação hospedeiro/patógeno, pode estar relacionado com a capacidade de adaptação de *C. tropicalis* a variações ambientais presentes nos micronichos no corpo do hospedeiro.

3.5 MORFOGÊNESE

C. tropicalis é capaz de desempenhar papel ativo no processo infeccioso através da manifestação de fatores de virulência (CAUCHIE et al., 2017). Dentre os fatores de virulência descritos para a espécie, a capacidade de formação de hifas verdadeiras, característica compartilhada com *C. albicans*, destaca-se como importante de mecanismo mecânico de invasão de tecidos e evasão do sistema imune (D’OSTIANI et al., 2000; VYLKOVA; LORENZ, 2017).

Formas leveduriformes são relacionadas com a disseminação na corrente sanguínea e colonização da região mucosa, enquanto as formas hifais com a capacidade de invasão de tecidos e formação de biofilme (DALLE et al., 2010; JACOBSEN et al., 2012). Em infecções sistêmicas ambas as formas podem ser encontradas. Além disso, o reconhecimento diferencial de hifas por células do sistema imune humano, faz com que seja desencadeada resposta pró-inflamatória, trazendo dano ao hospedeiro (GOW, 2013).

Durante a resposta imune do hospedeiro, fagócitos internalizam *C. tropicalis* e expõem o patógeno a espécies reativas de oxigênio e nitrogênio, redução de pH e privação de nutrientes. Para sobreviver e evadir, a levedura precisa acionar mecanismos de resistência ao estresse e flexibilidade metabólica (BASSO et al., 2017). Além da tolerância ao estresse, o escape da fagocitose é mediado pela transição blastoconídio-hifa, o que pode causar, em último estágio, a morte da célula imune (MCKENZIE et al., 2010; VYLKOVA; LORENZ, 2017). Além do rompimento físico do fagócito pela hifa, há relatos que sugerem que a forma filamentosa pode desencadear piroptose na célula do hospedeiro (UWAMAHORO et al., 2014; WELLINGTON et al., 2014).

Galán-Ladero et al. (2013) avaliaram a filamentação entre isolados de *C. tropicalis* obtidos de diferentes sítios anatômicos de pacientes internados em um hospital terciário. Os autores descreveram altos níveis de filamentação para 76,6% dos isolados. Wapinski et al. (2007) relataram que pelo menos 55 dos 105 genes envolvidos na filamentação de *C. albicans* são conservados em *C. tropicalis*.

Células de *C. tropicalis* sob cultivo em meio indutor de filamentação (spider) e advindas de colônias de fenótipo “enrugado” apresentam superexpressão do regulador transcricional WOR1 (PORMAN et al., 2013). Homólogos de Wor1p também foram encontrados em *Saccharomyces cerevisiae* (CAIN et al., 2012) e *Histoplasma capsulatum* (NGUYEN; SIL, 2008), controlando a transição morfológica dentro dessas espécies. Esse achado pode sugerir a existência de um gene ancestral comum encontrado no genoma de *C. tropicalis* (PORMAN et al., 2013).

Wor1 também participa como regulador mestre da transição “branco-opaca”, onde as células são maiores e alongadas, enquanto as colônias têm

aparência enrugada (SLUTSKY et al., 1987). Além da morfologia, esses dois tipos diferentes de células exibem diferenças dramáticas em relação à colonização e infecção de sítios específicos, além de respostas a variáveis ambientais, sinais nutricionais e capacidade de reprodução sexuada (MANCERA et al., 2015). Em *C. tropicalis*, a superexpressão de *WOR1* direciona as células para a fase opaca que está envolvida na formação do biofilme e na morfogênese (PORMAN et al., 2013).

Antagonista de *WOR1, EFG1* também participa da morfogênese em *C. tropicalis.* A deleção de ambos os alelos do gene *EFG1* aponta que Efg1p é essencial para a filamentação, formação de biofilme e troca branco-opaca em *C. tropicalis*, semelhante a *C. albicans*, indicando conservação na função deste gene ortólogo (PIERCE et al., 2013). Efg1 também se mostra importante para a regulação da colonização do trato gastrointestinal humano. A alta expressão de *EFG1* nesse nicho promove a emergência de subpopulações capazes de evadir do sistema imune (D'ENFERT, 2009; PIERCE et al., 2013).

Uma vez que transições morfológicas são desencadeadas por variáveis ambientais, a morfogênese caracteriza-se como importante estratégia de sobrevivência para *C. tropicalis* (JACOBSEN et al., 2012). Vias de transdução de sinal foram identificadas ligando sinais ambientais à ativação dos chamados genes específicos de hifas, que contribuem para a desregulação do ciclo celular, estabelecimento de polaridade e mudanças na parede celular (SUDBERY, 2011; HUANG, 2012). A morfogênese está sob o controle de uma intrincada rede de reguladores transcricionais que atuam negativa ou positivamente na expressão desses genes (SUDBERY, 2011). A regulação positiva é mediada em quase todas as condições indutoras de filamentos pelos fatores de transcrição Efg1, Flo8 e Ndt80 (CAO et al., 2006; SELLAM et al., 2010).

A formação de hifas proporcionará ao fungo o acesso a diferentes nichos no hospedeiro, o que por sua vez amplia a aquisição de recurso e permite o acesso a regiões com menor competição. Além disso, o menor reconhecimento e escape do sistema imune permite que o fungo tenha maior sucesso da colonização e infecção do hospedeiro (HUBE, 2004; KUMAMOTO, 2008).

3.6 MECANISMOS DE ADAPTAÇÃO AO ESTRESSE

O conjunto de fatores de virulência manifestados por *C. tropicalis* promove a patogenicidade desse microrganismo. Dentre esses fatores estão a capacidade de formação de hifas verdadeiras, s*witching* fenotípico, secreção de proteases e capacidade de formação de biofilme (CALDERONE; CLANCY, 2012). Além dos fatores de virulência e intimamente relacionado com a patogenicidade, está a plasticidade de resposta a variáveis ambientais. Dentre esses atributos estão a capacidade metabólica de assimilar os nutrientes do hospedeiro, a resistência a estresses fisiologicamente relevantes impostos em microambientes do hospedeiro, a tolerância às temperaturas elevadas do hospedeiro e a construção de uma parede celular robusta (BROWN et al., 2012; GOW; HUBE, 2012; LEACH et al., 2012; BROWN et al., 2014; ENE; BROWN, 2014). O metabolismo fornece a plataforma da qual todos os outros atributos de aptidão dependem, gerando os precursores e a energia necessária para a biossíntese da parede celular, produção de antioxidantes, reparo macromolecular e redobramento de proteínas (BROWN, 2014).

Os patógenos fúngicos são movidos pela necessidade de assimilar nutrientes, sobreviver e se multiplicar. No curto prazo, isso requer flexibilidade para se adaptar às mudanças ambientais. Após a disseminação para um novo hospedeiro, uma célula fúngica tenta assimilar os nutrientes locais, contrariar qualquer estresse ambiental local e, se possível, escapar de defesas do hospedeiro. Há indícios de que esses processos adaptativos estão inextricavelmente ligados. Consequentemente, o resultado da infecção depende da robustez fisiológica do patógeno fúngico dentro dos nichos do hospedeiro, bem como da eficácia das defesas do hospedeiro nesses nichos (BROWN et al., 2014).

A adaptação ao estresse é pautada em três princípios fundamentais: (1) A capacidade de detectar sinais ambientais; (2) A capacidade de transduzir esses sinais para regular os processos celulares que medeiam a adaptação ao estresse e; (3) A manifestação de respostas adaptativas que permitem que as células sobrevivam ao estresse. Esses processos adaptativos neutralizam ou desintoxicam o estresse inicial e reparam ou removem o dano molecular causado por esse estresse (BROWN et al., 2017).

Durante o processo de fagocitose, células de *Candida* exibem padrões de expressão que refletem a privação de carbono, ativando enzimas envolvidas na β-oxidação de ácidos graxos, no ciclo de glioxilato e na gliconeogênese (RUBIN-BEJERANO et al., 2003; LORENZ et al., 2004; FRADIN et al., 2005). O metabolismo também promove a virulência de *C. albicans* indiretamente, aumentando a adaptação ao estresse. A resistência ao estresse é necessária para a virulência de *C. albicans*: ela aumenta a sobrevivência das células fúngicas em nichos hospedeiros, reduzindo sua vulnerabilidade a estresses ambientais locais e à morte fagocítica (ARANA et al., 2007; BROWN et al., 2012; PATTERSON et al., 2013).

A adaptação metabólica parece ser coordenada ativamente com a regulação de fatores de virulência essenciais por meio de redes de sinalização complexas. Por exemplo, o eixo de sinalização adenilil ciclase-PKA-Efg1 controla o metabolismo do carbono, bem como a morfogênese de levedura-hifa, troca fenotípica branco-opaca e resistência ao estresse (DOEDT et al., 2004; MORSCHHAUSER, 2000; GIACOMETTI et al., 2009). Outro exemplo da interconexão das vias metabólicas de resposta ao estresse é a ativação, em *C. albicans*, de genes de resposta ao estresse oxidativo após a exposição à glicose. Durante a permanência da corrente sanguínea do hospedeiro, a levedura encontrará concentrações de glicose superiores às dos tecidos circundantes, no entanto a exposição a espécies reativas de oxigênio e nitrogênio também poderá ocorrer pela fagocitose por macrófagos e neutrófilos. A ativação de vias de resposta intimamente ligadas com vias metabólicas poderá então trazer vantagem adaptativa para a espécie (MIRAMON et al., 2013; BROWN et al., 2014).

3.7 RESPOSTA AO ESTRESSE OSMÓTICO

A resposta ao estresse osmótico desempenha um papel crucial na virulência fúngica e na suscetibilidade a antifúngicos. O estresse osmótico leva à rápida perda de água, redução do tamanho das células e queda na pressão de turgor (KLIPP et al. 2005; MAVOR et al. 2005; KUHN; KLIPP 2012). Gerenciar as mudanças no equilíbrio da água é um desafio fundamental para os fungos na maioria dos ambientes. O modelo experimental clássico para isso tem sido a

imposição de choque hiperosmótico por meio da adição de sorbitol ou sais como o NaCl (HOHMANN, 2002). Isso resulta em uma diminuição repentina na pressão de turgor intracelular necessária para o crescimento do fungo. O fungo deve restaurar sua pressão de turgor antes de retomar o crescimento e, para isso, ativa a síntese e o acúmulo de osmólitos intracelulares como o glicerol (KUHN; KLIPP 2012).

As respostas aos estresses osmóticos, oxidativos, nitrosativos e da parede celular são reguladas por uma gama de diferentes vias do MAPK (MONGE et al., 2006; BROWN et al., 2012; BROWN et al., 2014). Como todos os módulos de proteína quinase ativada por mitogenação (MAPK), o módulo Hog1 MAPK compreende três camadas de quinases; o (s) MAPKKK (s) no topo da via fosforila e ativa um MAPKK, que então fosforila e ativa o MAPK terminal (HOHMANN et al., 2007).

Os sinais ambientais induzem cascatas de quinases que, em última análise, levam à ativação de fatores de transcrição que regulam a resposta adaptativa ao estresse (MONGE et al., 2006). Hog1 (mitogen-activated protein kinase), uma quinase que é ativada em resposta a estresses osmóticos, oxidativos, térmicos, de metais pesados e da parede celular, tendo funções essenciais na resposta ao estresse central de *C. albicans* (SMITH et al., 2004; SMITH et al., 2010). Este MAPK regula o acúmulo de glicerol por meio da ativação transcricional de GPD1 e GPP1 em resposta ao estresse osmótico por meio dos fatores de transcrição Hot1, Msn2 e Msn4 (REP et al., 2000) e controlando a atividade da aquagliceroporina Fps1 (LEE et al., 2013). No entanto, Hog1 também medeia o atraso transitório na progressão do ciclo celular após choque hiperosmótico por fosforilação de Sic1 e Hsl1 e por regulação negativa das ciclinas G1 e G2 (YAAKOV et al., 2009).

Uma vez que a adaptação osmótica é alcançada, a célula de levedura atinge essencialmente um novo estado homeostático no qual a pressão de turgescência foi reestabelecida em resposta às condições osmóticas externas (MUZZEY et al., 2009). Consequentemente, o sinal é diminuído e Hog1 é desativado. Dessa maneira, o bloqueio da progressão do ciclo celular é desfeito e o crescimento pode ser retomado.

A ativação de Hog1 pode ser mediada por meio de uma via de sinalização de dois componentes envolvendo Sln1 e Ste11 (MONGE et al., 2006; BROWN et al., 2014). Hog1 também regula negativamente Cek1, afetando assim a morfogênese, consequentemente os mutantes hog1 são hiperfilamentares (ALONSO-MONGE et al., 1999; EISMAN et al., 2006). A falta simultânea de ativação de Hog1 e Cek1 leva a um fenótipo letal sintético sob estresse osmótico, destacando a importância de ambas as vias de sinalização sob esta condição de estresse (HERRERO-DE-DIOS et al., 2014).

A adaptação ao estresse osmótico é especificamente importante em certos nichos de hospedeiros. Por exemplo, as concentrações de NaCl podem ser extremamente altas nos rins e na urina, e *C. albicans* precisa lidar com os fluxos de K+ durante a fagocitose (BROWN et al., 2014). Não surpreendentemente, os mutantes sem Hog1 são fortemente atenuados em um modelo de infecção sistêmica de camundongo (ALONSO-MONGE et al., 1999). Além do acúmulo de osmólitos, importante para a osmoadaptação, *C. tropicalis* apresenta transportadores Na+/K+ - ATPase que são ativados imediatamente após exposição ao ambiente hiperosmótico. As bombas de efluxo iônico apresentam alta eficiência na resistência ao choque osmótico (GARCIA et al., 1997).

Zuza-alves et al. (2016) apontam que isolados de *C. tropicalis* da região costeira apresentaram alta osmotolerância, capacidade de expressão de virulência in vitro e CIMs elevados para antigúngicos. Os autores sugerem que a persistência de *C. tropicalis* em ambientes com alta salinidade pode estar relacionada com a relação de isolados de *C. tropicalis* não previamente expostos a antifúngicos e a expressão de altos níveis de resistência a azóis e anfotericina B.

3.8 RESPOSTA AO ESTRESSE OXIDATIVO

Todos os fungos que crescem aerobicamente são expostos a ânions superóxidos gerados como um subproduto da respiração aeróbia na mitocôndria (CADENAS; DAVIES, 2000). No entanto, durante o processo de fagocitose por leucócitos e macrófagos do hospedeiro, *Candida* é exposta a espécies reativas

de oxigênio (ROS) e consequentemente ao estresse oxidativo. (KLIPP et al., 2005; MAVOR et al., 2005; KUHN; KLIPP, 2012).

Espécies reativas de oxigênio (ROS) são formas reduzidas de oxigênio altamente prejudiciais, que incluem o ânion superóxido O2-, o peróxido de hidrogênio (H_2O_2) e o radical hidroxila (OH). Essas moléculas reativas danificam proteínas, DNA e lipídios e podem desencadear a morte celular programada (BROTHERS et al., 2014). Outros produtos químicos tóxicos são subsequentemente derivados das ROS geradas pelo hospedeiro (BROWN et al., 2009). As ROS são produzidas pelo complexo NADPH oxidase, em um processo conhecido como explosão respiratória. Além disso, a produção de ROS em resposta à infecção por *C. albicans* mostrou levar ao recrutamento de fagócitos adicionais, criando um ambiente oxidativo tóxico para o fungo (BROTHERS et al., 2014).

As espécies de *Candida* tentam neutralizar esses estresses ativando respostas celulares que incluem a ativação de genes que codificam proteínas envolvidas na desintoxicação e reparação do estresse. Estes incluem catalase, superóxido dismutases, glutationa peroxidases e tioredoxinas (ENJALBERT et al., 2006; ENJALBERT et al., 2007; PRADHAN et al., 2017). Em *C. albicans* e *C. glabrata*, essas vias de estresse são amplamente reguladas pela proteína quinase ativada por estresse Hog1 (SMITH et al., 2004; ENJALBERT et al., 2006). Dessa forma, esses reguladores promovem a aptidão de *C. albicans* durante a infecção sistêmica. De fato, mutantes sem Hog1 exibem virulência atenuada em camundongos, bem como tolerância prejudicada a esses estresses in vitro e sobrevivência fagocítica (ENJALBERT et al., 2006; ENJALBERT et al., 2007).

Em *C. albicans*, a exposição ao H_2O_2 desencadeia a parada do ciclo celular mediada por Rad53. Curiosamente, neste fungo, tal parada do ciclo celular induzida por genotóxicos promove a formação de uma forma de crescimento de botão hiperpolarizado filamentoso (SHI et al., 2007; DA SILVA DANTAS et al., 2010). As enzimas peroxirredoxina Tsa1 e tioredoxina Trx1 são vitais para a ativação de Hog1 induzida por H_2O_2 em *C. albicans* (DA SILVA DANTAS et al., 2010). Assim, a oxidação de proteínas parece ser um mediador

importante da ativação SAPK de *C. albicans* após estresse oxidativo. (FURUKAWA et al., 2005).

A adaptação ao estresse oxidativo pode ser crucial para os estágios iniciais da infecção, mas menos importante após o estabelecimento da infecção sistêmica (BROWN et al., 2014; BROWN et al., 2009). Com isso, é imprescindível que além da adaptação *C. tropicalis* seja capaz de manifestar fatores de virulência que permitam o sucesso sobre as barreiras impostas pelo hospedeiro. Em resposta a exposição a H_2O_2, *C. albicans* é capaz de apresentar secreção aumentada de fatores de virulência (proteinase e fosfolipase) e alterações no conteúdo dos componentes da parede celular quitina e manana. Além disso, há relatos de indução na formação de biofilme de matriz exopolimérica espessa sob essas condições (ZHANG et al. 2007; VILLA et al. 2012; MORYL et al. 2014).

3.9 RESPOSTA DE PAREDE CELULAR

Um dos fatores de virulência mais importantes manifestados por *Candida* spp. é a arquitetura da parede celular. Essa estrutura é essencial para a colonização de diferentes nichos dentro do hospedeiro (GOW; HUBE, 2012). O esqueleto central da parede celular é composto pelo polissacarídeo β-1,3-glucano, covalentemente ligado à β-1,6-glucano e quitina. A camada externa contém manoproteínas glicosiladas reticuladas com β-1,6-glucanos. A quantidade relativa de cada componente flutua entre morfologias e em resposta a desafios externos, impactando as respostas imunológicas (GOW; HUBE, 2012; HOPKE et al., 2018; GARCIA-RUBIO et al., 2020).

A parede celular também é o primeiro ponto de contato com o sistema imune do hospedeiro (GIL-BONA et al., 2015; WARRIS; BALLOU, 2019). Os glicanos, glicolipídeos e proteínas da parede celular de fungos que estão ausentes nos mamíferos ativam uma variedade de mecanismos de reconhecimento imunológico, e a exposição dinâmica de tais padrões moleculares associados a patógenos (PAMPs) pode modular o reconhecimento imunológico (ERWIG; GOW, 2016). A exposição de alguns PAMPs por *C. albicans* pode mudar durante a infecção. A β-glucana na superfície do fungo é, entre outras, reconhecida pelo receptor de dectina-1 e essa interação é

importante para o início da fagocitose e outras atividades antifúngicas. Embora a exposição a β-glucana de *C. albicans* cultivada in vitro seja relativamente baixa, ela aumenta durante a infecção sistêmica de camundongos e após a exposição à caspofungina (WHEELER; FINK, 2006; WHEELER et al., 2008).

Como consequência, a perturbação da arquitetura da parede celular do fungo pode potencializar as respostas imunes e induzir estresse letal na parede celular (ROEMER; KRYSAN, 2014). ENE et al (2012) identificaram alterações na biossíntese de parede por *C. albicans* sob crescimento com diferentes fontes de carbono. Células com alterações na estrutura foram menos reconhecidas por macrófagos murinos, evidenciando o importante papel antigênico da parede celular.

A biossíntese de quitina também está relacionada com o reconhecimento e capacidade de infecção in vivo. Além disso, a quitina exibe efeitos anti-inflamatórios via NOD2, receptor toll-like 9 (TLR9) e receptor de manose, sugerindo que os conteúdos de quitina também influenciam diretamente a resposta imune (WAGENER et al., 2014). Mudanças dramáticas no proteoma da parede celular, portanto, podem ocorrer in vivo em resposta às condições de crescimento e sinais ambientais. Assim, o reconhecimento pelo sistema imunológico e as respostas anti-*Candida* subsequentes podem diferir dependendo do local anatômico (GOW, 2013).

Embora o termo parede celular indique uma estrutura rígida, a parede celular de *Candida* spp é altamente dinâmica, apresentando repostas rápidas a variáveis ambientais (ENE et al., 2012). A rápida adaptação desempenha um papel crítico nas interações patógeno-hospedeiro. Muitos estresses afetam a composição e a fluidez da membrana, que por sua vez afeta a composição da parede celular. Dentre os agentes indutores de alterações na parede celular estão: tensões osmóticas, oxidativas, térmicas e catiônicas, bem como a exposição a certos antifúngicos, que em última análise, afetam a estabilidade, a estrutura e a integridade da parede celular. A fixação a uma superfície e a indução de crescimento hiperpolarizado requerem um rearranjo constante da estrutura da parede celular (KLIS et al., 2014).

Sob estresse oxidativo, há estímulo da biossíntese de quitina, configurando mecanismo de resistência potencial ou tolerância contra os efeitos

deletérios do estresse. Os fungos respondem às paredes celulares danificadas aumentando os níveis de quitina para fortalecer a parede celular enfraquecida, bem como para manter sua integridade (HEILMANN et al. 2013).

Sob estresse osmótico e oxidativo há redução no conteúdo de manana com unidades de manose β-ligadas reduzidas. Esses achados corroboram com mudanças estruturais observadas nas cadeias laterais de glucanos (KOYAMA et al. 2009). Manana é conhecida por ser um componente significativo de *C. albicans* com potencial virulência e propriedades antigênicas (NELSON et al. 1991). O componente auxilia na adesão às células de mamíferos como a primeira etapa da infecção, que induz a produção de citocinas (MIYAKAWA et al., 1992; JOUAULT et al., 1994; TRINEL et al. 2002). Além disso, a reorganização dos componentes de parede acontece segundos após a exposição ao ambiente hiperosmótico, evidenciando o alto grau de dinamismo da parede em resposta a variáveis ambientais (ENE et al., 2012).

Não surpreendentemente, várias vias de sinalização estão, envolvidas na resposta ao estresse da parede celular, incluindo as três vias MAPK com seus fatores-chave Hog1, Cek1 e Mkc1 (BROWN et al., 2014; BROWN et al., 2009; Monge et al., 2006). Hog1 é um regulador de tensão central também relacionado com a resposta ao estresse osmótico e oxidativo (BROWN et al., 2014A). Os mecanismos de regulação envolvendo redes de interação genética complexas podem permitir respostas compensatórias às perturbações da parede celular. Por exemplo, a ativação da síntese de quitina suprime a atividade antifúngica das equinocandinas, que inibem a biossíntese de β-1,3-glucana (WALKER et al., 2008; MUNRO, 2013; WALKER et al., 2013). A remodelação de quitina também é coordenada por vários fatores de transcrição, incluindo Rim101, Bcr1 e Efg1 (SHERRINGTON et al., 2017; COTTIER et al., 2019).

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ARTIGOS

ARTIGO 1

THE IMPACT OF PHENOTYPIC *SWITCHING* IN *Candida tropicalis* RESPONSE TO HYPEROSMOTIC STRESS.

ABSTRACT

Candida tropicalis is related to high mortality rates in candidemia episodes. The osmotic response is crucial for yeast adaptation to host niches, and morphological plasticity promotes a variety of pathogen responses. Here, we demonstrate the role of phenotypic *Switching* on *C. tropicalis* response to osmotic stress. For this, phenotypic morphotypes of switch systems 49.07 and 100.10 (parental, variants crepe and rough, and revertants of crepe and rough) were exposed to 1 M NaCl for 10 min (osmotic shock) and 60 min (osmoadaptation). Cell viability was performed by UFC counting and propidium iodide staining. The expression of *EFG1, HOG1* and *WOR1* genes was evaluated by qPCR assay, and the amount of chitin, mannan and β-glucan was obtained by fluorimetric dyes staining. Morphotypes obtained by phenotypic *Switching* (75%) were more resistant to the osmotic stress than Parental (clinical strain). The morphotype Crepe showed less membrane damage and less cell volume reduction when compared to Parental strain. The phenotypic *Switching* impact in *C. tropicalis* cell wall components and gene expression as observed under untreated conditions. Under osmotic shock the regulatory genes (*EFG1* and *WOR1*) were up-regulated in the parental strain, suggesting that *C. tropicalis* is able to induce phenotypic *Switching* in response to stress. The *HOG1* gene was overexpressed in the Crepe variant of the 100.10 system in the untreated condition, which may be related, at least in part, to the better stress response for this morphotype. Phenotypic *Switching* promotes differences in *C. tropicalis* osmoadaptation. Revertants morphotypes of the system 100.10 showed overexpression of *EFG1* and *HOG1*, also showing chitin amount greater than its parental counterpart strain (unswitched strain). The response of both switch systems to gene regulation and cell wall alterations seems to be morphotype-dependent. We conclude that phenotypic *Switching* promoted variability to the *C. tropicalis* responses under osmotic stress reflected in cell wall changes and in the expression of genes related to phenotypic transitions and stress response. Comprehension of response to stress mediated by *Switching* can provide information to develop clinical strategies against *C. tropicalis* infections.

Keywords: *Candida tropicalis*, phenotypic *Switching*, resistance, viability, sodium chloride.

1 INTRODUCTION

Candida species are frequently isolated from superficial and invasive fungal infections (BROWN et al., 2012; ANTINORI et al., 2016). Blood infections by *Candida tropicalis* are commonly observed in tropical regions, with high rates of mortality (COLOMBO et al., 2007; TAN et al., 2015). *C. tropicalis* is a human commensal and opportunistic pathogen (ZUZA-ALVES et al., 2017; BARROS et al., 2018), showing a greater phylogenetic proximity with *Candida albicans* (BUTLER et al., 2009; ZUZA-ALVES et al., 2017). The presence of *C. tropicalis* on multiple niches shows the adaptability plasticity of this species (LIN; CHEN, 2018).

Within the host's body, pathogens find several niches and environmental variations and need to develop strategies to survive and adapt (LIN; CHEN, 2018). Osmotic stress is one of the stressful conditions that fungi need to face in oral infections and during skin and kidney colonization (PORMAN et al., 2013; HERRERO-DE-DIOS et al., 2014). Hyperosmotic exposition leads to rapid water loss, cell volume reduction and decrease in turgor pressure (PEMMARAJU et al., 2016).

Morphological plasticity is an important tool for fungi and allows the yeast/hyphae transition to occur as well as the manifestation of virulence factors (GOW et al., 2012; HUANG, 2012; NOBLE et al., 2017). Epigenetic transitions are responsible for the ability of cells to undergo heritable changes in cell type without an underlying change in the primary DNA sequence (CANTONE; PAPP, 2013; PAPP; PLATH, 2013). The phenotypic *Switching* event occurs naturally in isogenic microbial populations at higher frequencies than somatic mutations, producing phenotypically distinct cell subpopulations (CLAVERYS et al., 2009; FINKEL; MITCHELL, 2011). In *C. tropicalis* phenotypic *Switching* plays a role in distinct traits, including modulation of virulence and sexual mating capacity (PORMAN et al., 2013; MORALEZ et al., 2014).

The most described switch system in *C. albicans* and *C. tropicalis* is the white-opaque (PORMAN et al., 2013; ZHANG et al., 2016). White to opaque transitions are regulated by specific sets of transcription factors that regulate a large number of target genes (KADOSH; JOHNSON, 2005; NOBILE et al. 2012;

HERNDAY et al. 2013). The transcription factor Efg1 from the APSES family plays a central role in the transcription circuits underlying several of these morphological changes (MANCERA et al., 2015). In addition to *EFG1, WOR1* is the master regulator of transition white to opaque in *C. tropicalis* and also is related with morphogenesis capacity (PORMAN et al., 2013; ANDERSON et al., 2016).

Our group described phenotypic *Switching* systems that present morphological patterns different from white-opaque (FRANÇA et al., 2011; MORALEZ et al., 2014). Switched cells showed differences in the host-pathogen relationship, undergoing changes in the recognition of defense cells and survival to phagocytosis (PERINI et al., 2019). For pathogenic fungi such as *C. tropicalis*, cell wall components modulate recognition of host's immune cells, being the first point of contact with the host (GOW; HUBE, 2012; WAGENER et al., 2014). Cell wall also plays a critical role in pathogen survival to environmental variables suffering reorganization seconds after stress exposure and biosynthesis in prolonged stress (ENE et al., 2015). Osmotic stress affects the stability of the cell wall and promotes decrease in cell viability (POLKE et al., 2015).

Several signaling pathways are involved in the response to cell wall stress, including the three MAPK pathways with their key factors Hog1, Cek1, and Mkc1 (BROWN et al., 2009; BROWN et al., 2014). HOG1 participates to filamentation process, cell wall biogenesis and is a crucial factor to osmoadaptation in *C. albicans* (ALONSO-MONGE et al., 1999; ALONSO-MONGE et al., 2003; (HERRERO-DE-DIOS et al., 2014). After salt exposure, Hog1 mediates intracellular glycerol accumulation and restores the cell turgor pressure (HERRERO-DE-DIOS et al., 2014). Mutants to *HOG1* are avirulent in mouse systemic infections and more susceptible to phagocytosis, evidencing that importance of adaptation strategies to survive inside the host (ALONSO-MONGE et al., 1999; CHEETHAM et al., 2011).

In this study, we investigate the role of phenotypic *Switching* in the *C. tropicalis* hyperosmotic stress response. These event modulated cell wall responses and regulation of *HOG1, WOR1* and *EFG1* genes. The change in the stress response promoted the emergence of morphotypes less susceptible to stress and the cellular implications that stress provides. These studies provide

new insights into the variability mediated by phenotypic *Switching*, the regulation of genes involved in white-opaque transitions in different *Switching* systems, and the *Switching* response of clinical isolates to osmotic stress. Our data can provide information that allows understanding the *C. tropicalis* pathogenesis and support the development of strategies to deal with yeast.

2 MATERIAL AND METHODS

2.1 MICROBIAL STRAINS AND CULTURE CONDITIONS

The *C. tropicalis* switch systems 49.07 and 100.10 are composed of five morphotypes each: Parental – Clinical strain (smooth colony phenotype); phenotypic variants – Crepe and Rough (obtained of *Switching* in parental culture - Complex colony phenotype) and morphological revertants – RC and RR (obtained of reversion of variants to original phenotype - smooth phenotype) (MORALEZ et al., 2014).

All morphotypes used in this study were stored as frozen stocks with 20% glycerol at −80 °C and subcultured on YPD agar plates (1% yeast extract, 2% peptone, and 2% dextrose) at 28 °C. Morphotypes were routinely grown in YPD liquid medium at 28 °C in a shaking incubator and plated on YPD agar plates at 28 °C for 96 hours.

2.2 QUANTITATIVE ANALYSIS OF STRESS SENSITIVITY

The analysis of stress sensitivity was performed according to Nikolaou et al. (2009), with modifications. *C. tropicalis* morphotypes of the switch systems 49.07 and 100.10 ($1x10^8$ cells/mL) were exposed to 1 M of NaCl (YPD medium supplemented with salt) for 10 min and 60 min. After, cell were washed 3 times in deionized water and serial dilutions were performed. Cells were plated in YPD medium and number of CFU were measured for direct observation. The percentage of growth of each morphotype was calculated relative to their non-stress control for each stress condition and expressed in percentage of growth. Each experiment was performed in quadruplicate and repeated at least 3 times.

2.3 MEMBRANE DAMAGE

To respond to osmotic stress, Parental, Crepe and RC morphotypes ($1x10^8$ cells/mL) were exposed to 1 M NaCl for 10 and 60 min. Membrane damage assay was performed according to Tommioto-Pelissier et al. (2018), with modifications. Exposed and non-exposed cells of the *C. tropicalis* morphotypes ($1x10^6$ cells/mL) were washed and suspended in 100 µL of assay buffer 1x (Santa Cruz Biotechnology). To determine the membrane integrity, cells were directly incubated with 0.50 µg/mL of propidium iodide (PI) (Sigma, St. Louis, MO, USA) for 5 min. Immediately thereafter, cells were analyzed using an excitation wavelength of 480 nm and an emission wavelength of 580 nm. A total of 10.000 events were acquired in a BD AccuriTM C6 flow cytometer.

2.4 CELL VOLUME

Cell volume assay was performed according to Malavia et al. (2017), with modifications. Exposed (1M NaCl – 10 and 60 min) and non-exposed (control) cells ($1x10^6$ cells/mL) of Parental, Crepe and RC morphotypes were fixed with 10% of formaldehyde solution and imaged in microscope (Zeiss, Germany). Cells were analyzed using ImageJ v1.47 and cell volume determined using the formula $V = 4/3\ \pi\ ab^2$ where a is the radius of the major axis and b is the radius of the minor axis of the cell.

2.5 CELL WALL

Parental, Crepe and RC morphotypes ($1x10^6$ cells/mL) were exposed to 1 M NaCl for 10 and 60 min as described. Exposed and non-exposed cells were washed 3 times with ultrapure water and fixed with 10 % paraformaldehyde solution in PBS. Fixed cells were stained independently with 100 µg/mL of Calcofluor White (CFW), 50 µg/mL of Aniline Blue and 100 µg/mL FITC-Concanavaline A conjugated (FITC-CONA) (Okada; Ohya., 2016). Cells were incubated for 10 min at room temperature in the dark. Following incubation, cells were washed twice with PBS. Stained samples were analyzed using GloMax® Explorer (Promega) instrument with 415-445 nm emission filter and 405 nm excitation filter to Aniline Blue; 415-445 nm emission filter and 365 nm excitation

filter to CFW; 500-550 nm emission filter and 475 nm excitation filter to FITC-CONA. Data were expressed in fluorescence units

Cell wall porosity assay was performed according to Ene et al. (2012) with modifications. Aliquots of $1x10^8$ cells/mL for each exposed and non-exposed morphotype were incubated with shaking (200 rpm) for 30 min at 30 °C in 1 mL of either 10 mM Tris-HCl pH 7.4 (control), the same buffer containing 5 mg/ml-1 DEAE-dextran (500 kDa) or buffer containing 15 mg/mL poly-L-lysine (50 kDa). Cells were then pelleted by centrifugation and the A_{260} of supernatants measured in microtiter plate reader (Bio-Tek EL 808). Relative porosity was calculated using the following formula: relative porosity = 100 x (A_{DEAE} - A_{buffer}) / ($A_{poly\text{-}L\text{-}lysine}$ - A_{buffer}). Data were expressed in percentage of porosity. Each experiment was performed in triplicate on tree different occasions.

2.6 RNA EXTRACTION AND GENE EXPRESSION ANALYSIS (RT-QPCR)

The gene expression was performed in untreated condition (control) and treated for 10 and 60 min with 1M NaCl. Cells were homogenized, frozen in liquid nitrogen and RNA was extracted and purified using an RNA Mini Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. RNA was quantified and the quality assessed using a NanoDrop spectrophotometer (ThermoScientific, Loughborough, United Kingdom). cDNA was synthesized from 200 ng of extracted RNA using an RT-PCR kit (Invitrogen, Carlsbad, CA, EUA) in a GeneAmp® PCR (Eppendorf, Gradiente Mastercycler) following the manufacturer's instructions.

Primers used for quantitative PCR (qPCR) were as follows: *HOG1* (forward, 5'-TTGCCAGTGGATACTTGGAG–3'); *HOG1* (reverse 5'–GTTGTTGTTCAGCACCATCG–3'); *WOR1* (forward 5'-CCGTCTAATGTTATACCTGCATCAA-3'); *WOR1* (reverse 5'-TTCGTCGTACTTATGGTAATTGTTTTCT-3'); *EFG1* (forward 5-TTCAACTGCTGGACAACCAC-3'); *EFG1* (reverse 5'-TACCAGGAGGTTGGAATTGG-3'); *β-actin* (forward, 5′-GGGACGATATGGAGAAGATCTG-3′); and *β-actin* (reverse, 5′-CACGCTCTGTGAG GATCTTC-3′).

The cycling conditions consisted of 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 51 °C and 60 s at 60 °C. Each sample was analyzed in duplicate using a StepOnePlus™ Real-Time PCR System (Applied Biosystems®). The reactions were performed using Platinum® SYBR® Green qPCR Supermix-UDG (Invitrogen, Carlsbad, CA, USA) with a final volume of 20 µl. Relative gene expression was calculated using the $2^{-(\Delta-\Delta Ct)}$ method.

2.7 STATISTICAL ANALYSIS

Three independent experiments were performed in triplicate for all experiments. All data were previously submitted to the F test and the Shapiro-Wilk test for analysis of variance and normality test, respectively. Comparison between the groups were done by one-way ANOVA, followed by Tukey's test for multiple comparisons. For stress exposition analysis the t-paired test was used. Statistical significance was set at *P<0.05, **P<0.01, ***P<0.001. All statistical analyses were performed by GraphPad Prism 5 statistical software (GraphPad Software, Inc., USA, 500.288). Data were expressed as mean ± standard deviation.

3 RESULTS

3.1 PHENOTYPIC *SWITCHING* IS ASSOCIATED WITH CHANGES IN *C. TROPICALIS* OSMOTIC STRESS RESPONSE

Osmotic stress reduced cell viability in all morphotypes of *C. tropicalis* (clinical strain and strains derived from phenotypic *Switching*) compared to control condition, however, phenotypic *Switching* promoted changes in the stress response (*p<0.05*). The responses to osmotic shock (10 min exposure) and to osmoadaptation (ability to survive after 60 min of exposure) varied between switch systems 49.07 and 100.10 of *C. tropicalis* (Fig. 1).

Under osmotic shock, phenotypic *Switching* promoted the emergence of switched morphotypes (Crepe variant of 49.07 system and all morphotypes of 100.10 system) less sensible than the parental strain (*p<0.05*) (Fig. 1A,C). Thus 62.5% of morphotypes derived from morphological transitions was less sensible than their parental counterpart strains (unswitched strain) (*p<0.05*). The

phenotypic revertants showed the same response pattern as Parental for the 49.07 *Switching* system and an intermediate pattern for the 100.10 *Switching* system, being less sensible than the parental and high sensible than their morphological variants (*p<0.05*).

All morphotypes showed osmoadaptation capacity. The morphotypes Crepe and RC of 100.10 system showed greater cell viability than Parental strain (*p<0.05*), with 25% higher ability of osmoadaptation. Differently, the crepe revertant (RC) of the 49.07 system showed lower viability than the Parental strain (*p<0.05*). Revertant of rough (RR) showed greater viability than its counterpart Rough variant morphotype (Fig. 1B,D).

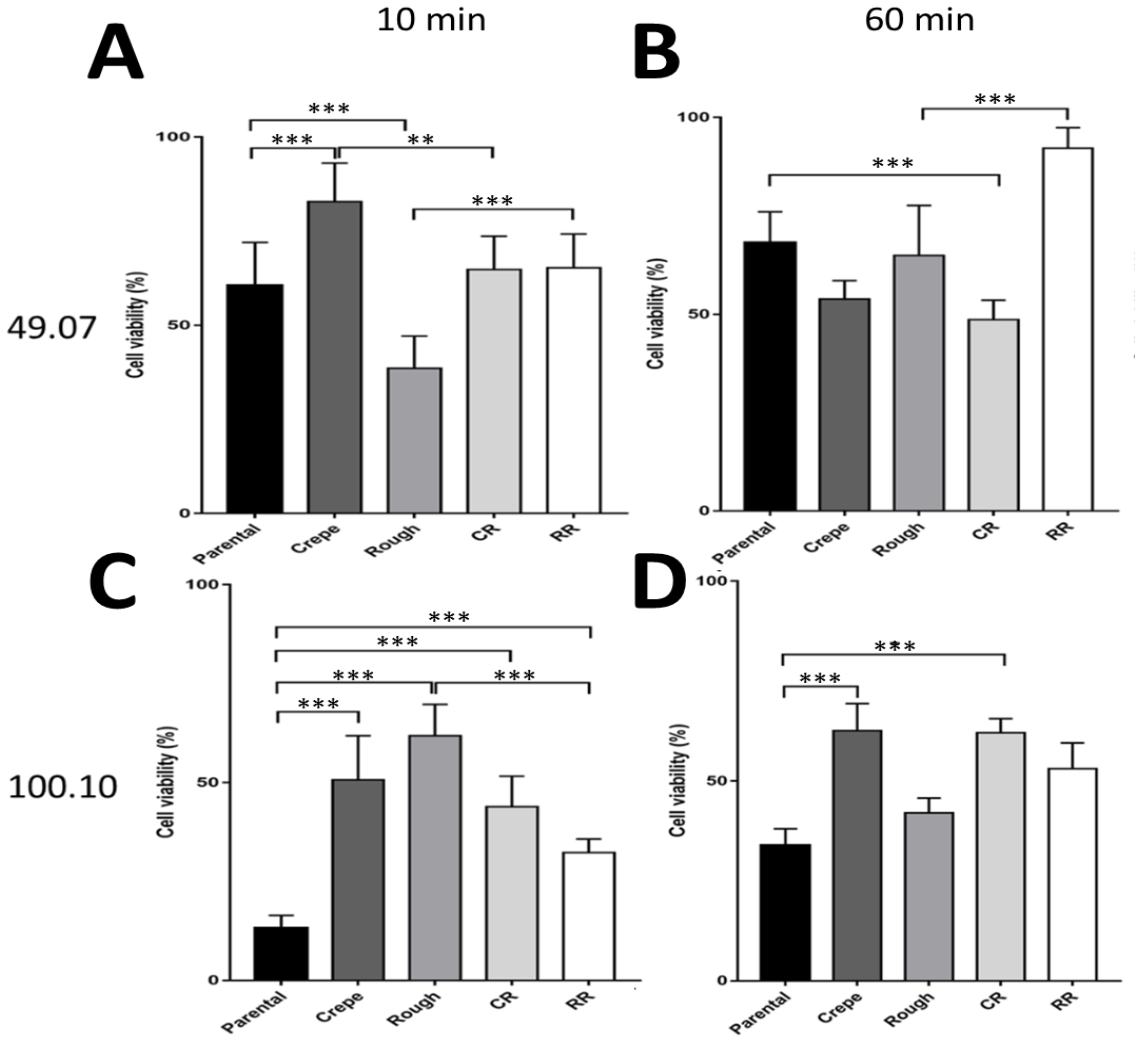

Fig 1. Sensitivity to osmotic shock and osmoadaptation (1M NaCl exposure) of *Candida tropicalis* 100.10 and 49.07 phenotypic *Switching* systems. (A) Cell viability of 49.07 morphotypes after 10 min of exposure; (B) Cell viability of 49.07 morphotypes after 60

min of exposure; (C) Cell viability of 100.10 morphotypes after 10 min of exposure; (D) Cell viability of 49.07 morphotypes after 60 min of exposure. Data were expressed as percentage of viability in relation to non-stressful condition. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ ANOVA with Tukey's post-test.

3.2 EFFECT OF OSMOTIC STRESS IN CELL VOLUME OF *C. tropicalis* MORPHOTYPES

Phenotypic *Switching* promoted changes in cell volume in *C. tropicalis* clinical strains. When compared to the Parental strain, 50% of the switch morphotypes tested showed differences in cell volume (49.07 switch morphotypes) ($p<0.05$) (Fig. 2A). Under untreated conditions, the Crepe variant of the 49.07 system showed lower cell volume, while the remaining morphotypes (Rough, CR and RR) exhibited higher volume than Parental strain ($p<0.05$). The revertant of crepe-CR of the system 100.10 showed lower cell volume than its Crepe counterpart morphotype ($p<0.05$) (Fig. 2B).

Osmotic shock (10 min exposure) promoted cell volume reduction in all morphotypes of both switch systems tested (49.07 and 100.10), except for the Crepe morphotype (49.07) ($p<0.05$) (Fig. 2A,B). The reduction in cell volume remained after 60 min of exposure ($p>0.05$). After 10 minutes of exposure to NaCl (1M), all switch morphotypes showed similar volume to that observed for the Parental strain. Under these condition, the Crepe variant of the 49.07 system presented greater volume than its revertant - RC. Under osmoadaptation condition, about 12.5% of the switched morphotypes showed changes in the cell volume". For the system 49.07, the Rough variant showed greater cell volume than the Parental strain ($p<0.05$) (Fig. 2A).

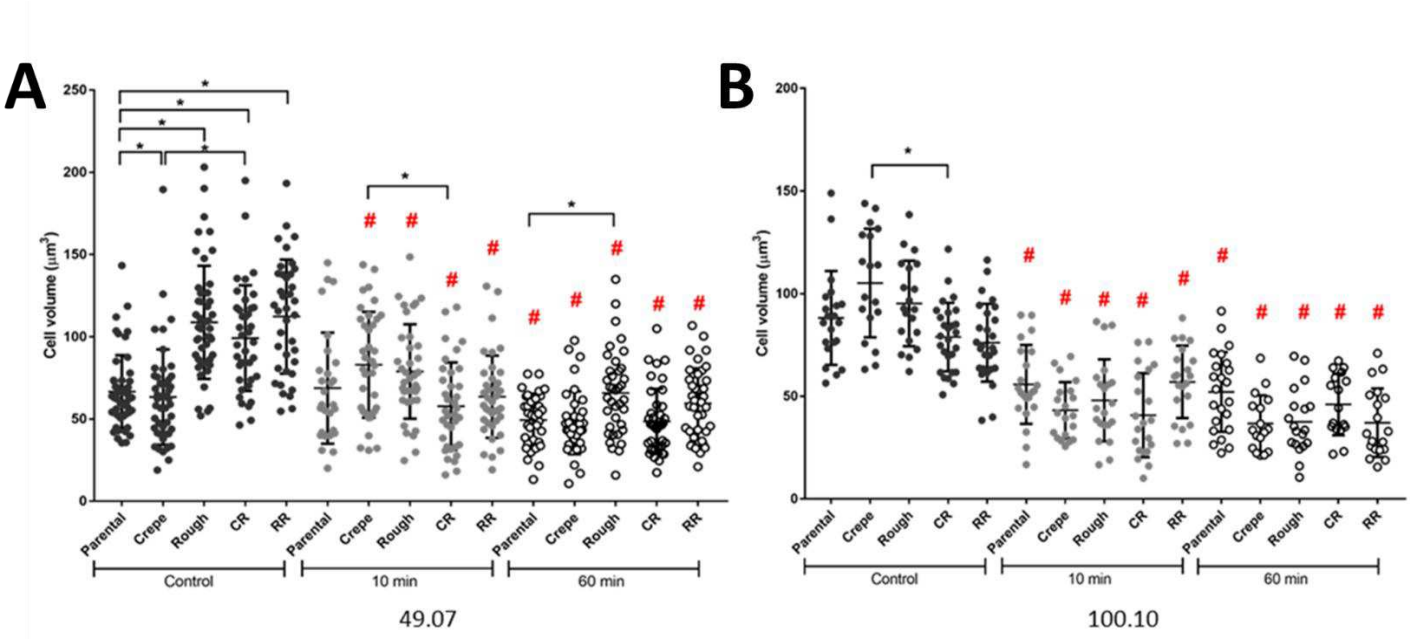

Fig 2. Cell volume changes mediated by phenotypic *Switching* in *Candida tropicalis* after 10 min and 60 min exposure to NaCl (1M). (A) 49.07 switch system; (B) 100.10 switch system. * p < 0.05 ANOVA with Tukey's post-test.

3.3 MEMBRANE DAMAGE IN *C. tropicalis* MORPHOTYPES CAUSED BY OSMOTIC STRESS

Iodide propidium dye indicate that osmotic stress caused membrane damage in all tested morphotypes, however, phenotypic *Switching* promoted the emergence of morphotypes that suffer less damage than Parental (Fig. 4C, D) (*p<0.05*). After osmotic shock (10 min exposure), the Crepe morphotype both of both *Switching* systems undergo less damage (*p<0.05*) compared to its parental strain and revertant – RC counterparts (*p<0.05*) (Fig. 3C, D).

Following 60 min exposure to NaCl (1M), the same pattern of response was observed for morphotypes of the system 100.10 (Fig. 3D). Differently, the Crepe variant of the system 49.07 showed an increase in membrane damage compared to its parental strain and revertant – RC counterparts (*p<0.05*) (Fig. 3C).Flow cytometry data indicated that prolonged exposure to osmotic stress promotes changes in the cellular pattern of the analyzed morphotypes (Fig 3.A,B).

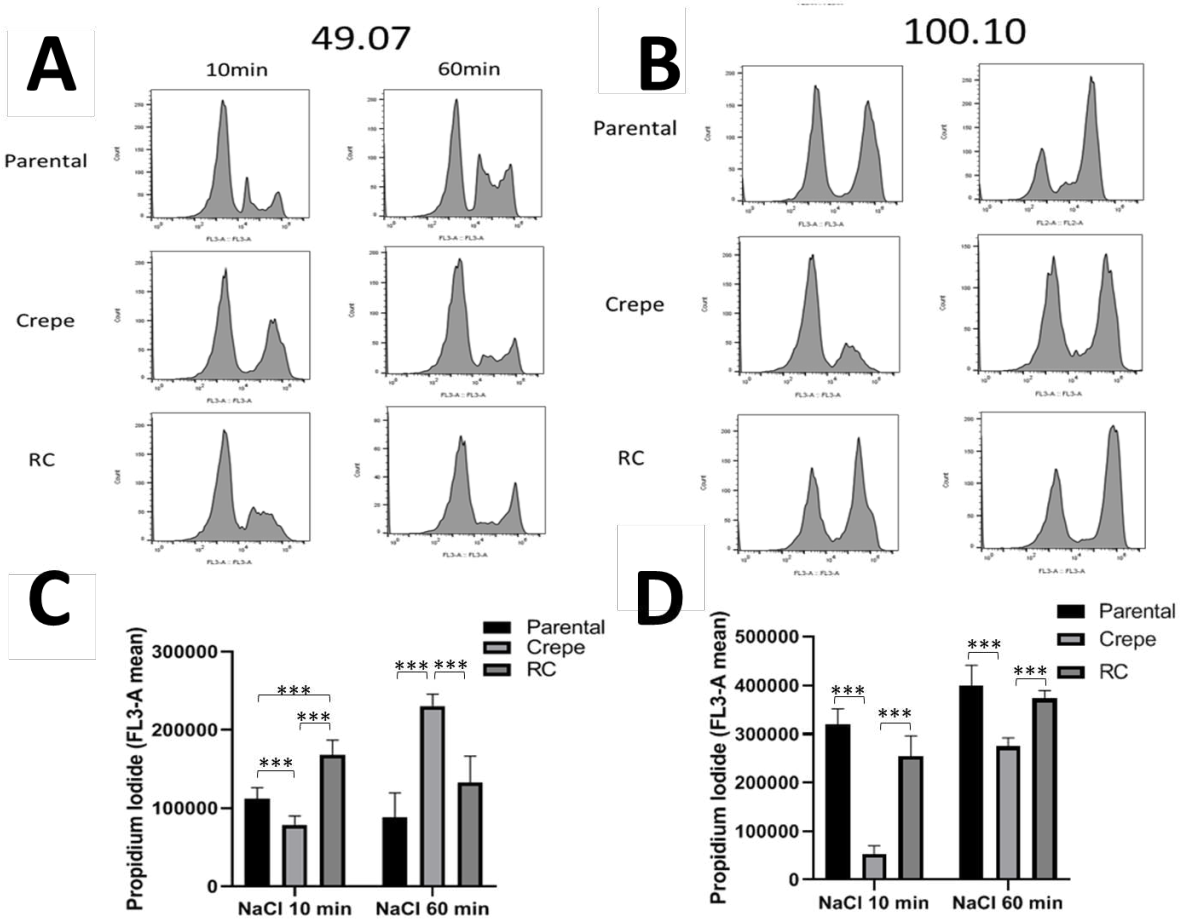

Fig 3. Cell complexity obtained by flow cytometry of *C. tropicalis* (A) 49.07 and (B) 100.10 morphotypes exposed to 1 M NaCl for 10 and 60 min – The X axis indicate the number of cells counted. Membrane damage caused by osmotic stress after 10 min and 60 min exposure to NaCl (1M) in *Candida tropicalis* morphotypes (parental strains and switched morphotypes). (C) Pattern of propidium iodide fluorescence. 49.07 switch system; (D) 100.10 switch system. *** p <0.001, ** p < 0.01, * p < 0.05 ANOVA with Tukey's post-test.

3.4 Effect of phenotypic *Switching* in the amount of cell wall compounds and porosity in response to osmotic stress

Phenotypic *Switching* promoted changes in the amount of cell wall compounds in untreated conditions (control). The Crepe Revertant – RC of both switch systems showed differences in the amount of chitin when compared to the Parental strain (Fig. 4A,E). The Crepe variant of switch system 100.10 showed increase in the amount of β-glucan and cell wall porosity (*p<0.05*) (Fig. 4G,H).

Changes in the amount of cell wall compounds and cell wall porosity compared to the control were observed after osmotic shock (10 min exposure)

(Fig. 4). Switched morphotypes of both *Switching* systems exhibited different responses to osmotic shock in comparison to the parental strains.

Responses of parental strains after osmotic shock comprise increased cell wall porosity for strain 49.07 (Fig. 4D), and increased mannan and β-glucan for strain 100.10 as well as decreased amount of chitin ($p<0.05$) (Fig 4.E,F,G). On the other hand, the switch-mediated responses that differed from Parental were increased amount of chitin (49.07 - RC) (Fig. 4A), increased mannan (Crepe - 49.07) (Fig. 4B) and increased cell wall porosity (RC - 100.10) ($p<0.05$) (Fig. 4H). Crepe revertant from the 100.10 system showed cell remodeling similar to the Parental strain, except for increased cell wall porosity (Fig. 4H).

Phenotypic *Switching* also promoted changes between morphotypes after osmotic shock. For the amounts of chitin and mannan in the cell wall, 75% and 50% of the tested switched morphotypes differ from the Parental ($p<0.05$), respectively (Fig. 4A,B,E,F). Differences between Crepe and its revertant were also noted. The amounts of β-glucan and mannan in the cell wall of the morphotype RC were higher in comparison to the amounts observed for the Crepe variant, for the systems 49.07 and 100.10, respectively (Fig 4. C,G). The Crepe variant showed greater cell wall porosity than RC for the 49.07 system (Fig. 4D).

Responses of parental morphotype (clinical strains) to osmoadaptation comprised decrease in the amount of chitin for both systems (49.07 and 100.10) (Fig 4.A,E), increase in the amount of mannan for system 49.07 (Fig 4.B), and increase in β-glucan and porosity for both systems (49.07 and 100.10) ($p<0.05$) (Fig 4.C,D,G,H). Switch morphotypes responses comprises increase in the amount of chitin (CR - 49.07); mannan (Crepe - 100.10) and decrease of β-glucan (Crepe - 100.10) ($p<0.05$).

After 60 min exposure to salt, all switched morphotypes showed higher amounts of chitin than the Parental strain (Fig. 4A,E). Decreased in β-glucan was observed in 50% of crepe variant of both systems (Fig. 4C,G). For cell wall porosity, 50% of the switch morphotypes had a less porosity than Parental ($p<0.05$) (Fig. 4D,H).

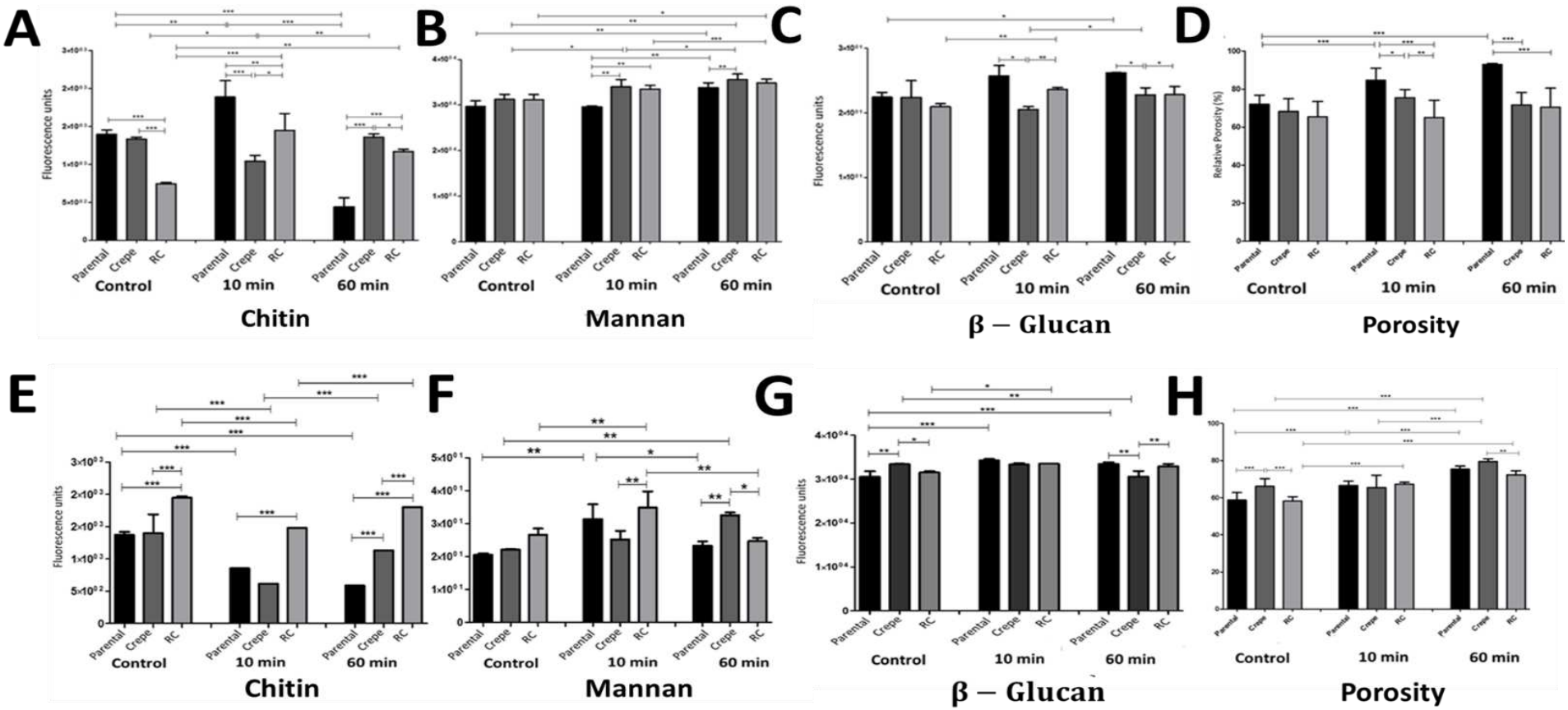

Fig 4. Cell wall compounds dynamics under osmotic stress (10 and 60 min) exposition for Parental, Crepe and RC morphotypes of 49.07 and 100 switch systems. (A) Amount of Chitin - 49.07 (stained whit CW), (B) Mannan – 49.07 (stained with FITC-CONA) and (C) β-Glucan – 49.07 (Stained with Aniline blue); (D) Cell wall porosity (49.07) based on the polycation-induced leakage of UV-absorbing. (E) Amount of Chitin – 100.10 (stained whit CW), (F) Mannan – 100.10 (stained with FITC-CONA) and (G) β-Glucan – 100.10 (Stained with Aniline blue); (H) Cell wall porosity (100.10) based on the polycation-induced leakage of UV-absorbing Control group – untreated with stress condition. Osmotic stress group (treated with 1M of NaCl for 10 and 60 min). Data were expressed in fluorescence units for cell wall compunds and relative data for porosity assay. *P< 0.05; **P<0.01; ***P<0.001.

3.5 Effect of phenotypic *Switching* on gene expression in response to osmotic stress.

Phenotypic *Switching* promoted alteration in gene expression of 75% of the switched morphotypes for *WOR1*; 75% for *EFG1* and 50 % for *HOG1* (*p<0.05*) (Fig. 5). In untreated condition, *WOR1* is upregulated in the Crepe morphotype of both systems, and downregulated in the revertant of crepe of the system 100.10, when compared to Parental strain (*p<0.05*) (Fig. 5A,D). The Crepe morphotype of the system 100.10 also showed greater expression compared to its revertant (*p<0.05*) (Fig. 5D). *EFG1* was upregulated in crepe and RC (49.07) (Fig 5B) and downregulated in RC for system 100.10 when compared with Parental strain (*p<0.05*) (Fig. 5E). *HOG1* was upregulated in Crepe morphotype (100.10) that showed greater expression than RC (*p<0.05*) (Fig. 5F).

Osmotic shock induces variation in the expression of tested genes. Furthermore, phenotypic *Switching* was capable of promoting the emergence of strains with greater expression than Parental strain in response to osmotic shock for all tested genes. The genes *WOR1*, *EFG1* and *HOG1* were upregulated in the Parental strain (49.07) in response to stress (Fig. 5). The Crepe morphotype showed a similar response pattern regulating *HOG1* and *WOR1*. Expression of *HOG1* on Crepe morhotype was greater than Parental (Fig. 5C). The Parental of the 100.10 system was downregulated for the *WOR1* gene after osmotic shock (Fig. 5D). For this system, RC morphotype showed increased expression of *WOR1* and *HOG1* (*p<0.05*) (Fig 5.D,F).

Responses to osmoadaptation comprised *WOR1* and *EFG1* overexpression of Parental (49.07) (*p<0.05*) (FIG. 5A,B). Crepe morphotype showed the same response, however, *HOG*1 is also overexpressed on this morphotype. For the 100.10 system the morphological revertant showed more pronounced upregulation of all genes tested (*p<0.05*).

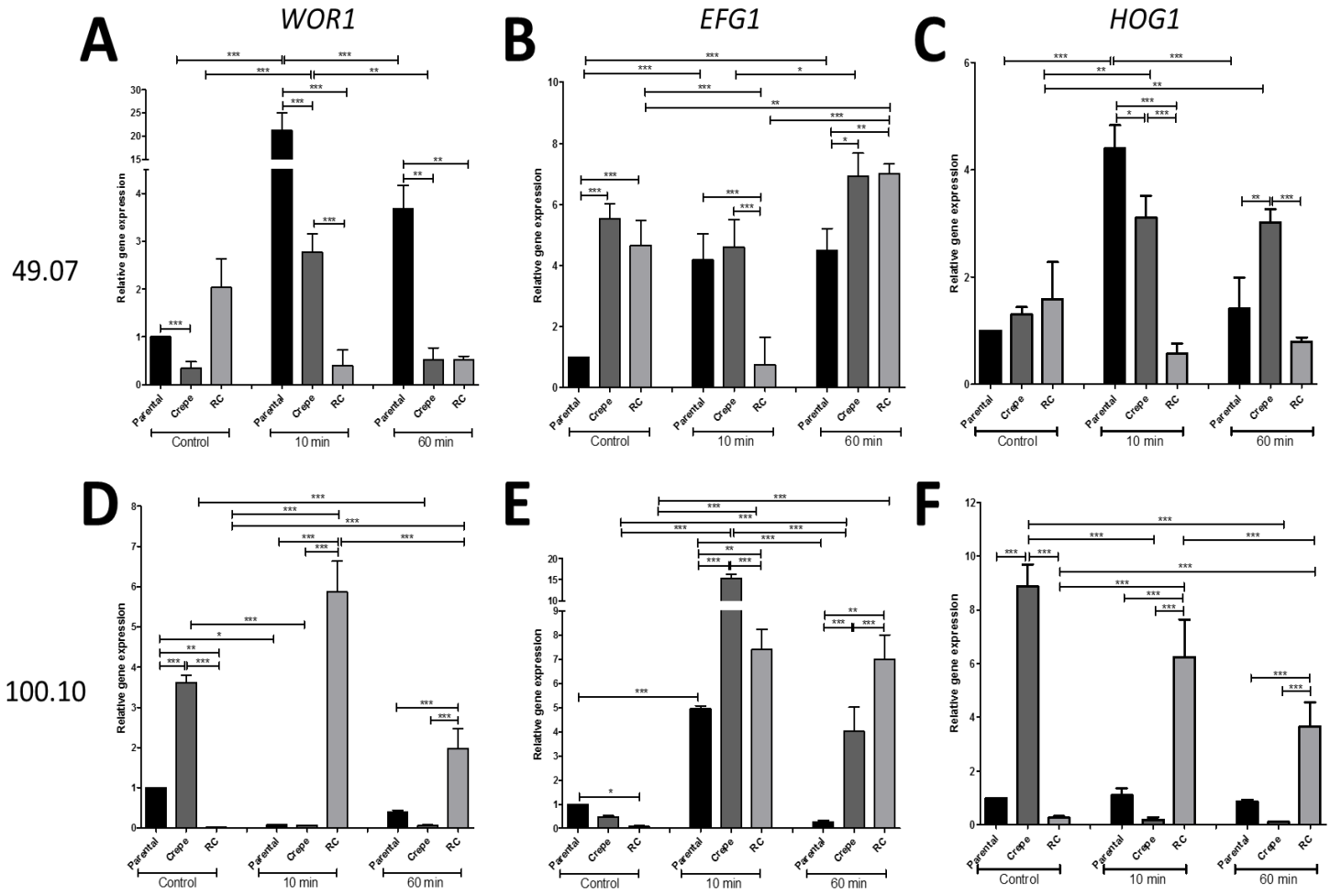

Fig 5. Relative gene expression of *C. tropicalis* switch system 49.07 (A) *HOG1*, (B) *WOR1,* (C) *EFG1* and 100.10 (D) *HOG1*, (E) *WOR1,* (F) *EFG1* in untreated (stress-free) condition (Control) and under osmotic stress (10 min and 60 min of exposure to 1M of H_2O_2). *P< 0.05; **P<0.01; ***P<0.001.

4 DISCUSSION

C. tropicalis is an important etiological agent related to high levels of mortality in candidemia cases (COLOMBO *et al*., 2007; KONTOYIANNIS *et al*., 2001). Modulation of virulence factors and virulence manifestation were associated with phenotypic *Switching* in *C. tropicalis* (MORALEZ *et al*., 2014; MORALEZ *et al*., 2016; PERINI *et al*., 2019; SOUZA *et al*., 2020). Here we demonstrated that phenotypic *Switching* also modulates cellular fitness in response to osmotic stress.

Pathogenic fungi such as *C. tropicalis* deal with changes in osmotic pressure within the human host (HERRERO-DE-DIOS; ALONSO-MONGE; PLA, 2014). High osmolarity levels (1M) can be found in the renal medulla (KWON *et al*.,

2009), and during phagocytosis *Candida* need to deal with K/Na fluxes (BROWN *et al*., 2014). Therefore, the ability to adapt to osmotic stress may be important for *Candida* colonization and virulence (HERRERO-DE-DIOS; ALONSO-MONGE; PLA, 2014). In the present work we report the raise of *C. tropicalis* morphotypes originated by phenotypic *Switching* that differ in response to osmotic stress compared to that observed for clinical strain (Parental).

Morphological variants of both switch systems tested (49.07 and 100.10) and morphological revertants of the 100.10 system showed more resistance to osmotic shock than Parental morphotype (clinical strain) (Fig. 1). Ene *et al*. (2015) showed that most reduction of cellular viability of *C. albicans* occurred after 5 to 10 min exposure of NaCl (1M). Here, we demonstrated this reduction in parental strain of *C. tropicalis*, however, phenotypic *Switching* promoted 62.5% morphotypes more resistant to osmotic shock than Parental. After osmotic shock, *C. albicans* shows cell volume reduction rapidly (30 to 60 s) and this volume is maintained after 10 min of salt exposure (ENE *et al*., 2015). Similarly, our data showed reduction in cell volume for all morphotypes tested, except for the Crepe variant of the system 49.07 (Fig. 2). Interestingly, Crepe showed increased cell volume after exposure to stress. After 60 min exposure (osmoadaptation), decrease in cell volume was maintained for all morphotypes. Crepe of both switch systems showed lower levels of iodide propidium dye (Fig. 3), indicating that osmotic shock promotes less damage on this morphotype, a characteristic that may be related to its greater survival. Responses to osmotic stress, in addition to resistance to osmotic shock, involve osmoadaptation. All morphotypes tested showed capacity to survive a 60 min of salt exposure. Phenotypic *Switching* promoted morphotypes more viable than Parental (Crepe and RC of 100.10 switch system). Crepe also showed less membrane damage on prolonged exposition.

Significant changes in cell wall architecture are related to osmotic shock response and cell volume decrease (ENE *et al*., 2015). The cell wall of *C. albicans* undergoes a dramatic increase in glucan and chitin immediately after exposure to salt (ENE *et al*., 2015). Here, phenotypic *Switching* promoted differences in content of cell wall compounds in untreated conditions (Fig. 4). Changes in the cell wall before exposure to salt may be related to the robustness of this structure and, consequently, to the cellular response to osmotic stress. After osmotic stress exposure morphotypes of *C. tropicalis* showed changes in the amount of wall compounds different from the

Parental response which may be related to the difference in cellular fitness of morphotypes.

Chitin represents the minor cell wall component in terms of biomass, however, this component is related to cell wall stability and rigidity (KLIS, 1994; KOLLÁR *et al*., 1997; MUNRO *et al*., 2001; SHEPHERD, 1987). On osmoadaptation, the Crepe variants and their morphological revertants of both switch systems showed increased chitin content. Increase of chitin layer was related to survival to osmotic stress (ENE *et al*., 2015). After prolonged exposure to salt, yeast cells may be able to biosynthesize cell wall compounds, unlike the reorganization caused by decreased on the cell volume caused by osmotic shock (ENE *et al*., 2015). Phenotypic *Switching* seems to be associated with alteration on the biosynthesis capacity of wall components in *C. tropicalis*, and as consequence a better response to environmental changes. Parental also showed an increase in cell wall porosity in response to osmotic shock and under osmoadaptation. Crepe and RC (both systems) did not show variations after osmotic shock and osmoadaptation (49.07). These data suggest that the modulation of the osmotic stress response mediated by *Switching* is related to structural and physiological characteristics of the cell wall. Once cell wall displays a high degree of biophysical and biochemical flexibility, which influences its ability to survive acute osmotic challenges, phenotypic *Switching* may be related to successful host niches through stress-response plasticity.

The Hog1 (High-osmolarity glycerol 1) has key functions in the core osmotic stress response in *C. albicans* (SMITH *et al*., 2004; SMITH; MORGAN; QUINN, 2010). In the present study, phenoytpic *Switching* promoted the emergence of *C. tropicalis* Crepe morphotype (100.10) with up-regulated *HOG1* under control conditions (no exposure to NaCl) (Fig 5). In *C. albicans*, exposure to hyperosmotic stress activates the Hog1 mitogen-activated protein kinase (MAPK) pathway. The pathway stimulates glycerol biosynthesis inducing intracellular osmolyte accumulation. Restoration of turgor pressure allows for osmoadaptation and growth resumption (KLIPP *et al*., 2005; NAVARRO-GARCÍA *et al*., 2005; SAN JOSÉ *et al*., 2006). In the present study, we demonstrated that the expression of *HOG1* is overexpressed in switched strain, which may be related to the better stress responses.

Under Osmoadaptation *HOG1* was upregulated in Crepe (49.07) and RC (100.10), but not in Parental strain. *HOG1* signaling is required to survival of *C.*

albicans under osmotic stress (KLIPP *et al*., 2005; NAVARRO-GARCÍA *et al*., 2005; SAN JOSÉ *et al*., 2006), however not essential immediately after osmotic shock (ENE *et al*., 2015). Besides that, *HOG1* mutants were avirulent in a systemic model of infection in mice and more susceptible to phagocytosis (ALONSO-MONGE *et al*., 1999; ARANA *et al*., 2007; CHEETHAM *et al*., 2011). Changes in regulation of *HOG1* expression mediated by *Switching* was demonstrated in present study, placing this event an important fitness promoter on *C. tropicalis*.

HOG1 pathway also participates in the hypha formation, cell wall biogenesis, and virulence (ALONSO-MONGE *et al*., 1999, 2003; SAN JOSÉ *et al*., 1996; SMITH *et al*., 2004). Our group described that phenotypic *Switching* in *C. tropicalis* are capable to alter the virulence manifestation and host-pathogen relationship (MORALEZ *et al*., 2014; MORALEZ *et al*., 2016; PERINI *et al*., 2019; SOUZA *et al*., 2020). Here we demonstrated cell wall alterations mediated by *Switching*. *EFG1* and *WOR1* act in the regulation of the white-opaque system in *C. albicans* and *C. tropicalis* and regulate the morphogenesis process and biofilm formation (LIN; CHEN, 2018). In the present study, we demonstrate the expression of these transcription factors in a non-white-opaque system and the expression profile of these genes under osmotic stress.

Phenotypic *Switching* promoted up-regulation of *WOR*1 in crepe morphotype (100.10) and *EFG1* in Crepe and RC (49.07) morphotypes. Under osmotic shock both genes were overexpressed in Parental morphotype (49.07). Up-regulation of genes involved in morphogenesis and phenotypic *Switching* by clinical isolates indicate that *C. tropicalis* may respond to osmotic stress by activating pathways that culminate in variability.

In the present work we demonstrated the effect of phenotypic *Switching* on the osmotic response of *C. tropicalis.* The response of cell wall alteration, in addition to the gene regulation of stress response pathways and morphological variations place the phenotypic *Switching* as an important promoter of strains more resistant to stress and, consequently, with greater potential for survival in the host.

5 CONCLUSION

In conclusion, phenotypic *Switching* promoted the emergence of morphotypes more resistant to osmotic stress. *Switching* promoted morphotype-dependent responses that result in adaptive variability, which reflect the main characteristics of the cell wall and gene expression. Understanding of *Switching*-mediated mechanisms of variability can provide important information for knowledge the pathogenesis of *C. tropicalis*.

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ARTIGO 2

PHENOTYPIC *SWITCHING* IN *Candida tropicalis* PROMOTES CHANGES TO OXIDATIVE STRESS RESPONSE AND PHAGOCYTOSIS BY HEMOCYTES AND MACROPHAGES

Abstract

Phenotypic *Switching* is related to the emergence of more virulent strains in *Candida tropicalis,* with alterations in host-pathogen relationships using *the Galleria mellonella* model. In this study, we evaluated the effect of phenotypic *Switching* on oxidative stress and stress response modulation in phagocytosis and gene expression. Phenotypic morphotypes of the *C. tropicalis* 49.07-switch system were exposed to 5 mM H_2O_2, and phagocytic assays employing hemocytes and macrophages were performed. The expression of *HOG1, WOR1*, and *EFG1* genes and changes in cell wall composition and porosity were examined. We showed the emergence of a variant morphotype (Crepe) that is more resistant to oxidative stress. Crepe was recognized by both the phagocytes tested, and pre-exposure to H_2O_2 promoted a decrease in phagocytic capacity. Hemocytes and macrophages showed the same pattern of phagocytosis. The Crepe morphotype showed high capacity for true hyphal formation (30%) in co-culture with hemocytes and under stress exposure. In addition, these morphologic variants showed upregulation of *HOG1, WOR1*, and *EFG1* under stress conditions. These genes were also upregulated in the parental strain during phagocytosis by hemocytes. Further, we demonstrated the expression of *WOR1* in a non-white-opaque phenotypic switch system. Exposure to stress increased the chitin content in the parental and Crepe morphotypes, and decreased cell wall porosity in the parental strain. The changes in hyphae formation capacity, upregulation of phenotypic *Switching*, and stress response genes induced by oxidative stress in the Crepe morphotype may be related to decreased recognition and phagocytosis by hemocytes and macrophages, which can alter the efficiency of the host immune response. Overall, we demonstrated alterations in fitness attributes in response to stress mediated by phenotypic *Switching* in *C. tropicalis*, characterizing this event as an important virulence factor in the species, as well as an important variable in the resistance to host defense strategies.

Keywords: *Candida tropicalis*, Phenotypic *Switching*, *Galleria mellonella,* Hydrogen peroxide.

INTRODUCTION

Candida tropicalis is one of the most important non-*C. albicans* species, in terms of epidemiology and virulence (Zuza-Alves et al., 2017). This species represents the second or third etiological agent of candidemia, especially in tropical regions (Costa et al., 2014; Da-Mata et al., 2017; Rodriguez et al., 2017), and shares high phylogenetic similarity with *C. albicans* (Butler et al., 2009; Zuza-Alves et al., 2017). The genetic similarity between *C. albicans* and *C. tropicalis* is so pronounced that these species share the ability to produce true hyphae (Zuza-Alves et al., 2017).

Morphogenetic ability is one of the most important steps for the establishment of blood infections by *Candida* and is considered a crucial step in the manifestation of virulence progression, including invasion of host tissues, endothelial rupture, and survival in phagocytic cell attack (Lackey et al., 2013). In *C. albicans*, the capacity of hyphae formation within the phagosome was shown to contribute to the ability of *C. albicans* cells to escape phagocytosis and kill macrophages (Marcil et al., 2002; Ghosh et al., 2009; McKenzie et al., 2010). *In vitro* coculture of *Galleria mellonella,* hemocytes and *C. tropicalis* cells promotes the phagocytosis of yeast cells, but also promotes a decrease in hemocyte number, suggesting the ability of *C. tropicalis* to kill these hemocytes (Perini et al., 2019).

Another virulence factor, phenotypic *Switching*, is directly related to the morphological transitions of *C. tropicalis* (Moralez et al., 2016). This reversible epigenetic event emerges naturally in a yeast population and occurs more frequently compared to somatic mutations (Slutsky et al., 1987). Phenotypic *Switching* is recognized as a strategy to promote phenotypic variability in isogenic populations, allowing microorganisms to adapt to environmental changes (Soll, 1992; Slutsky et al., 1987; Porman et al., 2011). In *C. tropicalis*, our group described the occurrence of changes in the virulence factors of clinical isolates under the effect of phenotypic *Switching* (França et al., 2011; Moralez et al., 2014; Moralez et al., 2016). These changes were observed in adhesion capacity and biofilm formation, hemolytic factor secretion (França et al., 2011), morphogenesis (Moralez et al., 2014), damage to epithelial cells, and virulence in *G. mellonella* (Moralez et al., 2016). Further, phenotypic *Switching* can also alter sensitivity to antifungal drugs (Moralez et al., 2014) and the host-pathogen relationship in *the G. mellonella* model (Perini et al., 2019).

In healthy mammalian hosts, the innate immune system is the first line of defense against *Candida* species (Dantas et al., 2015). Phagocytes, including macrophages and neutrophils, can produce reactive oxygen species (ROS) in response to *Candida* infection through a process known as the respiratory burst (Babior, 2004; Dantas et al., 2015). In the phagosome, other ROS compounds can interact with yeast, creating a toxic environment that induces oxidative stress (Brown et al., 2009; Dantas et al., 2015). Hydrogen peroxide (H_2O_2) can react with chloride ions (Cl^-) to form hypochlorous acid (HOCl) in a reaction catalyzed by myeloperoxidase (Brown et al., 2009).

The immune system of insects such as *G. mellonella*, exhibits a high degree of structural and functional similarity with the innate immune system of mammals (Kavanagh and Reeves, 2004). The cellular response of *G. mellonella* is mediated by hemocytes, which also exhibit phagocytic capacity (Ratcliffe, 1993). These cells use ROS as a means of attacking pathogens, both O_2^- (Glupov et al., 2001) and its dismutation product, H_2O_2 are known to be active in *G. mellonella* (Slepneva et al., 1999). Furthermore, as an alternative model for the use of mammalian hosts, *G. mellonella* has already proven to be efficient for virulence studies of the pathogenic fungus *C. tropicalis* (Moralez et al., 2016), showing highly sensitive humoral and cellular responses for this species (Perini et al., 2019).

In contrast, pathogens such as *C. tropicalis* require several virulence attributes to overcome the host immune system and establish infection (Zuza-Alves et al., 2017; Galocha et al., 2019). Resistance to oxidative stress is an important step for escape from immune cells, as ROS can induce programmed cell death in this yeast (Phillips et al., 2003). In addition, *C. albicans* cells can develop an oxidative stress response prior to phagocytosis (Miramón et al., 2012). Three signaling pathways of *C. albicans* are directly activated in response to ROS: the Cap1 transcription factor, Hog1 stress-activated protein kinase, and Rad53 DNA damage checkpoint kinase. Among these, Hog 1 is rapidly phosphorylated upon exposure to H_2O_2, and accumulates in the nucleus (Enjalbert et al., 2006).

Phenotypic *Switching* promotes the emergence of morphotypes with pronounced virulence factors as well as alterations in the response front of *G. mellonella* hemocytes that use reactive oxygen species to kill pathogens. In the present study, we analyzed the phenotypic *Switching* effects in response to oxidative stress.

To this end, we employed a *Switching* system (49.07) that was previously described by our group (Moralez et al., 2014). We evaluated the resistance to oxidative stress caused by H_2O_2, the gene regulation of morphogenesis and switch transitions (*WOR1* and *EFG1*) and stress response (*HOG1*), and the relationship between pre-exposure to H_2O_2 and phagocytosis by macrophages and hemocytes. These findings could provide information on the role of *Switching* in *C. tropicalis* pathogenesis and fitness attributes.

MATERIAL AND METHODS

Microbial strains and culture conditions

Morphotypes (original phenotype-parental, crepe variant, crepe revertant, rough variant and rough revertant) of the *Switching* phenotypic system 49.07 of *C. tropicalis* used in this study were stored as frozen stocks with 20% glycerol at −80 °C and subcultured on YPD agar plates (1% yeast extract, 2% peptone, and 2% dextrose) at 28 °C. Morphotypes were routinely grown in YPD liquid medium at 28 °C in a shaking incubator and plated on YPD agar plates at 28 °C for 96 hours.

Quantitative analysis of stress sensitivity

The analysis of stress sensitivity was realized according Nikolaou et al., (2009) with modifications. To semi-quantitatively compare the stress resistance to hydrogen peroxide (H_2O_2) of the *C. tropicalis Switching* system, the percentage of growth of each morphotype was calculated relative to their non-stress control for each stress condition.

C. tropicalis cells ($1x10^8$ cells/mL) were exposed to 5 mM of H_2O_2 for 10 min and 60 min, in broth. Cells were pelletized by centrifugation (4000 RPM, 5 min) and serial dilutions were performed. Cells were plated in YPD medium and number of UFC were measured for direct observation. Stress condition tested was counted and expressed as percentages of those on the corresponding control plates (same dilution without stress). Moreover, to measure relative growth, phenotypic morphotypes were plated in YPD medium-solidify containing 5 mM of H_2O_2. Amount of growth in the presence of stress was divided by the amount of growth observed for unstressed cells of the same morphotype and expressed as a percentage. Each experiment was realized in quadruplicate and repeated at least 3 times.

Cell wall staining for fluorimetry

Once the crepe morphotype showed a better response to oxidative stress, the following tests were performed with Parental and crepe morphotype. To evaluate the effect of phenotypic *Switching* in response to oxidative stress in *C. tropicalis*, this morphotypes cells exposed (10 min) and non-exposed to hydrogen peroxide (5 mM) were washed 3 times with ultrapure water and fixed with 10 % paraformaldehyde solution in PBS. Fixed cells ($1x10^6$ cells/mL) were stained independently with 100 µg/mL of Calcofluor White (CFW), 50 µg/mL of Aniline Blue and 100 µg/mL FITC-Concanavaline A conjugated (FITC-CONA) (Okada; Ohya., 2016).

Cells were incubated for 10 min at room temperature in the dark. Following incubation, cells were washed twice with PBS. Stained samples were analyzed using GloMax® Explorer (Promega) instrument with 415-445 nm emission filter and 405 nm excitation filter to Aniline Blue; 415-445 nm emission filter and 365 nm excitation filter to CFW; 500-550 nm emission filter and 475 nm excitation filter to FITC-CONA. Data were expressed in fluorescence units. Each experiment was performed in triplicate on tree different occasions.

Cell wall porosity assay

Cell wall porosity assay is based on the polycation-induced leakage of UV-absorbing compounds from cells (DE NOBEL et al., 1990). This assay compare leakage induced by small polycations (poly-L-lysine, which induces cell leakage independent of cell wall porosity) with the release caused by large polycations (such as DEAE-dextran, which cause limited leakage depending upon the degree of porosity of the cell wall).

The assay was preformed according Ene et al. (2012) with modifications. Exposed (H_2O_2 -10 min) and non-exposed cells are collected and washed twice with deionized water. Aliquots of $1x10^8$ cells were incubated with shaking (200 rpm) for 30 min at 30 °C in 1 mL of either 10 mM Tris-HCl pH 7.4 (control), the same buffer containing 5 mg/ml-1 DEAE-dextran (500 kDa) or buffer containing 15 mg/mL poly-L-lysine (50 kDa). Cells were then pelleted by centrifugation and the A_{260} of supernatants measured. Relative porosity was calculated using the following formula: relative porosity = 100 x (A_{DEAE} - A_{buffer}) / ($A_{poly\text{-}L\text{-}lysine}$ - A_{buffer}). Results are obtained of three independent experiments, each with three technical replicates.

Galleria mellonella **larvae manipulation and hemolymph collection**

G. *mellonella* larvae in the final stage without color alterations and with adequate weights (240–300 mg) were selected. The ventral region was cleaned with 70% ethanol before hemolymph collection.

To collect the hemolymph, we held the larvae in its ventral position, punctured one of the central pro-legs and collected 10 µl of the hemolymph. Adipose tissue and any liquid of a dark color were discarded. The collected hemolymph of ten larvae was transferred to a microtube containing 900 µL of IPS (insect physiological saline: 150 mM sodium chloride (Promega, USA), 5 mM potassium chloride (Promega), 10 mM Tris HCl (Promega) pH 6.9, 10 mM EDTA (Promega) and 30 mM sodium citrate (Sigma-Aldrich, USA) plus 10 mM N-ethylmaleimide (Sigma-Aldrich) (anticoagulant).

Tubes were placed on ice, and sample collection was carried out immediately to avoid cell melanization. After centrifugation at 2000 rpm and at 4 °C for 5 min, the supernatant was discarded, cells were washed with 500 µl of cold IPS and the contents of two tubes (or several, depending on the number of assays) were pooled together in a new tube. A second centrifugation was performed under the same conditions. The supernatant was again discarded, and the cells were resuspended in 1000 µl IPS. The number of cells was determined with a Neubauer chamber.

Peritoneal Macrophages

Mice weighing approximately 25–30 g and aged 6–8 weeks under sterile conditions were maintaining according to protocols approved by the Institutional Animal Care and Committee. The animals were euthanized by Ketamine-Xylazine overdose (100 mg/kg - 10 mg/kg, i.p.) followed by cervical dislocation and peritoneal macrophage were obtained. Macrophages (5 × 105 cells/mL) were recovered according Tommiotto-Pellissier et al. (2018) from the peritoneal cavity of BALB/c mice with cold PBS supplemented with 3% of FBS and then cultured in 24-well plates with 200 µL of RPMI 1640 medium (10% FBS) for 2 h (37 °C, 5% CO_2). This study was approved by the Ethics Committee for Animal Experimentation of the State University of Londrina (OF. CIRC. n°. 3715.2015.22).

Phagocytic assay

To analyze the effect of pre-exposition to oxidative stress on the phagocytosis by hemocytes and macrophages, *C. tropicalis* morphotypes (Parental and Crepe) were exposed to H_2O_2 for 10 min. Following, for enumeration of viable cells 0.5 mL of each sample suspension were mixed with 0.1 mL of 0.4% trypan blue stain (Sigma–Aldrich, USA). After 5 min, the number of viable cells was adjusted to a concentration of $5x10^6$ cells/mL.

The phagocytic assay was realized according to Tommioto-Pelisser et al. (2018) and Perini et al, (2019) with modifications. To adhere hemocytes and macrophages, 13-mm round coverslips that were treated with acetic acid were placed in a 24-well plate. Each well was filled with a volume corresponding to 1×10^5 phagocyte cells and was brought to a total volume of 1000 µL with RPMI 1640 medium (Gibco, USA) plus 10% fetal bovine serum (FBS, Gibco®, Brazil). The plates were incubated for 1 h at 37 °C with 5% CO_2 to allow cell adherence. Then, non-adherent cells were removed by washing with RPMI 1640 medium at room temperature, and the adhered cells were co-incubated with each morphotype strain at a 1:5 ratio.

Phagocytosis was allowed to occur for 2 h; samples were then washed to remove the non-adherent yeast. In sequence, samples were fixed with 1 mL cold methanol (Sigma-Aldrich, USA) for 20 min. Then, cells were stained with May-Grünwald (Laborclin, Brazil) for 15 min, washed with Sorenson's buffer (0.133 M Na_2HPO_4 and 0.133 M KH_2PO_4) and immersed in Giemsa dye (Laborclin, Brazil) for 15 min. Finally, the coverslips were washed again with Sorenson's buffer, air dried, and mounted on glass slides. Under an optical microscope (1000 times magnification), cells from 20 fields in three distinct experiments were analyzed and quantified according to the number hemocytes and macrophages containing internalized yeast cells.

Morphogenesis assay

To estimate the number of filamentous forms, direct counting of 20 fields of co-culture with macrophages and hemocytes was performed. Percentage of pseudohyphae and hyphae was relativized with a total number of yeasts cells.

RNA extraction and gene expression analysis (RT-qPCR)

The gene expression was tested on (1) control group - *C. tropicalis* morphotypes (Parental and Crepe colonies) not exposed to oxidative stress; (2) stressed group – phenotypic *Switching* morphotypes exposed to oxidative stress for 10 min; and (3) phenotypic *Switching* morphotypes not exposed (3.1 – Control) and exposed to oxidative stress for 10 min (3.2 – stressed group), after co-culture with hemocytes and macrophages. These cells were homogenized, frozen in liquid nitrogen and RNA was extracted and purified using an RNA Mini Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. RNA was quantified and the quality assessed using a NanoDrop spectrophotometer (ThermoScientific, Loughborough, United Kingdom). To phagocytosis analysis, RNA was extracted after co-incubation with phagocytes. cDNA was synthesized from 200 ng of extracted RNA using an RT-PCR kit (Invitrogen, Carlsbad, CA, EUA) in a GeneAmp® PCR (Eppendorf, Gradiente Mastercycler) following the manufacturer's instructions.

Primers used for quantitative PCR (qPCR) were as follows: *HOG1* (forward, 5'-TTGCCAGTGGATACTTGGAG–3'); *HOG1* (reverse 5'–GTTGTTGTTCAGCACCATCG–3'); *WOR1* (forward 5'-CCGTCTAATGTTATACCTGCATCAA-3'); *WOR1* (reverse 5'-TTCGTCGTACTTATGGTAATTGTTTTCT-3'); *EFG1* (forward 5-TTCAACTGCTGGACAACCAC-3'); *EFG1* (reverse 5'-TACCAGGAGGTTGGAATTGG-3'); *β-actin* (forward, 5′-GGGACGATATGGAGAAGATCTG-3′); and *β-actin* (reverse, 5′-CACGCTCTGTGAG GATCTTC-3′).

The cycling conditions consisted of 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 51 °C and 60 s at 60 °C. Each sample was analyzed in duplicate using a StepOnePlus™ Real-Time PCR System (Applied Biosystems®). The reactions were performed using Platinum® SYBR® Green qPCR Supermix-UDG (Invitrogen, Carlsbad, CA, USA) with a final volume of 20 µl. Relative gene expression was calculated using the $2^{-(\Delta-\Delta Ct)}$ method.

Statistical analysis

Three independent experiments were performed in triplicate for all experiments. All data were previously submitted to the F test and the Shapiro-Wilk test

for analysis of variance and normality test, respectively. Comparison between the groups were done by one-way ANOVA, followed by Tukey's test for multiple comparisons. For stress exposition analysis the t-paired test was used. Statistical significance was set at *P<0.05, **P<0.01, ***P<0.001. All statistical analyses were performed by GraphPad Prism 5 statistical software (GraphPad Software, Inc., USA, 500.288). Data were expressed as mean ± standard deviation.

RESULTS

Sensitivity of phenotypic *Switching* morphotypes to oxidative stress caused by hydrogen peroxide (H_2O_2)

To evaluate the ability of *C. tropicalis* to resist oxidative stress, morphotypes were exposed to 5 mM H_2O_2 for 10 min and 60 min (Fig. 1).

After 10 min of H_2O_2 exposure, all *C. tropicalis* morphotypes were viable, with growth capacity of up to 90% compared to the growth of non-exposed cells; however, phenotypic *Switching* promoted the emergence of strains (variant morphotypes) that were less sensible (20%) compared to the parental strain (clinical isolate). Switch variants (complex colony structure) showed more resistance compared to smooth morphotypes (parental and morphological revertants) after 10 min of stress exposure ($p < 0.001$). Interestingly, the rough revertant morphotype, despite the same phenotype, showed lower stress resistance than that in the parental strain ($p < 0.001$) (Fig. 1).

After 60 min of H_2O_2 exposure, the effect of phenotypic *Switching* on oxidative stress sensitivity was more pronounced. All morphotypes showed reduced viability when compared to non-exposed cells (Fig. 1). Viability was also lower than that after H_2O_2 exposure for 10 min ($p < 0.001$). The Crepe variant showed less sensitivity to oxidative stress ($p < 0.001$) compared to the parental strain and its Crepe revertant counterpart. In addition, the morphological revertants showed the same response pattern as the parental strain, which reflects, at least in part, that the return of variant phenotypes to the parental phenotype may be related to the reestablishment of the response pattern presented by the clinical isolate.

Moreover, to evaluate the effect of oxidative stress on the viability of *C. tropicalis* switch systems, we cultivated the morphotypes in the presence of a stressor for 96 h. Under these conditions, no growth was observed (data not shown).

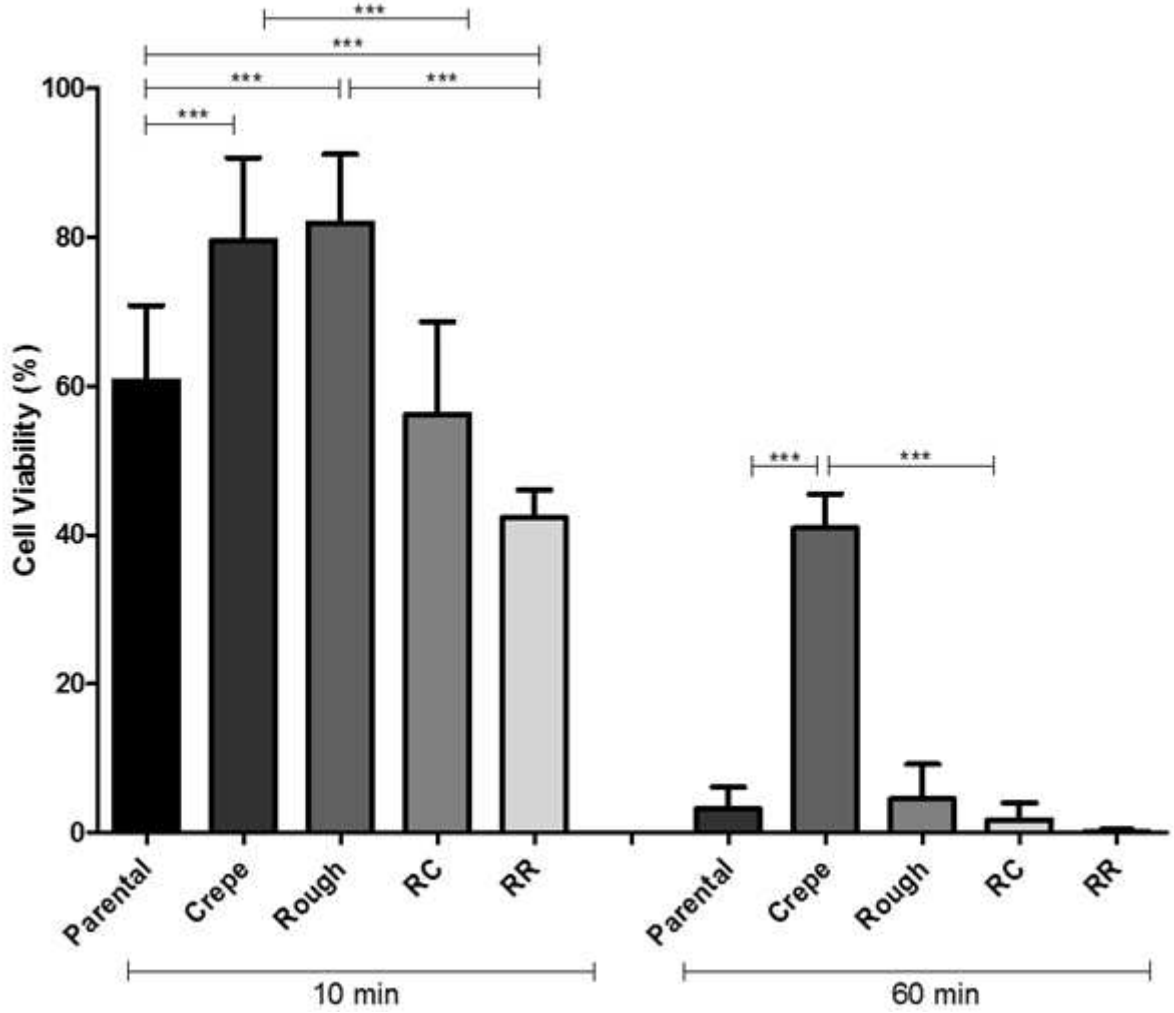

Fig 1. Oxidative stress sensitivity of *C. tropicalis Switching* system 49.07 after exposure to H_2O_2 for 10 min and 60 min. Data were showed in percentage of growth relativized to non-stress condition growth. Representative phenotypic *Switching* event. Parental morphotype (smooth colony), morphological variants – Crepe and Rough (structured colony) and morphological revertants (smooth colony like Parental strain). *P< 0.05; **P<0.01; ***P<0.001.

Effect of phenotypic *Switching* on HOG1, WOR1, and EFG1 gene expression in response to oxidative stress

As shown in Fig. 2, phenotypic *Switching* is associated with upregulation of *EFG1* (enhanced filamentous growth protein 1 – transcription regulator of phenotypic *Switching* and morphogenesis) by the Crepe variant, whereas the expression of *WOR1* (white-opaque regulator 1, the master regulator of white-opaque

transitions) was downregulated, compared to the expression observed in the parental strain. Oxidative stress exposure promotes the upregulation of *HOG1* (mitogen-activated protein kinase – stress response and *EFG1* in the parental strain. In the morphotype under the *Switching* effect (Crepe), the upregulation of tested genes was more pronounced after stress exposure. The Crepe morphotype showed an increase in *HOG1*, *WOR1*, and *EFG1* gene expression with greater expression compared to the parental strain. Interestingly, the expression of *WOR1* by the parental strain was repressed under oxidative stress conditions.

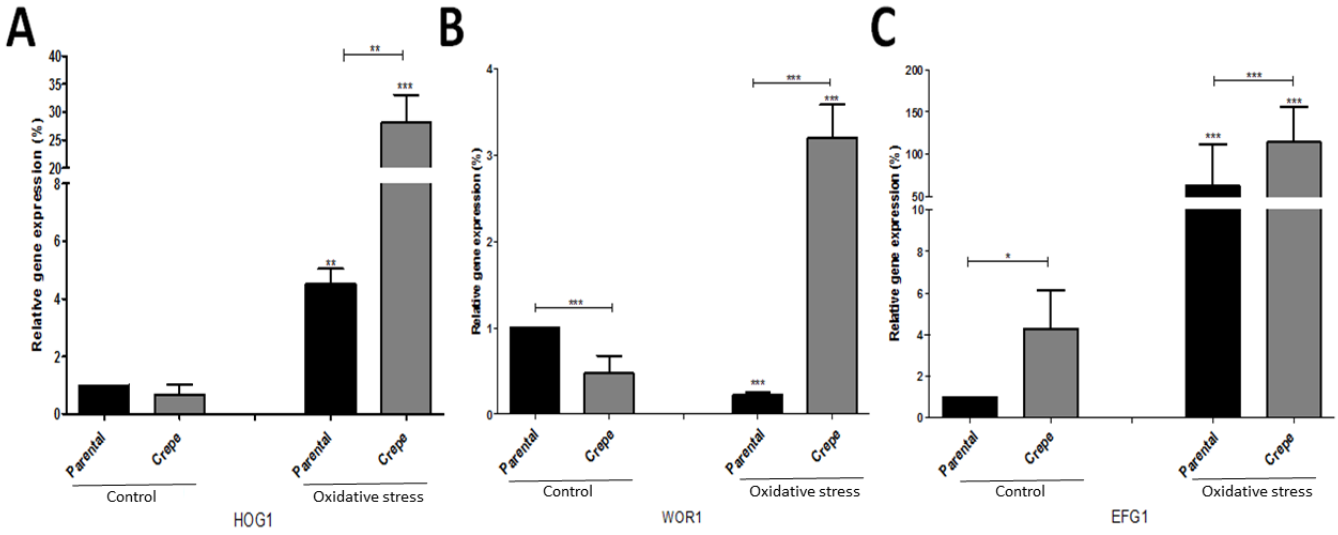

Fig 2. Relative gene expression of HOG1 (A), WOR1 (B) and EFG1 (C) of *C. tropicalis* Parental strain and Crepe switch variant untreated with stress condition (Control) and under oxidative stress (10 min of exposure to H_2O_2). *P< 0.05; **P<0.01; ***P<0.001.

Effect of oxidative stress in cell wall compounds

No phenotypic *Switching*-mediated changes were observed in cell wall compounds (chitin, mannan, and B-glucan). However, exposure to oxidative stress promoted an increase in chitin content in the parental and Crepe morphotypes (p < 0.001). Stress also increased the amount of mannan in the parental strain (p < 0.05) (Fig. 3). Although there were no changes in the amount of cell wall compounds mediated by *Switching*, this event may be related to the organization and distribution of these components.

Phenotypic *Switching* did not promote changes in cell wall porosity under untreated conditions; however, exposure to oxidative stress promoted a decrease in the parental cell wall porosity (p < 0.001). Under these conditions, the Crepe

morphotype showed higher cell wall porosity than that in the parental strain ($p < 0.01$) (Fig. 3).

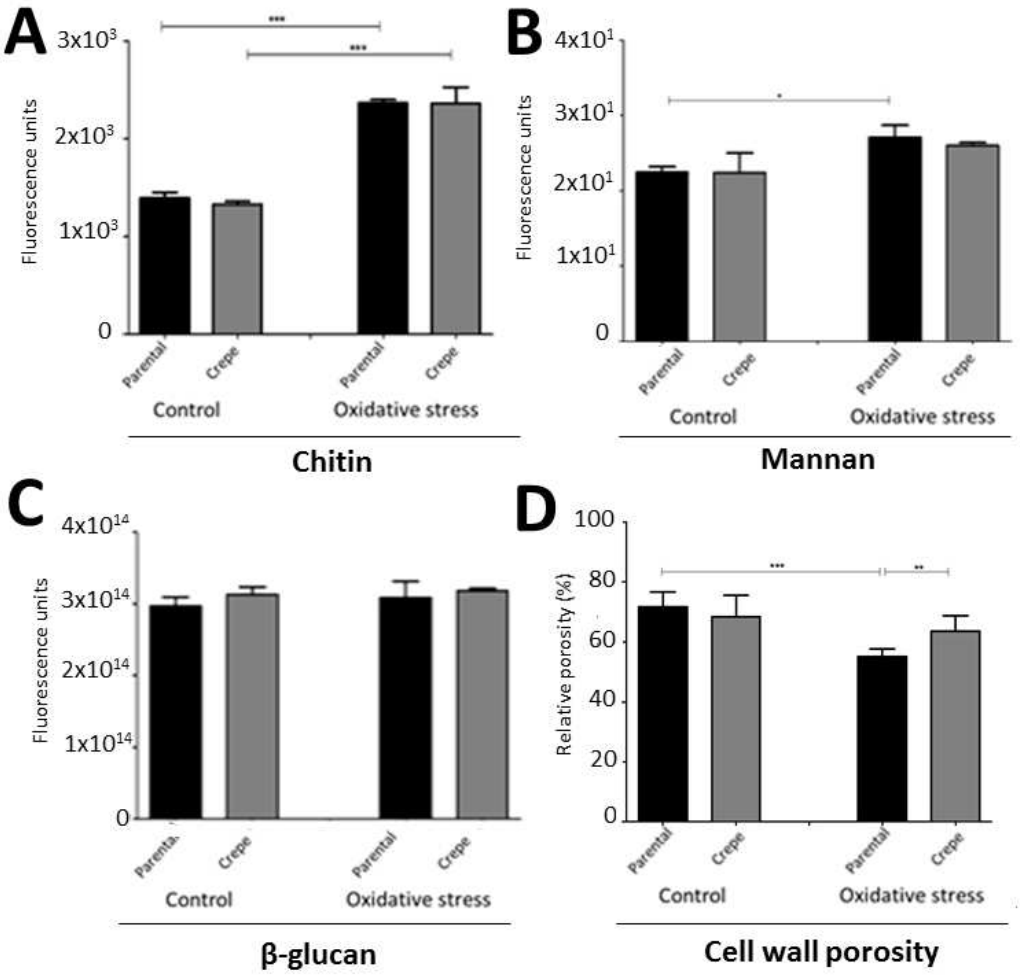

Fig 3. Cell wall compounds dynamics under oxidative stress exposition for Parental and Crepe morphotypes. (A) Amount of Chitin (stained whit CW), (B) Mannan (stained with FITC-CONA) and (C) β-Glucan (Stained with Aniline blue); (D) Cell wall porosity based on the polycation-induced leakage of UV-absorbing. Control group – untreated with stress condition. Oxidative stress group – treated with 5 mM of H_2O_2 for 10 min. Data were expressed in fluorescence units for cell wall compunds and relative data for porosity assay. *P< 0.05; **P<0.01; ***P<0.001.

Effect of *C. tropicalis* phenotypic *Switching* and oxidative stress exposure on phagocytosis by macrophages and hemocytes

To analyze the relationship between phenotypic *Switching*, oxidative stress, and phagocytosis ability, murine macrophages and *G. mellonella* hemocytes were co-incubated *in vitro* with parental and crepe variant cells, before and after hydrogen

peroxide exposure. Macrophages and hemocytes showed the same pattern of phagocytosis in both *C. tropicalis* morphotype (parental and crepe variant) cells (Fig. 4). Cells of the Crepe variant were more phagocytosed compared to cells of the parental strain, indicating that they are differently recognized by phagocytic cells. In contrast, after exposure of cells to oxidative stress, the same rate of phagocytosis was observed (Fig. 4 A,B). Cells of the crepe variant were less phagocytosed by both phagocytic cell types (macrophages and hemocytes) after exposure to H_2O_2 compared to the control cells (untreated) (Fig. 4 A,B).

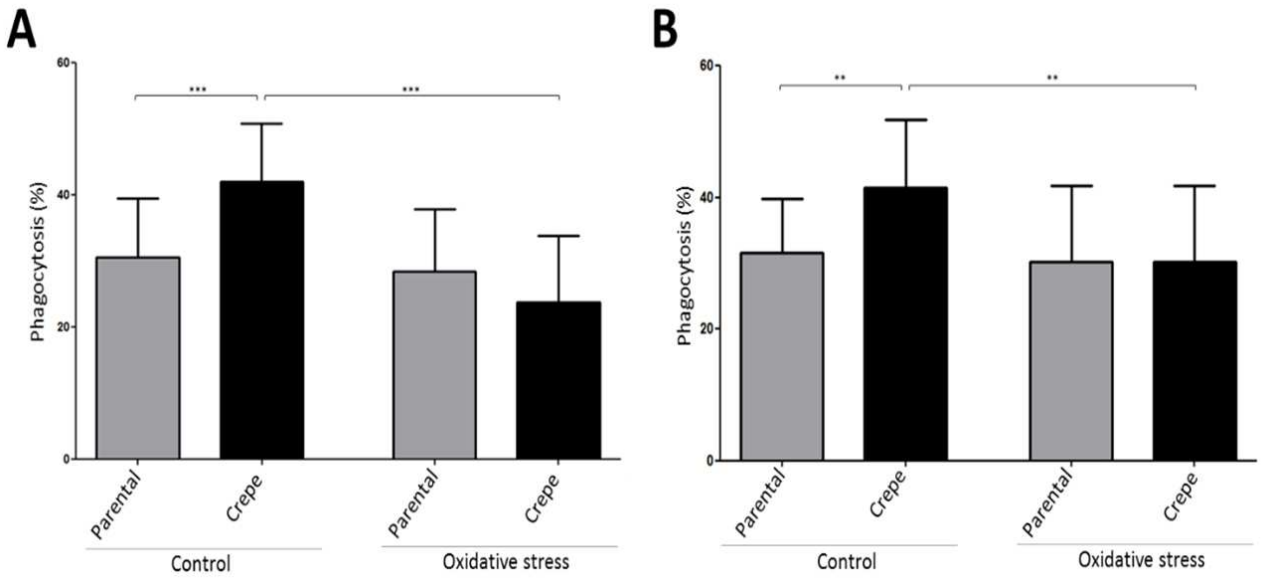

Fig 4. Phagocytosis by *Galleria mellonella* hemocytes (A) and *Mus musculus* macrophages (B) of *C. tropicalis* Parental strain and Crepe morphotype. Control group – untreated with stress condition. Oxidative stress group – treated with 5 mM of H_2O_2 for 10 min. *P< 0.05; **P<0.01; ***P<0.001.

Effect of hemocyte and macrophage phagocytosis on HOG1, WOR1, and EFG1 gene expression in *C. tropicalis* morphotypes

Real-time quantitative PCR analysis showed that the expression of *HOG1, WOR1*, and *EFG1* genes was distinct depending on the type of phagocytic cells used (Fig. 5). Phagocytosis by hemocytes upregulated the expression of all genes tested in both morphotypes; however, in the parental strain, their expression was higher than that in the Crepe morphotype ($p < 0.001$). The increased mRNA levels of genes that mediate phenotypic transition (*WOR1* and *EFG1*) and stress response (*HOG1*) may be related to the response of *G. mellonella* immune system cells. Phagocytosis by

macrophages showed only upregulation of the morphological regulator gene (*WOR1*) in the Crepe morphotype.

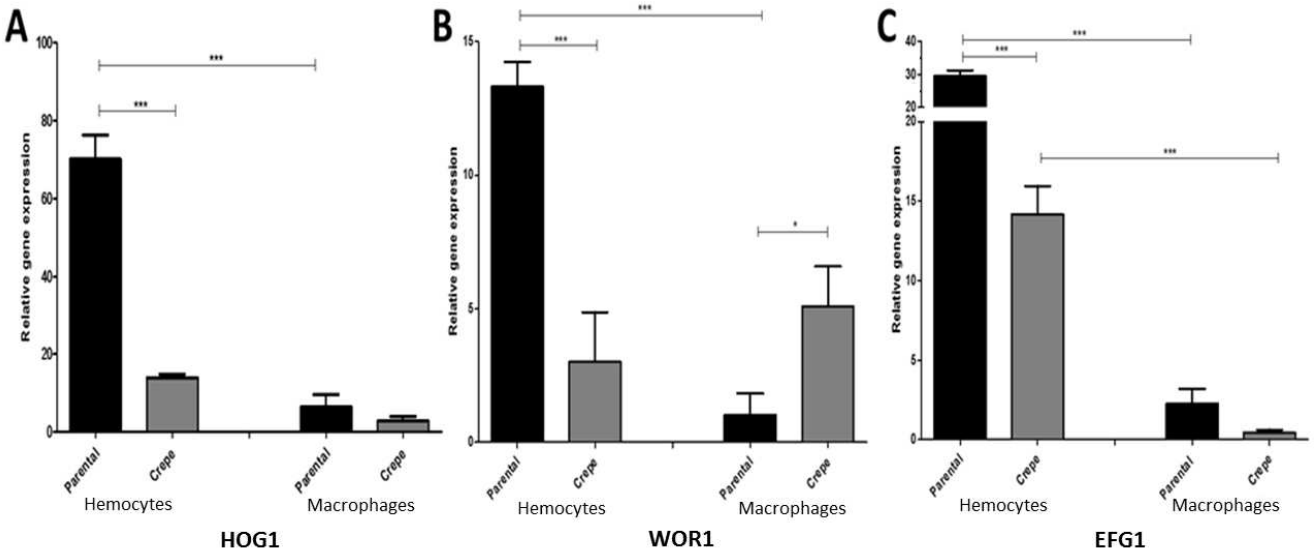

Fig 5. Relative gene expression of *HOG1* (A), *WOR1* (B) and *EFG1* (C) during phagocytosis event by hemocytes and macrophages on *C. tropicalis* Parental strain and Crepe morphotype. *P< 0.05; **P<0.01; ***P<0.001.

Gene expression by *C. tropicalis* morphotypes pre-exposed to oxidative stress during phagocytosis

During co-culture with phagocytic cells, pre-exposed *C. tropicalis* morphotypes showed differences in gene expression when compared with those in non-exposed cells (control group) (Fig. 6). In hemocyte phagocytosis, all the genes tested were downregulated upon exposure to oxidative stress. Although there was a decrease in expression, *WOR1* and *EFG1* expression was higher in the Crepe morphotype than that in the parental morphotype under these conditions. Phagocytosis by macrophages also promoted downregulation of *HOG1* and *EFG1* genes in both morphotypes pre-exposed to hydrogen peroxide. *WOR1* was upregulated and showed higher gene expression compared to the Crepe morphotype. The parental strain also showed higher expression of *EFG1* compared to Crepe.

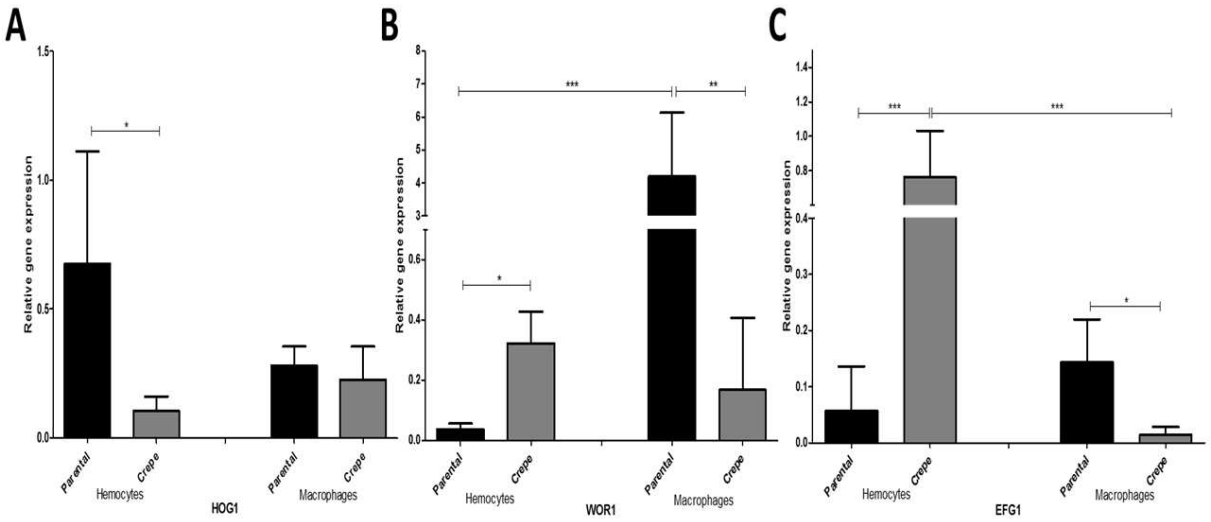

Fig 6. Relative gene expression of *HOG1* (A), *WOR1* (B) and *EFG1* (C) during phagocytosis event by hemocytes and macrophages on *C. tropicalis* Parental strain and Crepe morphotype pre-exposed to 10 min of H_2O_2. *P< 0.05; **P<0.01; ***P<0.001.

Morphogenesis mediated by phenotypic *Switching* in phagocytized *C. tropicalis* cells exposed and not exposed to oxidative stress

The filamentation process in some *Candida* species is directly related to immune evasion. In this study, we evaluated the morphogenetic ability of the parental strain and its Crepe variant when co-incubated with phagocytic cells.

When co-incubated with hemocytes, the Crepe variant showed higher filamentation compared to the parental strain ($p < 0.001$). This response was retained after oxidative stress exposure, and a large number of true hyphae were observed under these conditions ($p < 0.001$) (Fig.7A). The interaction of yeast cells with macrophages and the number of total filamentous forms did not differ between the parental strain and Crepe ($p > 0.05$); however, the parental strain showed more pseudohyphae than the Crepe morphotype ($p < 0.001$) (Fig.7B). Figure 7B shows that the parental morphotype pre-exposed to hydrogen peroxide showed an increase in pseudohyphal forms ($p < 0.001$).

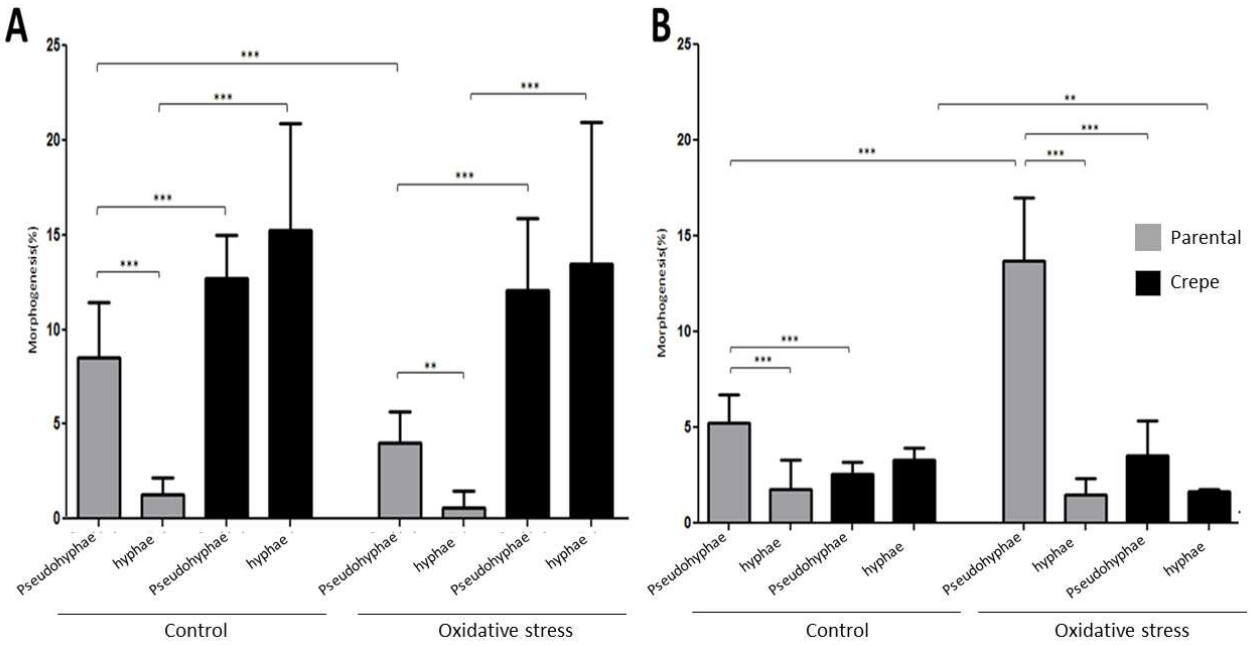

Fig 7. Morphogenesis capacity of *C. tropicalis* Parental strain and Crepe morphotype during phagocytosis by *G. mellonella* hemocytes (A) and *M. musculus* macrophages (B). Control group – untreated with stress condition. Oxidative stress group – treated with 5 mM of H_2O_2 for 10 min. *P< 0.05; **P<0.01; ***P<0.001.

DISCUSSION

According to reports of clinical surveillance programs (Wisplinghoff et al., 2014), the frequency of infections by *C. tropicalis* has increased, resulting in high mortality rates, which has made this yeast one of the most important of the *Candida* genus (Kontoyiannis et al., 2001; Colombo et al., 2007). Phenotypic *Switching* promotes the emergence of *C. tropicalis* strains with greater expression of virulence factors compared to clinical strains isolated from hospitalized patients (França et al., 2011, Moralez et al., 2014; Moralez et al., 2016). In a previous study, we reported that phenotypic *Switching* promotes the emergence of morphotypes with altered *G. mellonella* interaction profiles. This is reflected by changes in phagocytic capacity, escape from phagocytes, induction of melanization, and the humoral response of the larva (Perini et al., 2019). In this study, we showed that hemocytes and macrophages displayed equivalent phagocytic responses to *C. tropicalis* morphotypes (Fig. 4) as well as alterations in oxidative stress responses mediated by phenotypic *Switching*.

Phagocytic cells use oxidative strategies to eliminate yeast cells. In phagosomes, superoxide (O_2^-) is generated by reduced NADPH phagocyte oxidase

and can subsequently be converted to hydrogen peroxide (H_2O_2). Hydrogen peroxide can interact with chloride ions (Cl^-) to form hypochlorous acid (HOCl), a reaction catalyzed by myeloperoxidase, and creates a toxic environment to *Candida* through by oxidative stress (Dantas et al., 2015).

In this study, all *C. tropicalis* morphotypes showed growth after oxidative stress exposure (Fig. 1); however, phenotypic *Switching* promoted the emergence of strains that were more resistant to these conditions. Variant morphotypes were more resistant than the parental strains. The major resistance of the Crepe morphotype can be explained, at least partly, by the higher expression of *HOG1* compared to the parental morphotype after oxidative stress exposure (Fig. 2). In *C. albicans*, the *HOG1* gene is a critical component of the response to oxidative stress (Dantas et al., 2015). Unsurprisingly, several signaling pathways are involved in the stress response, including the MAPK pathway, which has Hog1 as one of its key factors. After exposure to H_2O_2, Hog1 is strongly phosphorylated and rapidly accumulates in the cell nucleus (ENJALBERT et al., 2006). Further, cells without Hog1 are more sensitive to reactive oxygen species, indicating that Hog1 activation is a critical component of the oxidative stress response in *C. albicans* (Alonso-Monge et al., 2003; Smith et al., 2004).

Phenotypic *Switching* promotes the emergence of isogenic mophotypes with alterations in virulence (Moralez et al., 2014; Moralez et al., 2016). Our data suggest that the effect of phenotypic *Switching* also interferes with fitness attributes, allowing success under stress. The inability to grow in the presence of hydrogen peroxide indicates that prolonged exposure to 5 mM H_2O_2 can cause irreversible damage to *C. tropicalis* cells.

As the Crepe phenotypic variant showed an increase in oxidative stress resistance, we investigated the relationship between phagocytosis and exposure to H_2O_2 in this morphotype and in the parental strain (Fig. 5). *Candida* cell types can escape defense cells, and after escape, they become available to be recognized and phagocytized by other defense cells. These strains are exposed to ROS in phagosomes and need to survive and develop escape strategies to achieve success in the host environment (Oliver et al., 2019). We thus analyzed the ability of phagocytosis under stress-free conditions and after 10 min of exposure to hydrogen peroxide. Upon co-incubation with both phagocytic cells (hemocytes and macrophages), the number of phagocytized Crepe morphotype cells pre-exposed to

oxidative stress was decreased. Thus, phenotypic *Switching* promotes an adaptive advantage for *C. tropicalis* morphotypes to escape recognition by immune innate system cells after oxidative stress exposure. The first point of recognition and contact with defense cells is the cell wall, and switch changes in this structure can be associated with the phagocytic capacity of macrophages and hemocytes (Dantas et al., 2015).

Phenotypic *Switching* did not alter the wall components in stress-free conditions (Fig. 3); however, after exposure to stress, there was an increase in chitin in both morphotypes and an increase in mannan in the parental morphotype. Upon stress exposure, the cell wall of parental morphotypes also showed reduced porosity (Fig. 3D). Increased chitin content is related to cell wall stability (Gow and Hube et al., 2012, Gow et al., 2017; Malavia et al., 2017) and altered immune system recognition (Wagener et al., 2014). Human peripheral blood monocytes exposed to ultrapure chitin demonstrated decreased recognition of *C. albicans* and decreased production of pro-inflammatory cytokines (Nagarajan et al., 2008). Furthermore, altered cell wall porosity upon exposure to oxidative stress indicates the impact of this variable on cellular biophysical properties. The decrease in the cell wall porosity of the parental morphotype is consistent with the increase in the amount of mannan (De Nobel et al., 1990; Ene et al., 2012). In this scenario, phenotypic *Switching* promoted stability in cell wall porosity under oxidative stress, as well as in the variation of mannan content.

The immune system uses several cells and substances to neutralize or eliminate *Candida* spp. during infectious processes. Therefore, immune evasion strategies are critical for the successful survival of *Candida* spp. within a host (Dantas et al., 2015). After phagocytosis, *C. albicans* can prevent death through the oxidative pathways activated by defense cells through the process of morphogenesis (Vylkova and Lorenz, 2017). In most cases, phagocyte membranes do not restrict hyphal extension, and morphological changes allow puncture of the phagosomal membrane and consequent escape from the immune system (D'Ostiani et al., 2000). In a previous study, co-culture of hemocytes and switched morphotypes of *C. tropicalis* were found to promote a decrease in hemocyte number, suggesting efficient mechanisms of phagocytosis escape promoted by phenotypic *Switching* (Perini et al., 2019). To examine whether the decrease in phagocyte cells may be related by hyphae formation, we analyzed the morphogenesis capacity in the presence of macrophages and

hemocytes (Fig. 7). As expected, phenotypic *Switching* promoted the emergence of variant morphotypes with a higher morphogenesis capacity upon co-incubation with hemocytes. Interestingly, the morphogenesis pattern of *C. tropicalis* upon co-cultivation with macrophages differed from that observed with hemocytes.

Studies on the exposure of *C. albicans* to H_2O_2 *in vitro* indicate the induction of the morphogenesis process (Miramón et al., 2012, Dantas et al., 2015); further, genes related to arginine biosynthesis are important for hyphae formation after phagocytosis (Jiménez-López et al., 2013). After exposure to hydrogen peroxide, the *C. tropicalis* Crepe morphotype maintained its morphogenesis capacity, and the pattern of true hyphae formation was similar to that observed under non-stress conditions. As co-culture with defense cells is already a stressful environment for *C. tropicalis* cells, responses such as filamentation may have been triggered, at least partly the induction through contact with macrophages and hemocytes.

The transcription program that accompanies the yeast-to-hyphal switch varies with environmental induction (Lane et al., 2001; Nantel et al., 2002, Kadosh et al., 2005). However, a “core” set of induced genes common to many hyphal induction modes can be defined, which includes genes encoding hyphal cell surface components (Martin et al., 2013). In this study, we showed that upregulation of *WOR1* and *EFG1* was mediated by phenotypic *Switching* after oxidative stress exposure (Fig. 5). Co-incubation with hemocytes also promoted the upregulation of these genes, and of *HOG1*, in the parental strain. Stimulation of morphogenesis (*WOR1*, *EFG1*), switch transitions (*EFG1*), and stress response (*HOG1*) during contact with host defensive cells indicate the ability to adapt. In addition, *WOR1* is a master regulator of white-opaque in *C. albicans* and *C. tropicalis,* and a morphogenesis regulator (Porman et al., 2011). Interestingly, we observed the action of this gene in switch forms that are not derived from white-opaque, suggesting that its action in global responses is mediated by phenotypic *Switching*.

Altered transcription of crucial genes in morphogenesis and stress response pathways, along with the capacity to evade immune system cells, indicates that phenotypic *Switching* in an important virulence factor in clinical isolates, and is a crucial event in the modulation of cellular fitness in *C. tropicalis*. *Candida* cells that successfully escape the immune system re-colonize the host, and can again be recognized and attacked by the immune system. As mentioned, pre-exposure of cells

to H_2O_2 promoted the emergence of a *C. tropicalis* variant strain that was less phagocytosed by hemocytes and macrophages (Crepe morphotype). Pre-exposure to oxidative stress altered the expression of *WOR*1 and *HOG1* during phagocytosis (Fig. 6). The parental strain showed increased expression of *EFG1* and *WOR1* in co-cultive with hemocytes whereas Crepe in contact with macrophages showed increased *WOR1* expression. These data suggest that morphogenesis pathways are crucial in the phenotypic *Switching*-mediated response of *C. tropicalis* to oxidative stress.

CONCLUSION

In conclusion, phenotypic *Switching* promotes a robust response to oxidative stress, acting in the emergence of a morphologic variant that is more resistant to hydrogen peroxide (Crepe morphotype) than the parental strain. Exposure to H_2O_2 promotes changes in the phagocytic capacity of hemocytes and macrophages, and activates the transcription response to morphogenesis genes, thus inducing filamentation, an important mechanism of phagocytosis escape. The transcriptional response of the *HOG1* gene mediated by *Switching* also indicates that this gene is crucial to the oxidative stress response in *C. tropicalis* morphotypes, and that *WOR1* is expressed in response mechanisms to stress and phagocytosis of a non-white-opaque phenotypic *Switching* system in *C. tropicalis*. Thus, phenotypic *Switching*, in addition to being an important virulence factor in *C. tropicalis*, is also crucial in the response of this species to host response mechanisms.

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ARTIGO 3

PHENOTYPIC *SWITCHING* IN *Candida tropicalis*: ROLE OF OXIDATIVE STRESS RESPONSE IN PHAGOCYTOSIS, CELL WALL DYNAMICS AND GENE EXPRESSION.

ABSTRACT

Phenotypic *Switching* is associated with modulation of virulence factors and host-pathogen interactions in *Candida tropicalis*, a fungus associated with high mortality rates. We aimed to evaluate the role of phenotypic *Switching* on oxidative stress response, and the effect of pre-exposure to H_2O_2 on phagocytosis and expression of genes related to stress response and morphogenesis. Morphotypes of the 100.10 switch system were exposed to 5 mM of H_2O_2 for 10 and 60 min and the viability assay and amount of cell wall compounds were performed. Phagocytosis was observed and the expression of *HOG1, EFG1* and *HOG1* genes was measured by qPCR in untreated and pre-exposed cells (10 min for H_2O_2). Phenotypic *Switching* promoted the emergence of a morphotype (Crepe) more resistant to oxidative stress than the parental strain (100% viability after 10 min of exposure). The resistance was reflected on less membrane damage and alteration on cell wall. Phenotypic *Switching* up-regulated *HOG1* and *WOR1* in the Crepe morphotype and oxidative stress induces an up-regulation in the *EFG1* gene in both morphotypes. Macrophages and hemocytes showed similar ability to phagocyte morphotypes, however pre-exposed Crepe cells were less phagocytosed by both phagocytes. Crepe showed the ability to produce true hyphae under phagocytes co-culture, a characteristic maintained after exposure to stress. Under co-culture with hemocytes Crepe upregulated *WOR1.* The emergence of morphotypes with altered response to oxidative stress and recognition of phagocytic cells modulated by changes in cell wall dynamics and expression of morphogenesis, stress response and phenotypic variations genes, reinforce phenotypic *Switching* as an important virulence factor capable of promoting increase in the fitness attributes of *C. tropicalis*.

Keywords: *Candida tropicalis*, phenotypic *Switching*, *Galleria mellonella*, hydrogen peroxide.

1 INTRODUCTION

Candida species are the most frequently isolated with human fungal infections (CALDERONE, 2002). *Candida tropicalis* is phylogenetically close to *Candida albicans*, sharing several morphological and virulence characteristics (BUTLER et al., 2009; ZUZA-ALVES et al., 2017). Mortality rates by *C. tropicalis* blood infections are similar or higher than *C. albicans* (KRCMERY; BARNES, 2002; WISPLINGHOFF et al., 2004; TORTORANO et al., 2006).

Phenotypic *Switching* is a virulence factor for some *Candida* species, it occurs naturally in isogenic populations, having higher frequencies than somatic mutations (SOLL, 2014). In *C. tropicalis*, phenotypic *Switching* promotes several systems, such as white-opaque, white-grey-opaque and systems that comprise complex colony morphology, called morphotypes (ZHANG et al., 2016; ZHENG et al., 2017; MORALEZ et al., 2014). These switched strains can show phenotypic plasticity in isogenic populations and promote the emergence of more virulent strains than the original counterpart (MORALEZ et al., 2014; SOLL, 2014; MORALEZ et al., 2016).

Previous studies have described the raise of switched morphotypes with increased expression of distinct virulence traits, including hemolytic capacity (MORALEZ et al., 2014), adhesion to polystyrene and HeLa cells (SOUZA et al., 2021), biofilm formation (MORALEZ et al., 2014; MORALEZ et al., 2020), virulence towards larva of *Galleria mellonella* (MORALEZ et al., 2016), and altered minimal inhibitory concentration for amphotericin B and fluconazole (MORALEZ et al., 2014). The phenotypic *Switching* also altered Interaction with *G. mellonella* hemocytes (PERINI et al., 2019).

In the host body, *C. tropicalis* encounters a hostile environment, being exposed to multiple stressors and to the immune system (DANTAS et al., 2015). Phagocytes can recognize fungal cells and produce reactive oxygen species (ROS) in response to yeast internalization, promoting fungal death (DANTAS et al., 2015). Furthermore, phagocytes can secrete ROS to attack pathogens (FROHNER et al., 2009).

The phagocytosis event depends on recognition mechanisms. The yeast cell wall is the first point of contact with the cells to the immune system (HOPKE et al., 2018; WARRIS; BALLOU, 2019). This dynamic structure undergoes several remodels

and is able to promote evasion and escape from the immune system to mask epitopes (HENRIET et al., 2013; CHOERA et al., 2018; WARRIS; BALLOU, 2019).

Phagocytosed *Candida* cells need to develop mechanisms to escape the phagosome. *C. tropicalis* is able to develop true hyphae, a factor associated with tissue invasion (JACOBSEN et al., 2012). Furthermore, phagosome escape is also related to hyphae formation. Morphogenesis within the phagocyte can either perforate the cell or induce pyroptosis, both culminating in host cell death (D'OSTIANI et al., 2000; GIL-BONA et al., 2015).

In the present study, we aimed to evaluate cellular alterations associated with phenotypic *Switching* of the *C. tropicalis* 100.10 system in response to the oxidative stress (exposure to H_2O_2), as well as, the role of exposure to stress in phagocytosis by *G. mellonella* hemocytes and *Mus musculus* macrophages. Furthermore, we evaluated the expression of important regulators of stress responses (*HOG1)* and morphological transitions (*EFG1* and *WOR1* genes), in response to phenotypic *Switching*, oxidative stress and phagocytosis.

2 MATERIAL AND METHODS

2.1 Microbial strains and culture conditions

Morphotypes (original phenotype-parental, crepe variant, crepe revertant, rough variant and rough revertant) of the *Switching* phenotypic system 100.10 of *C. tropicalis* used in this study were stored as frozen stocks with 20% glycerol at −80 °C and subcultured on YPD agar plates (1% yeast extract, 2% peptone, and 2% dextrose) at 28 °C. Morphotypes were routinely grown in YPD liquid medium at 28 °C in a shaking incubator and plated on YPD agar plates at 28 °C for 96 hours.

2.2 Cellular parameters by flow cytometry.

Cellular parameters were performed according to Tommioto-Pelissier et al., (2018) with modifications. Morphotype cells ($1x10^6$ cells/mL) were washed and suspended in 100 µL of assay buffer 1x (Santa Cruz Biotechnology). A total of 10,000 events were acquired in a BD AccuriTM C6 flow cytometer and FSC and SSC analyzed to measure volume and cell complexity.

To determine the membrane integrity, cells of morphotypes were directly incubated with propidium iodide (PI) (Sigma, St. Louis, MO, USA) (0.50 µg/mL) for 5 min. Immediately thereafter, *Candida* cells were analyzed using an excitation wavelength of 480 nm and an emission wavelength of 580 nm.

2.3 EFFECT OF PHENOTYPIC *SWITCHING* IN THE AMOUNT OF CELL WALL COMPUNDS AND POROSITY IN RESPONSE TO OXIDATIVE STRESS

To evaluate the effect of phenotypic *Switching* in response to oxidative stress in *C. tropicalis*, morphotypes cells exposed (10 min) and non-exposed to hydrogen peroxide (5 mM) were washed 3 times with deionized water and fixed with 10 % paraformaldehyde solution in PBS. Fixed cells ($1x10^6$ cells/mL) were stained independently with 100 µg/mL of Calcofluor White (CFW), 50 µg/mL of Aniline Blue and 100 µg/mL FITC-Concanavaline A conjugated (FITC-CONA) (Okada; Ohya., 2016).

Cells were incubated for 10 min at room temperature in the dark. Following incubation, cells were washed twice with PBS. Stained samples were analyzed using GloMax® Explorer (Promega) instrument with 415-445 nm emission filter and 405 nm excitation filter to Aniline Blue; 415-445 nm emission filter and 365 nm excitation filter to CFW; 500-550 nm emission filter and 475 nm excitation filter to FITC-CONA. Data were expressed in fluorescence units. Each experiment was performed in triplicate on tree different occasions.Cell wall porosity assay was performed according to Ene et al. (2012) with modifications. Aliquots of $1x10^8$ cells for each morphotype were incubated with shaking (200 rpm) for 30 min at 30 °C in 1 mL of either 10 mM Tris-HCl pH 7.4 (control), the same buffer containing 5 mg/ml-1 DEAE-dextran (500 kDa) or buffer containing 15 mg/mL poly-L-lysine (50 kDa). Cells were then pelleted by centrifugation and the A_{260} of supernatants measured. Relative porosity was calculated using the following formula: relative porosity = 100 x (A_{DEAE} - A_{buffer}) / ($A_{poly\text{-}L\text{-}lysine}$ - A_{buffer}).

To respond to oxidative stress, Parental and Crepe morphotypes ($1x10^8$ cells/mL) were exposed to 5 mM of H_2O_2 for 10 min. Exposed and non-exposed cells were washed 3 times with ultrapure water and fixed with 10 % paraformaldehyde solution in PBS. Staining cell wall and porosity was performed as described. Data were expressed in fluorescence units and percentage of porosity. Each experiment was performed in triplicate on three different occasions.

2.4 QUANTITATIVE ANALYSIS OF STRESS SENSITIVITY

The analysis of stress sensitivity was realized according Nikolaou et al., (2009) with modifications. To semi-quantitatively compare the stress sensitivity to hydrogen peroxide (H_2O_2) of the *C. tropicalis Switching* system, the percentage of growth of each morphotype was calculated relative to their non-stress control for each stress condition.

C. tropicalis cells ($1x10^8$ cells/mL) were exposed to 5 mM of H_2O_2 for 10 min and 60 min, in broth. After serial dilutions, cells were plated in YPD medium and number of UFC were measured for direct observation. Stress condition tested was counted and expressed as percentages of those on the corresponding control plates (same dilution without stress). Moreover, to measure relative growth, phenotypic morphotypes were plated in YPD medium-solidify containing 5 mM of H_2O_2. Amount of growth in the presence of stress was divided by the amount of growth observed for unstressed cells of the same morphotype and expressed as a percentage. Each experiment was realized in quadruplicate and repeated at least 3 times.

2.5 Galleria mellonella LARVAE MANIPULATION AND HEMOLYMPH COLLECTION

G. mellonella larvae in the final stage without color alterations and with adequate weights (240–300 mg) were selected. The collection of hemolymph of ten larvae was performed and the liquid transferred to a microtube containing 900 µL of IPS (insect physiological saline: 150 mM sodium chloride (Promega, USA), 5 mM potassium chloride (Promega), 10 mM Tris HCl (Promega) pH 6.9, 10 mM EDTA (Promega) and 30 mM sodium citrate (Sigma-Aldrich, USA) plus 10 mM N-ethylmaleimide (Sigma-Aldrich) (anticoagulant).

Tubes were placed on ice, and sample collection was carried out immediately to avoid cell melanization. After centrifugation at 2000 rpm and at 4 °C for 5 min, the supernatant was discarded, cells were washed with 500 µl of cold IPS and the contents of two tubes (or several, depending on the number of assays) were pooled together in a new tube. The cells were resuspended in 1000 µl IPS. The number of cells was determined with a Neubauer chamber.

2.6 PERITONEAL MACROPHAGES

Mice weighing approximately 25–30 g and aged 6–8 weeks under sterile conditions were maintained according to protocols approved by the Institutional Animal Care and Committee. The animals were euthanized by Ketamine- Xylazine overdose (100 mg/kg - 10 mg/kg, i.p.) followed by cervical dislocation and peritoneal macrophage were obtained. Macrophages (5 × 105 cells/mL) were recovered according Tommiotto-Pellissier et al. (2018) from the peritoneal cavity of BALB/c mice with cold PBS supplemented with 3% of FBS and then cultured in 24-well plates with 200 µL of RPMI 1640 medium (10% FBS) for 2 h (37 °C, 5% CO_2). This study was approved by the Ethics Committee for Animal Experimentation of the State University of Londrina (OF. CIRC. n°. 3715.2015.22).

2.8 PHAGOCYTIC ASSAY

To analyze the effect of pre-exposition to oxidative stress on the phagocytosis by hemocytes and macrophages, *C. tropicalis* morphotypes (Parental and Crepe) were exposed to H_2O_2 for 10 min. Following, for enumeration of viable cells, 0.5 mL of each sample suspension were mixed with 0.1 mL of 0.4% trypan blue stain (Sigma–Aldrich, USA). After 5 min, the number of viable cells was adjusted to a concentration of $5x10^6$ cells/mL.

The phagocytic assay was performed according to Tommioto-Pelisser et al. (2018) and Perini et al, (2019) with modifications. To adhere hemocytes and macrophages, 13-mm round coverslips that were treated with acetic acid were placed in a 24-well plate. Each well was filled with a volume corresponding to 1 × 10^5 phagocyte cells and was brought to a total volume of 1000 µL with RPMI 1640 medium (Gibco, USA) plus 10% fetal bovine serum (FBS, Gibco®, Brazil). The plates were incubated for 1 h at 37 °C with 5% CO_2 to allow cell adherence. Then, non-adherent cells were removed by washing with RPMI 1640 medium at room temperature, and the adhered cells were co-incubated with each morphotype strain at a 1:5 ratio.

Phagocytosis was allowed to occur for 2 h; samples were then washed to remove the non-adherent yeast. In sequence, samples were fixed with 1 mL cold methanol (Sigma-Aldrich, USA) for 20 min. Then, cells were stained with May-Grünwald (Laborclin, Brazil) for 15 min, washed with Sorenson's buffer (0.133 M Na_2HPO_4 and 0.133 M KH_2PO_4) and immersed in Giemsa dye (Laborclin, Brazil) for

15 min. Finally, the coverslips were washed again with Sorenson's buffer, air dried, and mounted on glass slides. Under an optical microscope (1000 times magnification), cells from 20 fields in three distinct experiments were analyzed and quantified according to the number of hemocytes and macrophages containing internalized yeast cells.

2.9 MORPHOGENESIS ASSAY

To estimate the number of filamentous forms, direct counting of 20 fields of co-culture with macrophages and hemocytes was performed. Percentage of pseudohyphae and hyphae was relativized with a total number of yeast cells.

2.10 RNA EXTRACTION AND GENE EXPRESSION ANALYSIS (RT-QPCR)

The gene expression was tested on (1) control group - *C. tropicalis* morphotypes (Parental and Crepe colonies) not exposed to oxidative stress; (2) stressed group – phenotypic *Switching* morphotypes exposed to oxidative stress for 10 min; and (3) phenotypic *Switching* morphotypes not exposed (3.1 – Control) and exposed to oxidative stress for 10 min (3.2 – stressed group), after co-culture with hemocytes and macrophages. These cells were homogenized, frozen in liquid nitrogen and RNA was extracted and purified using an RNA Mini Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. RNA was quantified and the quality assessed using a NanoDrop spectrophotometer (ThermoScientific, Loughborough, United Kingdom). To phagocytosis analysis, RNA was extracted after co-incubation with phagocytes. cDNA was synthesized from 200 ng of extracted RNA using an RT-PCR kit (Invitrogen, Carlsbad, CA, EUA) in a GeneAmp® PCR (Eppendorf, Gradiente Mastercycler) following the manufacturer's instructions.

Primers used for quantitative PCR (qPCR) were as follows: *HOG1* (forward, 5'-TTGCCAGTGGATACTTGGAG–3'); *HOG1* (reverse 5'–GTTGTTGTTCAGCACCATCG–3'); *WOR1* (forward 5'-CCGTCTAATGTTATACCTGCATCAA-3'); *WOR1* (reverse 5'-TTCGTCGTACTTATGGTAATTGTTTTCT-3'); *EFG1* (forward 5-TTCAACTGCTGGACAACCAC-3'); *EFG1* (reverse 5'-TACCAGGAGGTTGGAATTGG-3'); *β-actin* (forward, 5′-

GGGACGATATGGAGAAGATCTG-3′); and *β-actin* (reverse, 5′-CACGCTCTGTGAG GATCTTC-3′).

The cycling conditions consisted of 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 51 °C and 60 s at 60 °C. Each sample was analyzed in duplicate using a StepOnePlus™ Real-Time PCR System (Applied Biosystems®). The reactions were performed using Platinum® SYBR® Green qPCR Supermix-UDG (Invitrogen, Carlsbad, CA, USA) with a final volume of 20 µl. Relative gene expression was calculated using the $2^{-(\Delta-\Delta Ct)}$ method.

2.11 Statistical analysis

Three independent experiments were performed in triplicate for all experiments. All data were previously submitted to the F test and the Shapiro-Wilk test for analysis of variance and normality test, respectively. Comparison between the groups were done by one-way ANOVA, followed by Tukey's test for multiple comparisons. For stress exposition analysis the t-paired test was used. Statistical significance was set at *P<0.05, **P<0.01, ***P<0.001. All statistical analyses were performed by GraphPad Prism 5 statistical software (GraphPad Software, Inc., USA, 500.288). Data were expressed as mean ± standard deviation.

3 RESULTS

3.1 Effect of phenotypic *Switching* on cellular parameters

3.1.1 Cell Volume and Complexity

Phenotypic *Switching* promoted changes in cell volume and complexity (Fig. 1). The complex morphotypes (Crepe and Rough) showed lower FSC : SSC than smooth morphotypes (Parental, RC and RR) for FSC : SSC (Fig. 1A).

The structured morphotypes (Crepe and Rough) were smaller than the smooth morphotypes (Parental, RC and RR) (Fig. 1B). For cell size, the morphological revertants returned to the pattern presented by Parental, being larger than their respective variants (Crepe and Rough). Structured morphotypes also showed less cellular complexity than the parental strain (Fig. 1C). For this parameter, RR also showed less complexity than Parental.

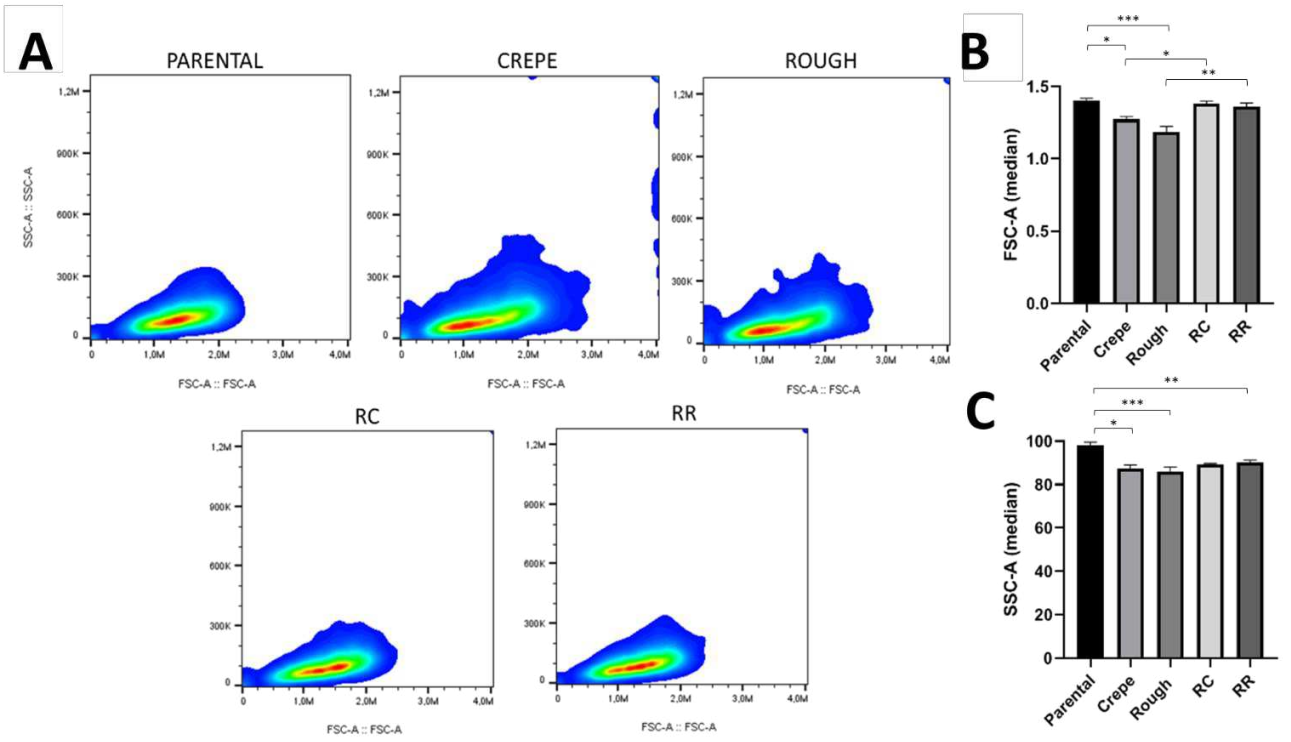

Fig 1. Carachterization of volume and cellular complexity of *Candida tropicalis* 100.10 switch system (Parental, Crepe, Rough, RC and RR morphotypes). (A) Relationship and FSC and SSC; (B) FSC – Cellular volume and (C) SSC – Cellular complexity. *P< 0.05; **P<0.01; ***P<0.001.

3.1.2. Cell Wall Dynamics

Phenotypic *Switching* promoted changes in cell wall structure of *C. tropicalis* morphotypes. As shown in Fig. 2, morphological variants (Crepe and Rough) showed an increase in cell wall porosity (*p<0.01*) (Fig 2.D), mannan content (Crepe) (Fig. 2B) and β-glucan (Rough) (Fig. 2C) than Parental strain. Crepe and Rough variants showed higher amounts of mannan than smooth revertants (RC and RR) (Fig. 2B).

Morphological revertants (smooth-like morphology) showed higher amount of chitin than the Parental and their morphological variants (*p<0.01*) (Fig. 2A). Crepe revertant (RC) also showed higher β-glucan amounts than Parental and Crepe morphotypes (*p<0.05*) (Fig. 2C).

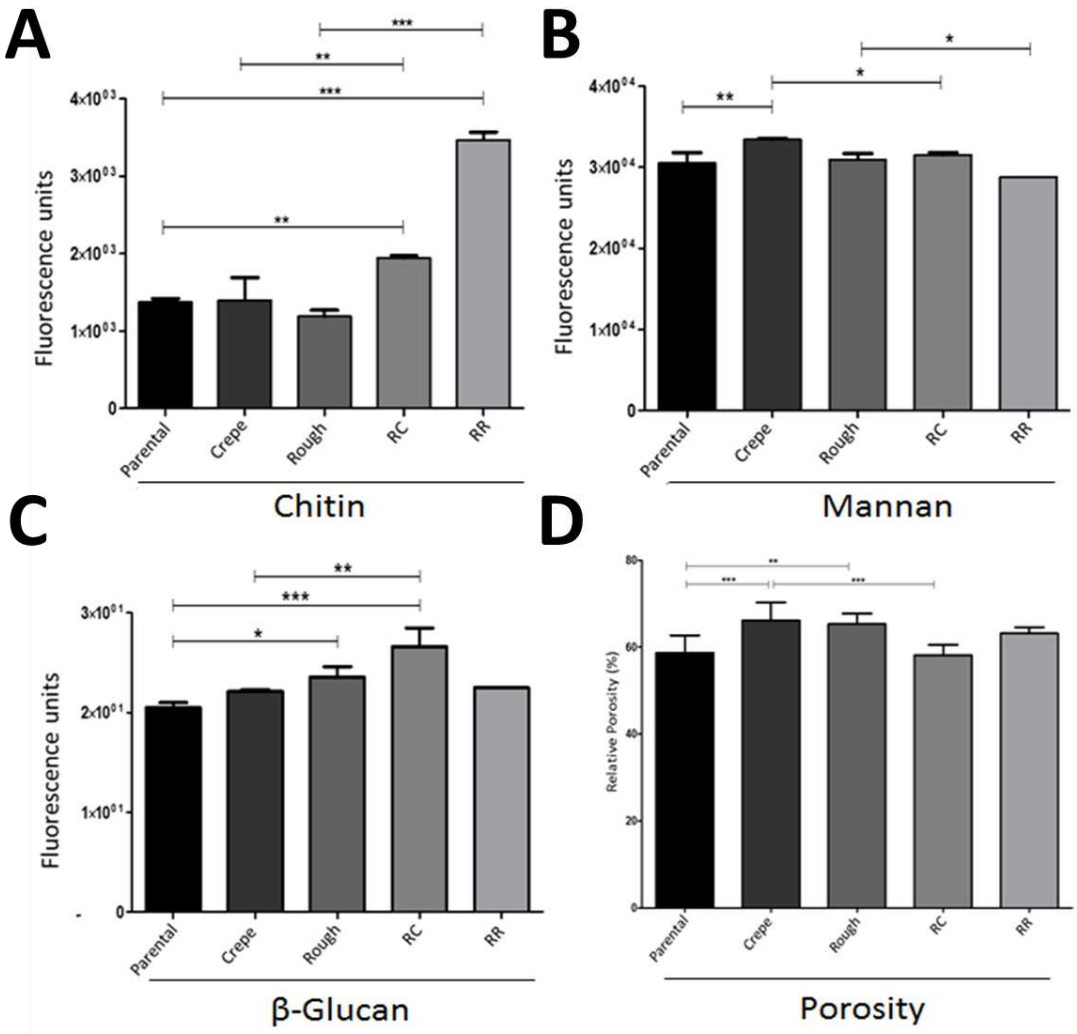

Fig 2. Cell wall characterization of *Candida tropicalis* 100.10 switch system (Parental, Crepe, Rough, RC and RR morphotypes). (A) Amount of Chitin (stained whit CW), (B) Mannan (stained with FITC-CONA) and (C) β-Glucan (Stained with Aniline blue); (D) Cell wall porosity based on the polycation-induced leakage of UV-absorbing. *P< 0.05; **P<0.01; ***P<0.001.

3.2. EFFECT OF PHENOTYPIC *SWITCHING* ON RESPONSE TO OXIDATIVE STRESS

3.2.1. Resistance

Morphotypes of *C. tropicalis* were exposed to hydrogen peroxide (H_2O_2) for 10 and 60 min. After 10 min of exposure, the Crepe morphotype exhibited the same rate of cell viability than that observed for the control (not exposed) ($p>0.05$), while its parental counterpart decreased the cell viability ($p<0.05$) (Fig. 3A). After 60 minutes of exposure to H_2O_2, the decrease in cell viability was more pronounced for all morphotypes ($p<0.05$). Under this condition, the Parental (clinical isolate) showed a

very low viability (2.5%), while the Crepe morphotype showed higher viability than its parental counterpart strain and its revertant morphotype (CR) (*p<0.05*) (Fig. 3A).

The propidium iodide dye assay showed that oxidative stress promoted more damage to the membrane of the parental strain than to the membrane of de crepe variant (*p<0.05*) (Fig. 3B). Furthermore, after being exposed to oxidative stress, Crepe and Parental showed differences in cell complexity (*p<0.05*) (Fig. 3C).

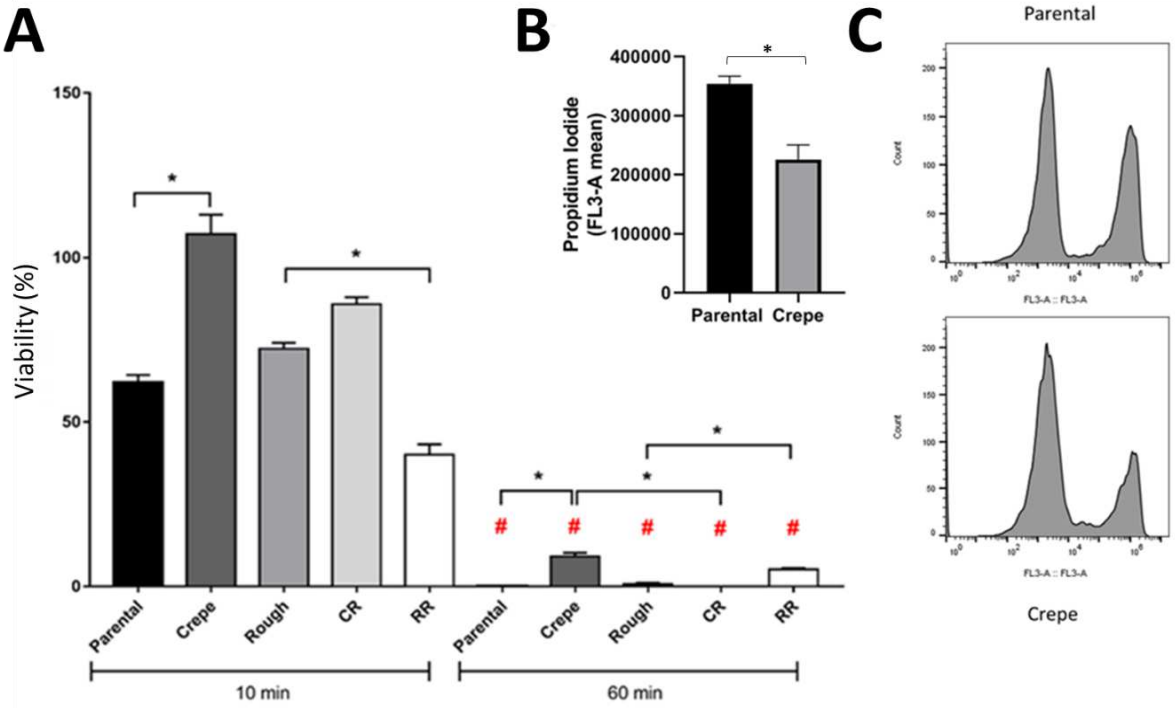

Fig 3. Oxidative stress sensitivity of the *Switching* system of *C. tropicalis* 100.10 (A) (Parental, Crepe, Rough, RC and RR morphotypes) after exposure to H_2O_2 for 10 min and 60 min. Data expressed as percentage of growth relative to growth in non-stressed conditions. (B) Propidium iodide staining showing membrane damage caused by oxidative stress and (C) cell complexity obtained by flow cytometry – 10 min exposure. *P<0.05; **P<0.01; ***P<0.001.

3.2.2. Gene Expression

Phenotypic *Switching* in untreated condition (control) promoted upregulation of *HOG1* (mitogen-activated protein kinase – stress response) and *WOR1* (white-opaque regulator 1, the master regulator of white-opaque transitions) genes. In this condition Crepe morphotype showed higher expression than Parental (*p<0.05*) (Fig. 3A). Oxidative stress promoted downregulation of *WOR1* gene and upregulation of *EFG1* in both morphotypes (Parental and Crepe). After stress exposure, Parental showed higher *EFG1* gene expression than Crepe (*p<0.05*) (Fig. 4C).

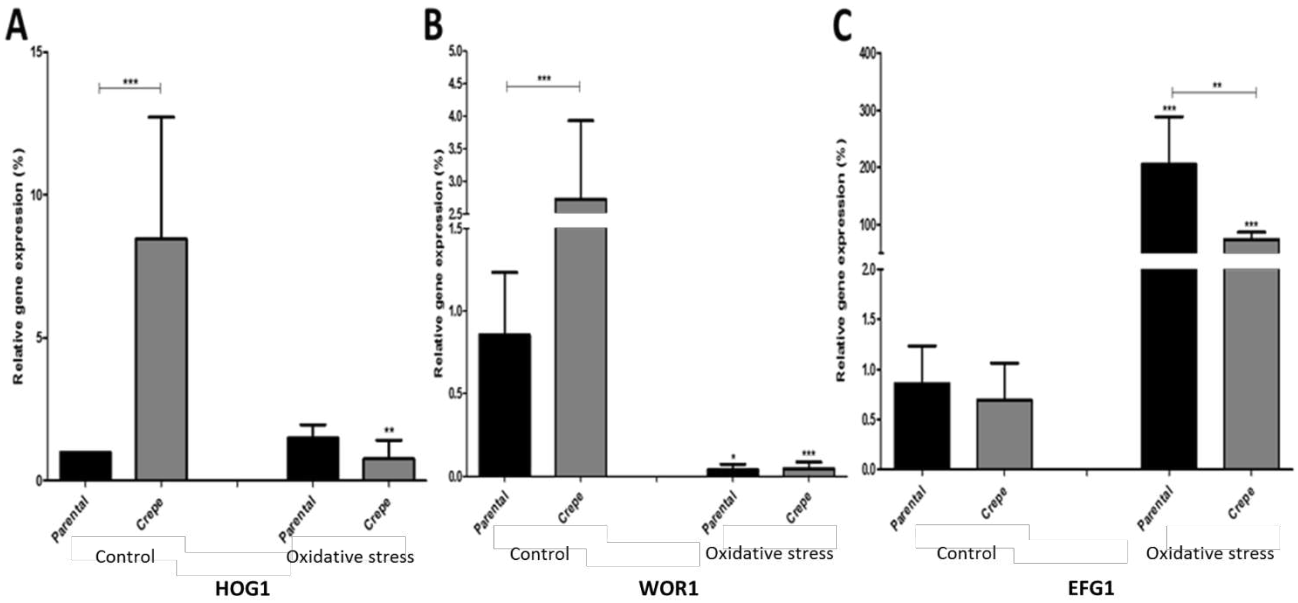

Fig 4. Relative gene expression of (A) *HOG1*, (B) *WOR1* and (C) *EFG1* of *C. tropicalis* Parental strain and Crepe switch variant untreated with H_2O_2 (Control) and under oxidative stress (10 min of exposure to H_2O_2). *P< 0.05; **P<0.01; ***P<0.001.

3.2.3. Cell Wall Compounds

Phenotypic *Switching* promoted increase in the amount of mannan and cell wall porosity (Crepe higher than Parental) (Fig. 5B,D), however, exposure to oxidative stress promoted additional alterations in the *C. tropicalis* cell wall (Fig 5). Exposure to hydrogen peroxide promoted a decrease in chitin content in the Parental strain (Fig. 5A), a decrease in mannan in Crepe (Fig. 5B) and an increase in the amount of β-glucan in both morphotypes (Fig. 5C). Cell wall porosity was also affected, increasing in both morphotypes (Fig. 5D).

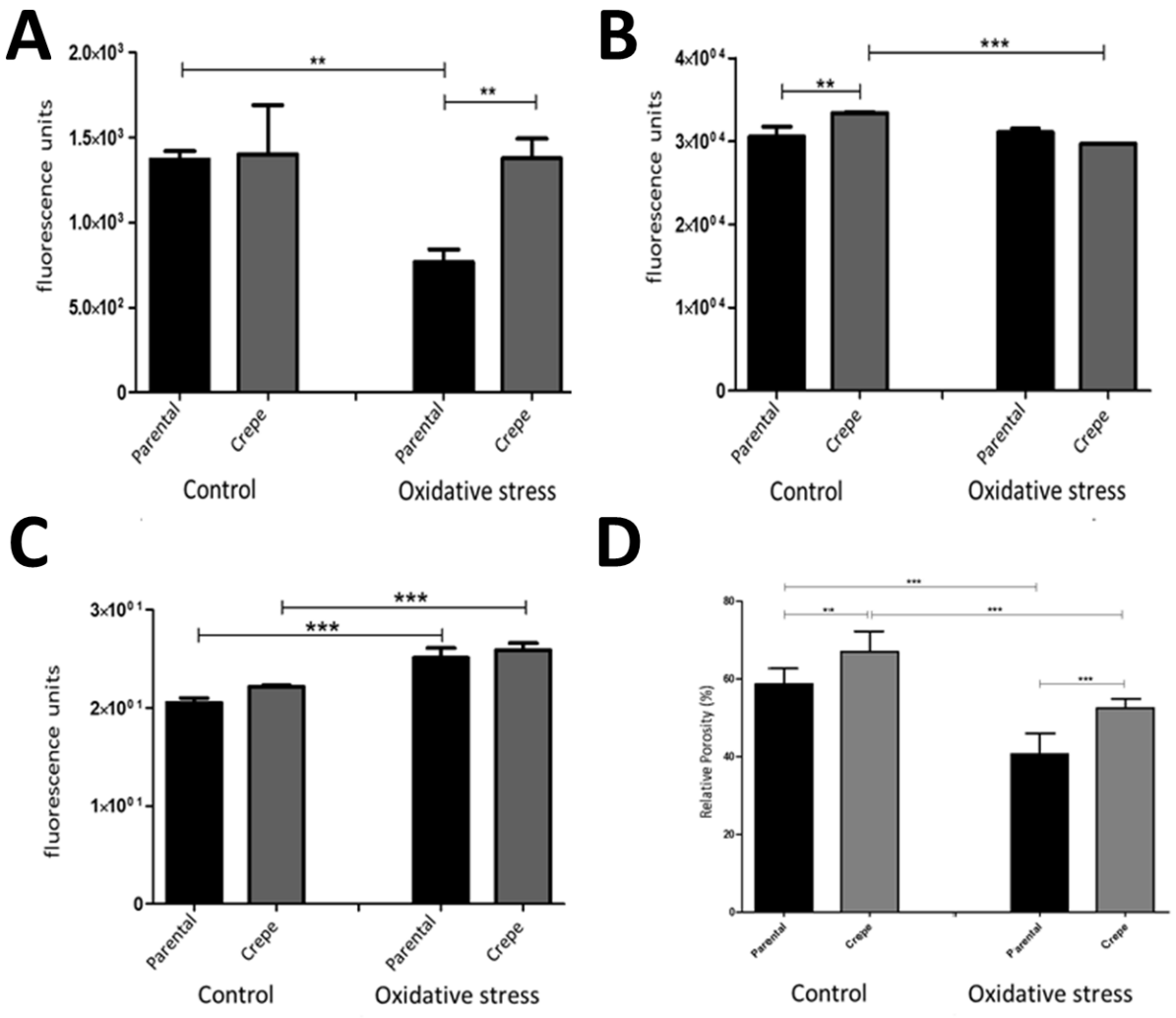

Fig 5. Cell wall characterization of *C. tropicalis* Parental strain and Crepe switch variant untreated with H_2O_2 (Control) and under oxidative stress (10 min of exposure to H_2O_2). (A) Amount of Chitin (stained whit CW), (B) Mannan (stained with FITC-CONA) and (C) β-Glucan (Stained with Aniline blue); (D) Cell wall porosity based on the polycation-induced leakage of UV-absorbing. *P< 0.05; **P<0.01; ***P<0.001.

3.3. Effect of co-culture with hemocytes and macrophages

3.3.1. Phagocytosis

Both cell types (hemocytes and macrophages) were able to phagocyte cells of the *C. tropicalis* morphotypes. There was no difference in the phagocytosis capacity of hemocytes and macrophages against the parental and crepe morphotypes (*p>0.05*). However, pre-exposure to hydrogen peroxide reduced the crepe phagocytosis by the two phagocytes cells (*p<0.05*) (Fig 6A,B). After pre-exposure to H_2O_2 Crepe was less phagocytosed than Parental strain.

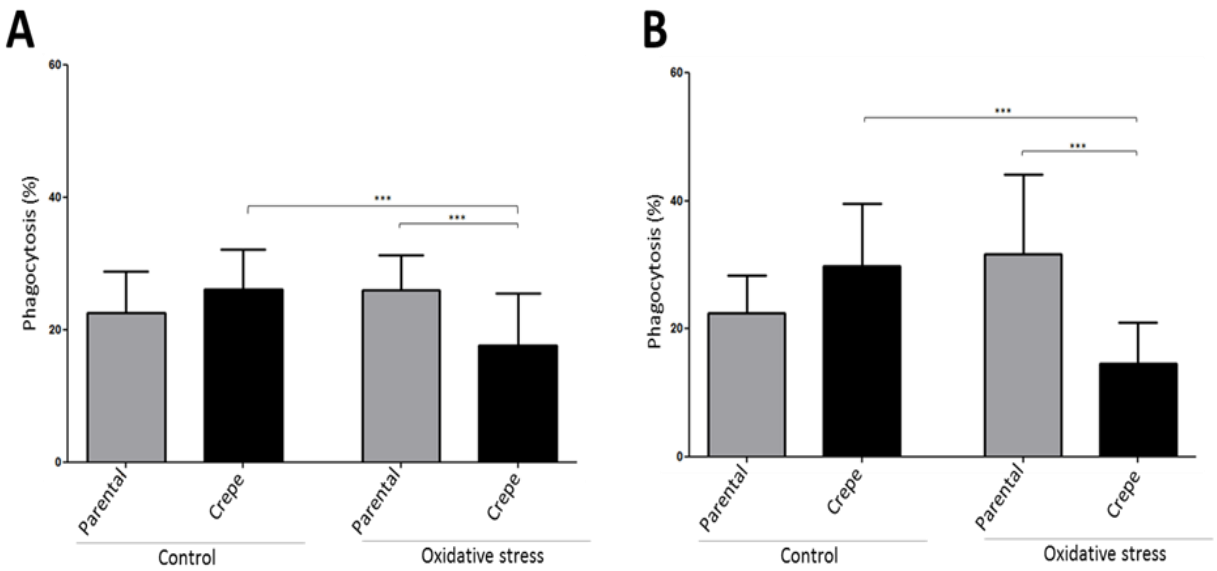

Fig 6. Phagocytosis by (A) *Galleria mellonella* hemocytes and (B) *Mus musculus* macrophages of *C. tropicalis* Parental strain and Crepe morphotype. Control group – untreated condition. Oxidative stress group – exposed to 5 mM of H_2O_2 for 10 min. *P< 0.05; **P<0.01; ***P<0.001.

3.3.2. Morphogenesis

Co-culture of *C. tropilcais* morphotypes with either hemocytes or macrophages induced the filamentation process (presence of true hyphae) in the crepe morphotype (*p<0.05*). The parental strain produced higher pseudohyphae percentage under this condition (*p<0.05*) (Fig 7A).

Pre-exposure to H_2O_2 does not change the morphogenesis capacity. Pre-exposed Crepe cells maintained the ability to form true hyphae and pre-exposed Parental produced more pseudohyphae than the Crepe morphotype (*p<0.05*) (Fig 7B).

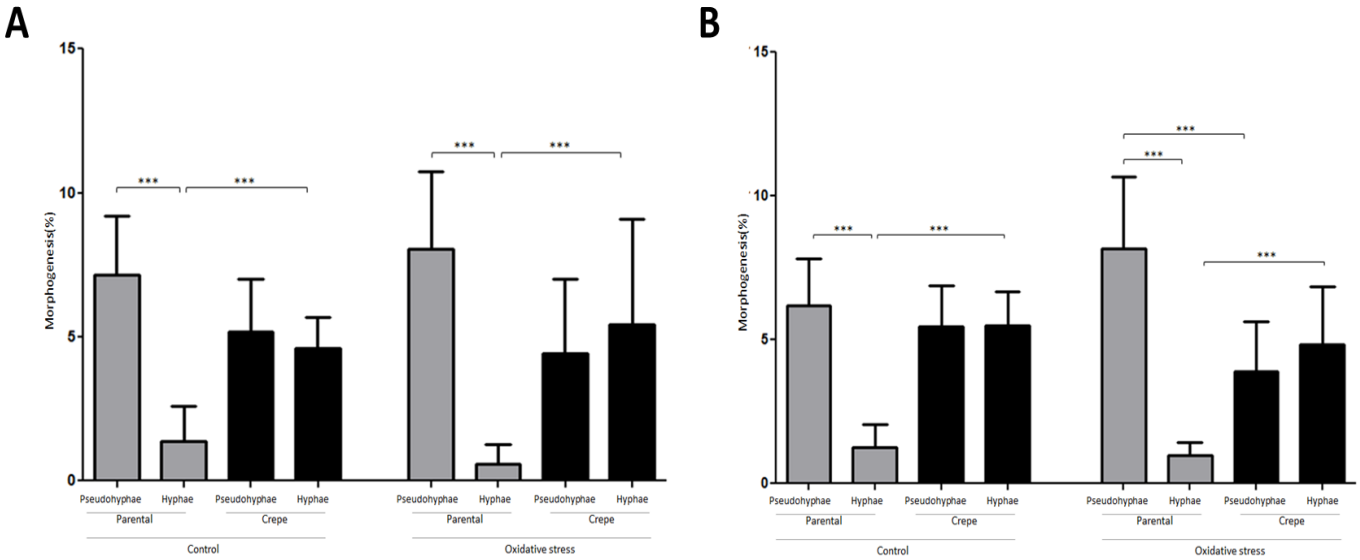

Fig 7. Morphogenesis capacity of *C. tropicalis* Parental strain and Crepe morphotype during phagocytosis by (A) *G. mellonella* hemocytes and (B) *M. musculus* macrophages. Control group – untreated with stress condition. Oxidative stress group – treated with 5 mM of H_2O_2 for 10 min. *P< 0.05; **P<0.01; ***P<0.001.

3.3.3. Gene Expression

Real-time quantitative PCR analysis showed that the expression of *HOG1, WOR1*, and *EFG1* genes was distinct depending on the type of phagocytic cells used (Fig 8). *HOG1* was upregulated in Parental strain in co-culture with hemocytes (Fig 8A). The same response was observed to *EFG1* for Crepe morphotype. (Fig 8C). *WOR1* was downregulated in both morphotypes (Fig 8B). Co-culture of morphotypes with macrophages showed induction of expression of *EFG1* in both morphotypes, once more Crepe showed higher expression than Parental (Fig 8C). WOR1 was downregulated in both morphotypes (Fig. 8C).

Pre-exposure to H_2O_2 (10 min) before hemocytes co-culture induced upregulation of *HOG1* in Crepe and downregulation in Parental strain (Fig 8D). *WOR1* is upregulated in both morphotypes (Fig 8E), however Crepe showed higher expression than Parental. *EFG*1 was downregulated in both morphotypes (Crepe higher expression than Parental) (Fig 8F, *p<0.05*). Pre-exposed *Candida* cells before macrophages co-culture showed overexpression of *HOG1* gene (Fig. 8D). Parental strain expression was higher than Crepe morphotype. *WOR1* was downregulated in both morphotypes and *EFG1* was upregulated in Crepe while it was downregulated in Parental strain (Fig 8. E-F).

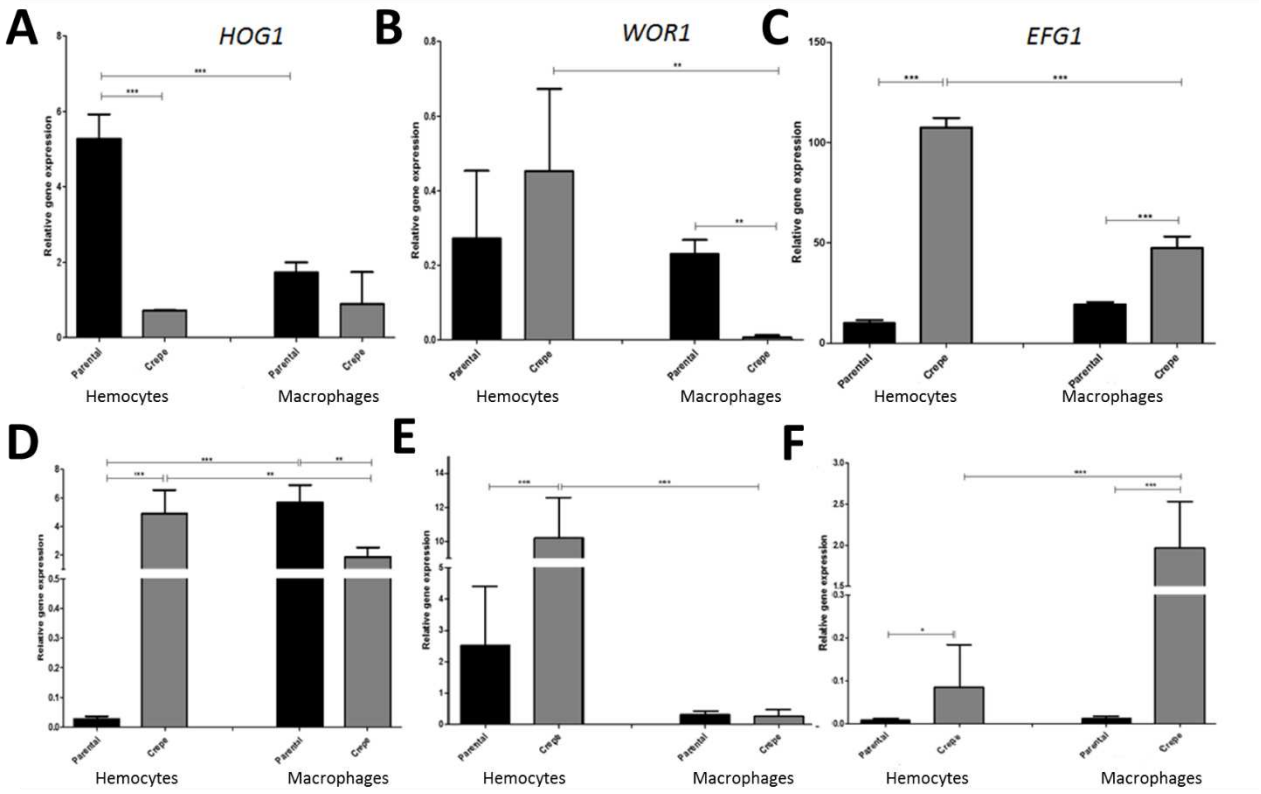

Fig 8. Relative gene expression in untreated stress condition of (A) *HOG1*, (B) *WOR1* and (C) *EFG1* and pre-exposed to 5 mM of H2O2 for 10 min of (D) *HOG*1, (E) *WOR*1 and (F) *EFG*1 during phagocytosis event by hemocytes and macrophages on *C. tropicalis* Parental strain and Crepe morphotype. *P< 0.05; **P<0.01; ***P<0.001.

4 DISCUSSION

C. tropicalis is an important human pathogen and frequently isolated from bloodstream infections (KONTOYIANNIS, et al., 2001; COLOMBO et al., 2007). Phenotypic *Switching* emerges as virulence factor for this species, generating phenotypic variability in isogenic populations (SOLL, 2009; PORMAN et al., 2011). In 100.10 isolate phenotypic *Switching* modulates alterations in virulence factors (MORALEZ et al., 2014; SOUZA et al., 2020), however it is unclear the effect of this event at a cellular level.

Phenotypic *Switching* is associated with the decrease of the cell morphological complexity for Crepe and Rough variants, as shown by flow cytometry (Fig 1). The altered morphological complexity may be related with a greater number of filamentous forms by structured colonies (MORALEZ et al., 2014). For instance, the

crepe variant showed an increase on the amount of cell wall mannan and cell wall porosity, and Rough showed an increase in β-glucan and cell wall porosity (Fig. 2). The cell wall is the most external structure of *Candida* cells, and the cellular integrity depends on its arrangement, to protect against environmental changes and immune responses. Moreover, the cell wall is the first point of contact with the host immune cells (GIL-BONA et al., 2015; WARRIS; BALLOU, 2019). The alteration in cell wall mediated by phenotypic *Switching* places this event as an important cellular fitness factor against environmental variables.

Changes in the host-pathogen interaction with *G. mellonella* mediated by phenotypic *Switching* has already been described (PERINI et al., 2019). Hemocytes showed capacity to phagocyte *C. tropicalis* cells (PERINI et al., 2019), besides that, the production of reactive oxygen species (ROS) like H_2O_2 was detected in *G. mellonella* hemocytes (SLEPNEVA et al., 1999). Here, after 10 min of exposition, Crepe did not show decrease in cell viability, indicating stress response mechanisms for this morphotype (Fig. 3). All others morphotypes showed reduction in CFU counting. Although there was a decrease in viability of all morphotypes after 60 min, the Crepe variant still remained more viable than Parental and its Revertant (RC). The propidium iodide assay indicates that crepe suffers less damage to the membrane due to oxidative stress. Furthermore, oxidative stress promoted cell wall changes in *C. tropicalis* morphotypes. The emergence of resistant morphotype may be related, at least in part, by the alteration of the cell wall modulated by phenotypic *Switching*.

One regulator of the response network is MAPK compounds that help the fungi cells to rapidly and accurately remodel the architecture and composition of the cell wall (WARRIS; BALLOU, 2019). The cell wall integrity and dynamism signaling network was conserved in *Candida* and is able to recognize stresses and transduce them in cell wall responses (LAMBOU et al., 2010; DANTAS et al., 2015).

HOG1 is a crucial component in the MAP kinase pathway that is related to the ability of cells to adapt to environmental changes (SMITH et al., 2010; DANTAS et al., 2015). Phenotypic *Switching* in *C. tropicalis* blood isolate (100.10) modulated the expression of *HOG*1 and *WOR*1 genes. In *C. albicans*, mutants to *HOG1* exhibit high sensitivity to oxidative stress (ALONSO-MONGE et al., 2003; SMITH et al., 2004); besides, *HOG1* stimulation after exposure to H_2O_2 has been described (ENJALBERT et al., 2006). *HOG1* also regulates cell wall chitin deposition and ß-glucan unmasking

(BOGDAN, 2010), an important mechanism to mask cell epitope and make recognition difficult by immunological cells (WARRIS; BALLOU, 2019). In *C. tropicalis*, *WOR1* is a master regulator of white-opaque transitions. This transcription factor also regulates the transition to filamentous forms in *C. albicans* and *C. tropicalis*. (PORMAN et al., 2011). The up-regulation of *HOG1* and *WOR1* mediated by phenotypic *Switching* may be related with the emergence of a more resistant morphotype to oxidative stress.

In mammalian hosts, the first line of defense against *Candida* infection is phagocytosis by macrophages and neutrophils (DANTAS et al., 2015). In Insects, hemocytes develop this function (BERGIN et al., 2005). The immune system of insects and mammals exhibits a high degree of structural and functional similarity (SALZET, 2001; BERGIN et al., 2005). Phagocyte cells produce reactive oxygen species to attack internalized pathogens such as *C. tropicalis* (DANTAS et al., 2015). In the present work, hemocytes and macrophages showed similar capacity to phagocyte *C. tropicalis* morphotypes. Similar responses were described to neutrophils and hemocytes (BERGIN et al., 2005). Phenotypic *Switching* does not promote changes in phagocytosis capacity under untreated conditions (stress free), however stress exposure for 10 min promotes decrease in crepe cells phagocytes. Decrease in phagocytosis may be related to alterations in the cell wall in response to stress. Crepe showed alterations in the amount of mannan, glucan and porosity after H_2O_2 exposure. The dynamics of the cell wall was regulated by conserved mechanisms of integrity signaling and is one of the most important evasion mechanisms of fungi. The alteration of cell surface to enhance or limit immune recognition and maintain the cell structure (WARRIS; BALLOU, 2019).

Co-culture with hemocytes and macrophages promoted up-regulation of *EFG1* gene on Crepe morphotype. The stress exposition also up-regulated *EFG1* gene in both morphotypes (Parental and Crepe). *EFG1* is highly conserved in *C. albicans* and *C. tropicalis* and is an important regulator of the filamentation process in these species (PORMAN et al., 2011). The stimulation of expression of this gene can be related with the ability to form true hyphae in the crepe morphotype in co-cultivation with both phagocytes. Several studies linked response to oxidative stress to the hyphae formation capacity (ALONSO-MONGE et al., 1999; ARANA et al., 2007; BROWN et al., 2009). The ability to switch to yeast form to hyphae is essential to virulence manifestation (BERMAN, 2006; MAYER et al., 2013), and formation to

hyphae forms was linked to evasion of phagocytic cells (SUDBERY et al., 2004; JACOBSEN et al., 2012). Perini et al. (2019) showed that co-culture of *C. tropicalis* morphotypes promoted a decrease in hemocytes number presented in hemolymph. Crepe morphotype of 49.07 switch system was capable to reduce drastically the hemocytes number, however this morphotypes was more phagocytosed. In phagocytosis, the capacity of formation of hyphae in 100.10 Crepe morphotype may bring adaptive advantage against the immune system of the host.

Pre-exposition to oxidative stress before co-culture with phagocytic cells showed variable responses regarding gene expression. The stimulation of morphogenesis genes and stress response genes may be related to the yeast cells surviving after phagocytosis. Cells that punctured phagocytes by hyphae formation, can be phagocytosed by other immune cells (STOJANOVIC et al., 2016; POLONI et al., 2020). Stimulation of response mediated by pre-exposition to oxidative stress may give a light to mechanisms to success in the host.

5 CONCLUSION

Phenotypic *Switching* influences the response to oxidative stress and the interaction with phagocytic cells. The switched morphotypes showed alterations in cell wall, cell size, morphogenesis and gene expression mediated by stress response and immune cells co-culture. The knowledge of physiological changes and stress response may be important for the development of clinical strategies against *C. tropicalis*.

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CONCLUSÃO

O presente trabalho pode concluir que *Switching* fenotípico é capaz de promover a emergência de morfotipos em *C. tropicalis* com resposta alterada ao estresse osmótico e oxidativo. O evento modulou a expressão de genes centrais nas vias de regulação de resposta ao estresse e morfogênese, promoveu alterações na parede celular e na capacidade de morfogênese. A compreensão das alterações de fitness celular mediadas por *Switching* fenotípico podem auxiliar na melhor compreensão da patogênese de *C. tropicalis*.