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Tese de Doutorado

Rafael Wesley Bastos

INFLUÊNCIA DE ANTIFÚNGICOS AMBIENTAIS SOBRE A TOLERABILIDADE AOS ANTIFÚNGICOS CLÍNICOS, MORFO-FISIOLOGIA E VIRULÊNCIA EM *Cryptococcus gattii* e *C. neoformans*

Belo Horizonte
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Tese apresentada ao Programa de Pós-Graduação em Microbiologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito para obtenção do título de Doutor em Ciências Biológicas (Microbiologia).

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"Se eu vi mais longe, foi por estar sobre ombros de gigantes".

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RESUMO

Cryptococcus gattii e *C. neoformans* são os principais agentes etiológicos da criptococose. Por esses patógenos serem encontrados associados a plantas e devido ao aumento da utilização de agroquímicos, nesse trabalho nós avaliamos a hipótese de que a exposição a antifúngicos ambientais poderia afetar a tolerância a antifúngicos clínicos e a virulência de *Cryptococcus* spp. A exposição aos agroquímicos tebuconazol (TBZ) e piraclostrobina (PCT) aumentou a tolerância (resistência cruzada) de algumas linhagens de *C. gattii* e *C. neoformans* ao fluconazol (FCZ), itraconazol e ravuconazol, mas o mesmo efeito não foi observado quando metalaxil foi usado. Algumas linhagens continuaram apresentando resistência cruzada aos azólicos clínicos mesmo após o contato com os agroquímicos ter cessado. Além da alteração da tolerância antifúngica, TBZ e PCT causaram alterações morfológicas em *Cryptococcus*, sendo que algumas linhagens formaram pseudohifas em contato com os agroquímicos. Células de *C. gattii* R265 e *C. neoformans* H99 expostas a TBZ foram menos virulentas em modelo murino do que células não expostas. Contudo, leveduras recuperadas de animais infectados com células previamente expostas a TBZ continuaram apresentando tolerância ao azólicos clínicos. Essa tolerância *in vivo* foi confirmada quando o tratamento com FCZ não foi capaz de diminuir a carga fúngica no pulmão dos animais infectados com células expostas a TBZ. O aumento da tolerância aos antifúngicos pode ser explicado pelo aumento da expressão de *ERG11* e das bombas de efluxo, *PDR11* (*AFR1*) e *MDR1*. Em relação a exposição à PCT, apesar das células de *C. gattii* R265 expostas a esse agroquímico e depois cultivadas em meio sem a droga por 10 passagens (células 10p) serem menos virulentas em modelo animal murino comparada às células não-expostas,. elas demonstraram ser mais tolerantes ao tratamento *in vivo* com FCZ. Essa tolerânica pode ser atribuída a maior expressão de bombas de efluxo e a diminuição da virulência poderia ser atribuída a menor expressão de genes relacionados com vias de aquisição de íons, como mostra a análise transcriptômica. Conclui-se, portanto, que a exposição aos agroquímicos TBZ e PCT aumenta a tolerância a azólicos clínicos, altera a morfologia e diminui a virulência de *Cryptococcus gattii* e *C. neoformans*.

Palavras- Chave: agroquímicos, criptococose, bombas de efluxo, resistência cruzada, pseudohifa.

ABSTRACT

Cryptococcus gattii and *C. neoformans* are the main etiological agents of cryptococcosis. Because these pathogens are found associated with plants and due to the increased use of agrochemicals, in this work we evaluated the hypothesis that exposure to environmental antifungal could affect tolerance to clinical antifungal and virulence of *Cryptococcus* spp. Exposure to agrochemicals tebuconazole (TBZ) and pyraclostrobin (PCT) increased the tolerance (cross-resistance) of some *C. gattii* and *C. neoformans* strains to fluconazole (FCZ), itraconazole and ravuconazole. The same effect did not happen when metalaxyl was used. Some strains presented cross-resistance to clinical azoles even after contact with agrochemicals had ceased. In addition to altering antifungal tolerance, TBZ and PCT caused changes in the morphology of *Cryptococcus*, with some strains forming pseudohyphae in contact with agrochemicals. Cells of *C. gattii* R265 and *C. neoformans* H99 exposed to TBZ were less virulent in murine model than non-exposed cells. However, cells recovered from animals infected with cells previously exposed to TBZ maintained the increased tolerance to clinical azoles. In addition, treatment with FCZ was not able to decrease the fungal load in the lung of animals infected with TBZ-exposed cells. Increased antifungal tolerance may be explained by increased expression of *ERG11* and efflux pumps, *PDR11* (*AFR1*) and *MDR1*. Regarding PCT exposure, *C. gattii* R265 cells exposed to PCT and then cultured in medium without the drug for 10 passages (10p cells) were less virulent in a murine animal model, and treatment with FCZ was ineffective in animals infected with these cells, unlike what happened to animals infected with non-exposed cells. Transcriptomic analysis of 10p and non-exposed cells showed that increased tolerance can be attributed to increased expression of efflux pumps and the reduction of virulence could be attributed to lower expression of genes involved in acquisition of ions pathway. It is concluded, therefore, that the exposure to the agrochemicals TBZ and PCT increases tolerance to clinical azoles, alters the morphology and decreases the virulence of *Cryptococcus gattii* and *C. neoformans*.

Keywords: agrochemicals, cryptococcosis, efflux pumps, cross-resistance, pseudohyphae

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LISTA DE ABREVIAÇÕES E SIGLAS

5-FC: 5-flucitosina

10p: Dez passagens

A: Adaptada

AFR1*: ATP-Binding Cassette Transporter*

AIA: Ácido Indol-Acético

AIDS: Síndrome da Imunodeficiência Humana

ANB (*AMB*): Anfotericina B

ASD (*SDA*): Ágar Sabouraud Dextrose

ATCC: *American Type Culture Collection*

BHE: Barreira Hemato-Encefálica

BMDM: *Bone Marrow-Derived Macrophages*

CEUA/UFMG: Comissão de Ética no Uso de Animais da Universidade Federal de Minas Gerais

CFP: Coleção de Fungos Patogênicos

CIM (*MIC*): Concentração Inibitória Mínima

MCA: *Maximum Concentration Achieved*

COBEA: Colégio Brasileiro de Experimentação Animal

ERG11: *Lanosterol 14α-Demethylase*

FCZ: Fluconazol

FRAC: *Fungicide Resistance Action Committee*

GXM: Glucoronoxilomanano

GalXM: Galactoxilomanana

HIV: Vírus da Imunodeficiência Humana

ITZ: Itraconazol

LBA (*BAL*): Lavado Bronco-Alveolar

MDR1*: Putative ABC multidrug resistance transporter with similarity to Ste6*

mtDNA: DNA Mitocondrial

MTX: Metalaxil

NA: Não-adaptada

PDR11*: Pleiotropic Drug Resistance 11*

PCT: Piraclostrobina

PSZ: Posoconazol

QOI: Q*uinone Outside Inhibitor*

ROS: Espécies Reativas de Oxigênio

RNS: Espécies Reativas de Nitrogênio

RPMI-1640 - *Roswell Park Memorial Institute*

RVZ: Ravuconazol

SNC: Sistema Nervoso Central

SOD: Superóxido Dismutase

TBZ: Tebuconazol

VRZ: Voriconazole

WHO: *World Health Organizatin*

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1 – INTRODUÇÃO

1.1 - *Cryptococcus gattii* e *C. neoformans*

Cryptococcus é um gênero de fungo pertencente ao filo Basidiomycota, ao subfilo Agaricomycotina e à classe Tremellomycetes (Chen *et al.*, 2014). No entanto, *Cryptococcus* é um grupo polifilético, uma vez que seus representantes ocorrem em diferentes ordens de basidiomicetos - Tremellales, Trichosporonales, Filobasidiales e Cystofilobasidiales - segundo estudos filogenéticos baseados na análise da sequência da região D1/D2 do DNA, que codifica a subunidade maior do ribossomo. Atualmente, esse gênero possui mais de 80 espécies descritas, sendo que *C. neoformans* e *C. gattii*, que pertencem a ordem Tremellales e a família *Tremellaceae*, destacam-se por usa importância na medicina humana e veterinária (Fell *et al*, 2000; Chen *et al.*, 2014). Indivíduos dessas duas espécies são compostos, na fase anamorfa, por células leveduriformes esféricas ou globosas envoltas por uma cápsula polissacarídica. A cápsula possui três componentes principais, glucoronoxilomanano (GXM; composto por ácido glucurônico, xilose e manose), galactoxilomanana (GalXM, composto por glactose, xilose e manose) e manoproteínas (manose e, em menor quantidade, xilose e galactose) (Bielska e May, 2016; Watkins *et al.*, 2017).

Diferenças estruturais do polissacarídeo capsular GXM, que constitui cerca de 90% da cápsula, permitem a classificação do complexo *C. neoformans*/*C. gattii* em cinco sorotipos (A, B, C, D e AD). As cepas com sorotipos A e D e o híbrido AD pertencem a *C. neoformans*, sendo que as cepas do sorotipo A foram nomeadas *C. neoformans* var. *grubii*, e as cepas do sorotipo D, *C. neoformans* var. *neoformans.* Já as cepas com sorotipos B e C são classificadas como *C. gattii*. (Franzot *et al.*, 1999; Hagen *et al.*, 2015). É possível, também, classificar *C. neoformans*/*C. gattii* em oito genótipos moleculares: *C. neoformans* é dividido nos genótipos VNI a VNIV e *C. gattii* em VGI a VGIV. O genótipo VGII abriga, ainda, três subtipos: VGIIa, VGIIb e VGIIc, que empregam diferentes circuitos transcricionais e apresentam diferentes fenótipos de virulência (Chaturvedi e Chaturvedi, 2011).

Nos últimos anos, novos estudos demonstraram evidências contra a classificação de *C. neoformans*/*C. gattii* em apenas duas espécies (Bovers *et al.*, 2008; Ngamskulrungroj *et al.*, 2009). Tais evidências levaram Hagen e colaboradores (2015) a

proporem a divisão dos sorotipos em sete espécies haplóides (Tabela 1). Essa classificação, entretanto, tem gerado grande discussão entre a comunidade científica, o que fica claro em dois artigos recentemente publicados: o primeiro indo contra a nova classificação e defendendo a classificação antiga sugerida por Kwown-Chung e colaboradores (2002) em duas espécies (Kwon-Chung *et al*., 2017); e a segunda contra-argumentado a favor da nova classificação (Hagen *et al*., 2017). Apesar do debate, ainda prevalece a antiga classificação, sendo também utilizados os termos "complexo *C. gattii*" e "complexo *C. neoformans*", sugeridos por Kwon-Chung e colaboradores (2017) para se referir a esses micro-organismos (Kwon-Chung *et al*., 2017).

Tabela 2. Nomenclatura atual e proposta das espécies do complexo *C. neoformans*/*C. gattii*.

Nome da espécie atualmente	Genótipo	Nome da espécie proposta
Cryptococcus neoformans var. *grubii*	VNI, VNII e VNIII	*Cryptococcus neoformans*
C. neoformans var. *neoformans*	VNIV	*Cryptococcus deneoformans*
C. gattii	VGI	*Cryptococcus gattii*
C. gattii	VGII	*Cryptococcus deuterogattii*
C. gattii	VGIII	*Cryptococcus bacillisporus*
C. gattii	VGIV	*Cryptococcus tetragattii*
C. gattii	VGIV/VGIIIc	*Cryptococcus decagattii*

Adaptado Hagen *et al*., 2015

O ciclo de vida de *Cryptococcus* spp. é composto por dois estágios, sexual e assexual. *Cryptococcus neoformans* e *C. gattii* são ditas variedades anamórficas (assexuadas), enquanto *Filobasidiella neoformans* e *F. bacillispora* são ditas variedades teleomórficas (sexuadas), respectivamente (Barnett, 2010; Chen *et al*., 2014). Na fase assexual, as leveduras (Figura 1A) de *Cryptococcus* spp. se dividem por meio de brotamentos. Já na fase sexuada há a formação de hifas verdadeiras (Figura 1B). Sobre estímulos específicos (baixa temperatura e pressão de CO_2, ausência de luz, presença de metais como cobre e carência de nitrogênio) as células de *mating* opostos (MAT α e a) se fundem (plasmogamia) e começam a produzir hifas bissexuais dicarióticas (dois núcleos) com núcleos haplóides. Em seguida, algumas dessas hifas, em sua parte terminal, produzem uma basídia, onde ocorrem os eventos de fusão dos núcleos (cariogamia). É na basídia, também, que ocorrem os eventos de meiose que culminam

na formação dos esporos haploides sexuados (basidiósporos), que são considerados propágulos infecciosos. Além disso, a frutificação monocariótica também forma hifas verdadeiras. Nesse caso, células de mesmo *mating* (normalmente MAT α) se fundem para formar uma hifa monocariótica (um núcleo) diploide. A partir daí, os eventos de formação da basídia e, consequentemente dos esporos, ocorre de maneira semelhante ao ciclo sexual (Kronstad *et al.*, 2011; Watkins *et al.*, 2017).

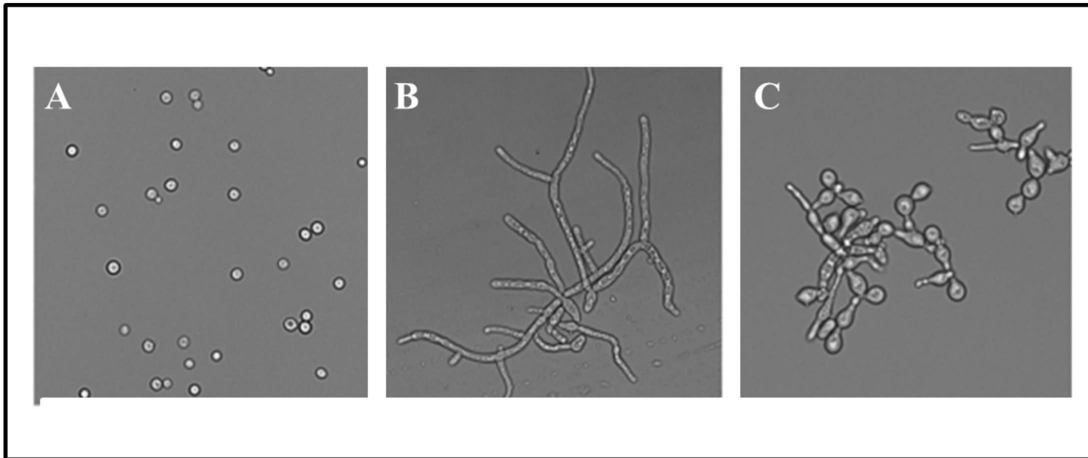

Figura 1. *Cryptococcus* spp. podem apresentar três tipos morfológicos principais: A) Leveduras; B) Hifas verdadeiras e C) Pseudohifas. Fonte: Lin *et al.*, 2015.

Além de formar leveduras e hifas verdadeiras (Figura 1), *C. neoformans* e *C. gattii* podem produzir pseudohifas (Lee *et al.*, 2012; Feretzaki *et al.*, 2014; Pereira *et al.*, 2016) (Figura 1B), que são formadas quando a levedura produz brotos e esses não se desprendem da célula mãe (Trevijano-Contador *et al.*, 2016). Já foi relatado o isolamento de pseudohifas de *Cryptococcus* no líquor de pacientes com criptococose (Williamson *et al.*, 1996; Gazzoni *et al.*, 2010), quando as leveduras são expostas a condições de limitação de nitrogênio (Lee *et al.*, 2012), estresse por drogas antifúngicas *in vitro* (Pereira *et al.*, 2016) e quando esse fungo interage com amebas (Bunting *et al.*, 1979).

Apesar de *Cryptococcus* ser conhecido por ser um patógeno de mamíferos, parece que a infecção criptocóccica acontece de forma acidental, pois esse fungo não necessita dos animais para completar seu ciclo de vida. Acredita-se que os fatores de virulência das espécies patogênicas desse gênero, como melanização, termotolerância, encapsulação e alteração morfológica, foram selecionados ao longo da evolução por meio da interação entre o fungo e o ambiente e/ou predadores naturais e tem um papel dual na sobrevivência desse fungo (Casadevall e Perfect, 1998; Casadevall *et al.*, 2003;

Lin *et al.*, 2015). Portanto, é de extrema importância estudar como esse patógeno se comporta no ambiente para se entender a sua patogênese nos animais.

1.2- Interação *Cryptococcus*-ambiente

Cryptococcus neoformans já foi isolado em diversas localidades, sendo considerado ubíquo. Aves, como pombos, canários e periquitos são consideradas reservatórios dessa espécie, por isso a levedura é encontrada em ninhos e nos excrementos desses animais, onde ela encontra compostos nitrogenados, como ureia e creatinina (Negroni, 2012).

Apesar de ser um reservatório e participar da dispersão dessa espécie, aves normalmente não são infectadas por *C. neoformans*. Inicialmente imaginava-se que isso não ocorria devido a elevada temperatura corporal desses animais que pode alcançar 42°C, o que limitaria o crescimento do fungo. Entretanto, Johnston e colaboradores (2016), ao demonstrarem que *C. neoformans* é capaz de crescer a 42°C, provaram que a temperatura sozinha não explicava o refratismo da doença em aves. Os autores, então, verificaram a capacidade de *C. neoformans* de sobreviver a fagocitose por macrófagos aviários em diferentes temperaturas (37, 39 e 42 °C). Os resultados mostraram que quanto maior a temperatura de incubação dos fagócitos, menor é a sobrevivência do patógeno à fagocitose. Portanto, postula-se que aves são refratárias às infecções por *C. neoformans* por esse não ser capaz de sobreviver dentro de fagócitos na temperatura corporal desses animais (Johnston *et al.*, 2016).

Além de ser encontrado em fezes de aves, *C. neoformans* também já foi isolado em vegetais, como eucalipto (*Eucalyptus* spp.), ipê Rosa (*Handroanthus heptaphyllus*), amendoeiras (*Prunus dulcis*) e em madeira em decomposição (Lazera *et al.*, 1993; Lazera *et al.*, 1996; Mseddi *et al.*, 2011; Noguera *et al.*, 2015). Alguns autores, porém, propõem que o isolamento dessa espécie em vegetais seria devido à presença de fezes de aves nas árvores (Mseddi *et al.*, 2011), o que tem sido refutado por outras pesquisas (Cogliati *et al.*, 2016).

Cogliati e colaboradores amostraram 3763 árvores coletando 6432 amostras desses sítios em 12 países europeus, encontrando *C. neoformans* em 177 dessas árvores (*Ceratonia*, *Eucalyptus*, *Olea*, *Pinus*, *Plantanus*, *Prunus* e *Quercus*). Eles concluíram que as árvores são também um importante e estável nicho para *C. neoformans*,

propondo que as excretas de aves seriam um nicho secundário e temporário para essa espécie (Cogliati *et al.*, 2016).

Inicialmente, pensava-se que *C. gattii* era restrito a regiões tropicais e subtropicais, especialmente América do Sul, África e Austrália. Contudo, nas duas últimas décadas, surtos envolvendo essa espécie aconteceram no hemisfério norte, especificamente em Vancouver (Columbia Britânica-Canadá) e no noroeste do Pacífico dos Estados Unidos da América (Chen *et al.*, 2014; Bielska e May, 2016). As linhagens desse surto chamaram a atenção da comunidade científica por serem mais virulentas (Cheng *et al.*, 2009) e mais resistentes a drogas do que linhagens de *C. gattii* de outras localidades (Gast *et al.*, 2013). Estima-se que 218 pessoas foram infectadas (5,8 pessoas infectadas por milhão por ano) em Vancouver com 19 mortes (Galanis *et al.*, 2010; Bielska e May, 2016). Em Washington e Oregon (EUA), 83 pessoas foram infectadas com taxa de mortalidade de 33% (Harris *et al.*, 2011), mais do que o dobro da taxa de letalidade de outras linhagens (13%) (Chen *et al.*, 2012).

Ao contrário de *C. neoformans*, há um consenso entre os pesquisadores que o principal habitat de *C. gattii* está associado a vegetais. Essa espécie já foi isolada em mais de 50 espécies de árvores, sendo encontrada na casca e em cavidades dos troncos, frutos, solo debaixo das árvores e madeira em decomposição (Chaturvedi e Chaturvedi, 2011; Negroni, 2012; Noguera *et al.*, 2015; Cogliati *et al.*, 2016). No Brasil, amostras de *C. gattii* foram isoladas a partir de amostras de *Eucalyptus* spp., árvores nativas, madeira em decomposição e em poeira de livros em bibliotecas (Lazéra *et al.*, 1998; Montenegro e Paula, 2000; Chaturvedi e Chaturvedi, 2011; Leite *et al.*, 2012). Brito-Santos encontraram *C. gattii* também na poeira de casas no Amazonas. Eles sugeriram que o fungo poderia ter alcançado a parte interna das habitações por meio dos materiais usados para a construção das moradias (Brito-Santos *et al.*, 2015).

Como e por que *C. neoformans* e *C. gattii* se associam com plantas, e como esse nicho ambiental influencia seus ciclos de vida, permanece um mistério (Xue *et al.*, 2007). Na tentativa de responder essas questões, Xue e colaboradores (2007) estudaram a interação entre *C. gattii*/*C. neoformans* e a planta modelo *Arabidopsis thaliana* ("agrião de parede"). Eles observaram que a presença da planta aumenta a eficiência de *mating* em *C. gattii* e que esse fungo pode causar nanismo e clorose nesses vegetais, demonstrando um comportamento parasítico. Essa infecção só foi observada quando uma mistura de *C. gattii* de *matings* opostos foram inoculados no vegetal, o que indica que os eventos de *mating* podem causar sintomas nos vegetais. Interessantemente, foi

demonstrado que o hormônio vegetal ácido indol-acético (AIA) induz eventos de *mating* em *C. gattii,* mas não em *C. neoformans*. A partir desse resultado os autores concluíram que a interação planta-fungo seja mais importante para a primeira espécie completar seu ciclo do que para a segunda, o que justificaria a maior frequência de isolamento de *C. gattii* em vegetais (Xue *et al.*, 2007). Essa ideia de maior frequência de isolamento de *C. gattii* em vegetais, porém, tem sido refutada por autores que mostraram que a frequência de isolamento de *C. neoformans* em árvores foi 13X maior do que de *C. gattii* (Cogliati *et al*., 2016). Nesse mesmo trabalho, os autores encontraram *matings* opostos de linhagens de *C. neoformans* e *C. gattii* co-habitando a mesma árvore, o que parece ser raro em linhagens de *C. gattii* isoladas em árvores na Austrália (Halliday *et al*. 1999). Dessa forma, o estudo de Cogliati e colaboradores (2016) reforça a hipótese de que o contato com vegetais seja importante para ambas as espécies se reproduzirem sexuadamente e completarem seus ciclos de vida (Cogliati *et al*., 2016), o que também fica claro em outro estudo que demonstrou que *Cryptococcus* spp. são capazes de produzir filamentos (reprodução sexuada) em meios de cultura feitos a partir de diversos materiais de plantas (Springer *et al*., 2017).

A interação entre *Cryptococcus* e plantas também pode ocasionar mudanças morfológicas que afetam a virulência fúngica. Springer e colaboradores (2010) demonstram que *C. gattii* consegue colonizar os tecidos vegetais por meio da formação de fibrilas extracelulares, a partir de sua cápsula, e que a formação dessas estruturas possibilita um aumento de virulência do fungo em modelo murino. Esse trabalho demonstrou como a interação com o ambiente, especificamente, com os vegetais, pode influenciar a mofo-fisiologia e virulência de *C. gattii* (Springer *et al.*, 2010).

Outra interação que também parece influenciar a virulência dessas leveduras é a com as amebas (Coelho *et al.*, 2014). *Acantamoeba castellanii* é uma ameba de vida livre que habita os solos, onde ela pode predar *Cryptococcus* spp (Bunting *et al.*, 1979). Alguns estudos indicaram que a interação entre esses dois organismos foi importante para a seleção de fatores de virulência que ajudariam *Cryptococcus* spp a infectar e sobreviver à fagocitose por macrófagos em uma possível infecção, já que o fungo precisa utilizar os mesmos fatores para sobreviver à fagocitose pelas amebas (Casadevall *et al.*, 2003). Assim, a interação com amebas pode ser considerada uma pressão seletiva que ajudou esse fungo a desenvolver uma sofisticada estratégia de patogênese intracelular (Coelho *et al.*, 2014).

1.3 - Interação *Cryptococcus*-hospedeiro

C. neoformans e *C. gattii* podem infectar seres humanos e animais causando uma micose sistêmica conhecida como criptococose. A fonte dessa infecção é exógena e a porta de entrada no hospedeiro é por meio dos pulmões (Negroni, 2012): os basidiósporos ou leveduras dessecadas, por meio da poeira (vento) atingem o hospedeiro pelo trato respiratório, de onde as leveduras podem se disseminar para outros órgãos (Li e Mody, 2010; Huang *et al.*, 2015). Essa infecção pode se apresentar de duas formas distintas: "(i) criptococose oportunística, cosmopolita, associada a condições de imunodepressão, causada principalmente por *C. neoformans* e (ii) criptococose primária, endêmica em áreas tropicais e subtropicais, ocorre em hospedeiros aparentemente imunocompetentes, causada por *C. gattii*. Ambas causam meningoencefalite, de evolução grave, fatal se não tratada, acompanhada ou não de lesão pulmonar evidente, fungemia e focos secundários para pele, ossos, rins, supra-renal, entre outros"(Ministério da Saúde, 2012).

1.3.1 - Histórico e epidemiologia

A criptococose foi reportada pela primeira vez pelos médicos alemães Busse e Buschkeem em 1884, em uma mulher de 31 anos com lesão na tíbia. Como Busse considerou o patógeno isolado semelhante à levedura *Saccharomyces*, ele chamou o quadro clínico de "Saccharomycosis hominis", contudo, nenhum dos dois autores nomeou o patógeno, sendo que esse ficou conhecido como "levedura de Busse". Coincidentemente, na mesma época, o médico italiano Francesco Sanfelice descreveu uma levedura encapsulada isolada de suco de pêssego e a identificou como *Saccharomyces neoformans*, relatando, também, que esse fungo era patogênico para animais experimentais e que tinha características semelhantes às da "levedura de Busse". Em 1901, Vuilleman reviu estes isolados e transferiu-os para o gênero *Cryptococcus* porque estas leveduras não eram capazes de fermentar açúcares e nem produzir ascósporos, características básicas do gênero *Saccharomyces*. A partir de 1900 surgiram inúmeros relatos em humanos e também em outros animais, com progressivo reconhecimento do agente como patógeno relacionado à infecção do sistema nervoso central (Barnett, 2010).

C. gattii, por sua vez, foi primeiramente descrito depois de ser isolado de um paciente com meningite no antigo Zaire, centro da África. O líquor desse paciente se apresentava de forma atípica, com leveduras alongadas capsuladas, semelhantes a grãos de arroz, ao lado das formas usuais arredondadas. Esse isolado foi denominado *C. neoformans* var. *gattii*, depois identificado como sorotipo B (Vanbreuseghem e Takashio, 1970).

Antes da década de 80, a infecção criptocóccica era uma infecção sistêmica incomum, que ocorria em pacientes em terapia com corticóides ou diagnosticados com diabetes melito, doença de Hodgkin, lúpus eritematoso sistêmico, dentre outros (Mitchell e Perfect, 1995). Entretanto, com a ascensão da infecção pelo HIV (Vírus da Imunodeficiência Humana), a incidência da criptococose aumentou significativamente, sendo que a infecção por esse vírus esteve associado a mais de 80% de casos de criptococose em todo o mundo (Hajjeh *et al.*, 1999). Já em 2009, o CDC (*Control Desease Center*) estimou que a neurocriptococose acometia em torno de 1 milhão de pessoas com HIV anualmente, resultando em aproximadamente 600 mil mortes. As estimativas foram recentemente atualizadas para 223100 casos/ano de meningite criptocócica e mais de 81% (181000) de mortalidade (Rajasingham, *et al.*, 2017, Williamson *et al.*, 2017). A meningite criptocócica, nos dias atuais, tem sido associada a 15% das mortes de pacientes com HIV (Rajasingham,*et al.*, 2017). A maioria dos casos diagnosticados ocorre na África, Ásia e na América (Park *et al.*, 2009; Chen *et al.*, 2014).

No Brasil, segundo o Ministério da Saúde (2012), a criptococose é a micose sistêmica que mais causou internações entre os anos de 2000-2007. Por ser considerada, principalmente, uma infecção oportunística, essa micose acompanha os casos de aumento da população de risco, que são pacientes com AIDS (Síndrome da Imunodeficiência Humana), em terapias contra leucemias e linfomas e que fazem uso de corticoides (Ministério da Saúde, 2012). Contudo, a infecção por *C. gattii* ocorre como infecção primária, não estando necessariamente associada com o aumento dessa população (Chaturvedi e Chaturvedi, 2011).

A criptococose é mais prevalente, no Brasil, na região sudeste, provavelmente pelo maior número de casos de pacientes com HIV relatados. No entanto, no Norte e Nordeste, são encontrados casos de infecção criptocóccica em pacientes HIV-negativos, sem evidência de imunodepressão, ocasionados por *C. gattii*. Dessa forma, a

criptococose nessa região é considerada endêmica (Ministério da Saúde, 2012; Chen *et al.*, 2014).

A taxa de mortalidade por criptococose no Brasil varia de 26-70% (Moreira *et al.*, 2006; Mora *et al.*, 2012; Mezzari *et al.*, 2013; Souza *et al.*, 2013). Em estudo recente, sendo 85% dos pacientes HIV-positivos, uma taxa de 58,5% (n=41) foi encontrada mesmo após os pacientes receberem tratamento considerado adequado (Aguiar *et al.*, 2017). Um dos motivos relatados pelos autores para essa elevada taxa de mortalidade foi a virulência das linhagens de *C. neoformans*. Isso salienta a importância de se estudar e compreender como as diferentes linhagens são capazes de infectar e causar doença nos hospedeiros, a fim de criar terapias cada vez mais direcionadas e específicas contra a criptococose.

1.3.2 - Patogênese

A infecção por *Cryptococcus* spp. é adquirida pela inalação de basidiósporos, produzidos durante o ciclo sexuado, ou células dessecadas da levedura que se depositam nos alvéolos, comprometendo inicialmente o pulmão de onde podem disseminar-se para outros órgãos (Datta *et al.*, 2009; Gibson e Johnston, 2015). Leveduras dessecadas presentes no ambiente são propágulos infecciosos devido ao seu pequeno tamanho (3 µm), bem como os esporos (1-2 µm), em relação àquelas crescendo ativamente (4-10 µm), que são grandes demais para invadirem os alvéolos (Kronstad *et al.*, 2011; Watkins *et al.*, 2017).

Dentro do hospedeiro, as leveduras dessecadas ou os esporos transformam-se em leveduras capsuladas, estimuladas pela temperatura de 37ºC. O hospedeiro desenvolve um complexo linfonodal pulmonar primário e na maioria dos casos, a inalação de *C. neoformans* produz uma infecção pulmonar assintomática autolimitada, e as leveduras permanecem latentes dentro desse complexo, morrem ou, com um posterior evento de imunossupressão, são reativadas e causam doença. Essa infecção pode também causar sintomas pulmonares no hospedeiro, em caso de imunossupressão ou de um grande inóculo da levedura. No pulmão, as manifestações vão desde infecção assintomática até pneumonia grave (Chaturvedi e Chaturvedi, 2011; Negroni, 2012; Chen *et al.*, 2014). No caso de *C. gattii*, pouco é conhecido sobre as fases iniciais da sua interação com o hospedeiro. É sabido, porém, que o contato com os propágulos infecciosos dos genótipos VGI e VGII causam doenças em pacientes imunocompetentes, já VGIII e

VGIV parecem acometer mais imunossuprimidos, tendo patogênese provavelmente semelhante a *C. neoforomans* (Chen *et al*., 2014).

Ambas as espécies de *Cryptococcus* parecem ter predileção para a migração para o sistema nervoso central (SNC), onde elas podem causar meningite ou meningoencefalite. Os principais sinais e sintomas são cefaleia, febre, neuropatia craniana (danos nos nervos cranianos), alteração da consciência, letargia (perda temporária ou completa da sensibilidade do movimento), perda de memória, sinais de irritação meníngea e coma (Nadrous *et al.*, 2003).

Para alcançar o SNC, esses fungos precisam atravessar a barreira hematoencefálica (BHE). O mecanismo pelo qual eles penetram a BHE não é totalmente entendido, embora várias possibilidades tenham sido propostas, incluindo passagem entre células endoteliais vizinhas (entrada paracelular), transporte para o sistema nervoso central dentro de fagócitos infectados (modelo Cavalo de Tróia), ou por absorção e travessia através de células endoteliais (transcitose) (Chang *et al.*, 2004; Charlier *et al.*, 2009; May *et al*., 2016; Sorrell *et al*., 2016).

Vários fatores do patógeno possibilitam a interação *Cryptococcus*-hospedeiro e consequente infecção, sendo os principais: termotolerância e presença de cápsula, das enzimas lacase, urease, fosfolipase B, superóxido dismutase (SOD) e do açúcar trealose. Resumidamente, a termorolerância permite ao fungo se mutiplicar dentro do hospedeiro; a cápsula participa da evasão/supressão da resposta imune do hospedeiro; a atividade da lacase é responsável pela produção de melanina que protege o micro-organismo contra o estresse oxidativo e, assim como a fosfolipase B e a urease, é importante para a disseminação do fungo e para a transmigração para o SNC; já a trealose e a SOD são importantes no processo de antioxidação, sendo a trealose, ainda, essencial no processo de termotolerância (Krontad *et al*., 2011; Chen *et al*., 2014; Bielska e May, 2016; May *et al*., 2016; Watkins *et al*., 2017).

Por meio desses fatores de virulência e de outros, *Cryptococcus* spp. causa infecções graves, principalmente a meningoencefalite, que se não tratadas apropriadamente levam o hospedeiro a morte (Ministério da Saúde, 2012).

1.3.3 - Tratamento da criptococose

O tratamento da criptococose está intrinsecamente ligado ao sítio de infecção, ao estado imunológico do paciente e a disponibilidade de drogas (Perfect *et al.*, 2010; Coelho e Casadevall, 2016). Normalmente, os antifúngicos utilizados são o polieno anfotericina B (ANB) combinado com o azólico fluconazol (FCZ) e/ou 5-flucitosina (análogo de pirimidina)(Nooney *et al.*, 2005; WHO, 2011; Coelho e Casadevall, 2016; Perfect *et al*., 2017). Para neurocriptococose, a terapia é dividida em três fases: indução, consolidação e erradicação. Na primeira fase (indução), recomenda-se o uso de ANB + 5-flucitosina por duas semanas seguida pela administração de FCZ por mais oito semanas (fase de consolidação) e depois por mais 6-12 meses e/ou até a restauração da imunidade do hospedeiro (fase de erradicação) (Perfect *et al.*, 2010; Perfect e Bicanic, 2015). Já em casos de pneumonia leve a moderada, recomenda-se o uso de FCZ ou itraconazol (ITC) por 6-12 meses (Perfect *et al.*, 2010). O ITC não é recomendado para pacientes com neurocriptococose por não ter boa penetração no líquor (Subramanian e Mathai, 2005). Outros azólicos, como voriconazol (VRC) e posoconazol (PSC) (novos azólicos) são indicados como terapia alternativa quando o FLC se encontra indisponível ou é contraindicado (Perfect *et al.*, 2010; Chen *et al.*, 2013).

Para os casos de criptococose por *C. gattii*, estudos realizados por Chen e colaboradores (2013) indicaram que o melhor regime terapêutico seria: (i) para neurocriptococose, seis semanas de terapia com ANB + 5-FC, seguida da administração de FLC por 18 meses e, (ii) para infecção pulmonar, tratamento com ANB + 5-FC por duas semanas e 12 meses de FCZ (Chen *et al.*, 2013). Nos países onde a ANB não está disponível, como nos países africanos, a Organização Mundial de Saúde (*World Health Organization* -WHO) recomenda o uso de FLC + 5-FC, ou monoterapia com altas doses de FLC (WHO, 2011). Outros antifúngicos, como alilaminas e a nistatina, não são recomendados por não serem absorvidos suficientemente ou por serem muito tóxicas (Coelho e Casadevall, 2016). Já a nova classe de antifúngicos, equinocandinas, representada principalmente pela caspofungina, não é usada no tratamento da criptococose, pois os agentes causadores são intrinsicamente resistentes a esse grupo de antifúngicos (Huang *et al.*, 2016).

O tratamento padrão da criptococose, descrito anteriormente, é o uso de ANB + 5-FC e, em seguida, uso de FLC (Perfect *et al*., 2010). Day e colaboradores (2013) em um estudo com quase 300 pacientes, mostraram que a combinação entre ANB+5-FC era

mais eficiente que a administração de ANB sozinha ou ANB combinada com FLC. Contudo, nosso grupo recentemente demonstrou que a combinação de ANB + “alta dose” de FLC (150 mg/Kg de animal) é mais eficiente que o tratamento com os antifúngicos separados, e com ANB + “baixa dose” de fluconazol (15 mg/Kg de animal) em modelo murino de criptococose por *C. gattii* (Santos *et al.*, 2017). Esse estudo abre novas perpesctivas para os regimes de administração dos antifúngicos já usados contra a criptococose.

A ANB pertence à classe dos polienos e é comercializada na forma de deoxicolato e em três outras formulações lipídicas, anfotericina B lipossomal, complexo lipídico de anfotericina B e dispersão coloidal (Nett e Andes, 2016). Esse polieno tem um amplo espectro de ação e age na membrana plasmática da célula fúngica ao se ligar ao ergosterol e formar poros. A formação dessas estruturas promove um aumento da permeabilidade extracelular a cátions, o que resulta na morte do fungo (Cannon *et al.*, 2009; Nett e Andes, 2016). Além da formação de poros, ANB pode sofrer auto-oxidação (Lamy-Freund *et al.*, 1985) ou induzir a produção de espécies reativas de oxigênio (ROS), assim como espécies reativas de nitrogênio (RNS) (Ferreira *et al.*, 2013). Esses radicais livres conduzem a peroxidação lipídica nas membranas celulares, o que culmina em mais danos à célula fúngica e morte (Ferreira *et al.*, 2013). Embora altamente eficaz, a ANB apresenta efeitos nefrotóxicos e hepatotóxicos que limitam o seu uso e as formulações lipídicas são indicadas nos casos de pacientes com histórico de problemas renais, uma vez que essas formulações são menos tóxicas (Nett e Andes, 2016).

A flucitosina é uma pirimidina fluorada (5-fluorocitosina) e, por ser um análogo de pirimidina, ela é transportada para dentro da célula pela permease de citosina e, então, convertida a fluorouracil pela citosina deaminase. O fluorouracil prejudica a síntese de ácido nucleico, e, consequentemente, interfere na síntese de proteínas (Waldorf e Polak, 1983). Alguns problemas estão envolvidos com o uso da 5-FC, como sua baixa meia vida, o que cria a necessidade do paciente ter que tomar quatro doses diárias dessa droga, a sua toxicidade para a medula óssea e fígado e sua disponibilidade, já que apenas duas empresas produzem essa droga no mundo e ela está indisponível em várias localidades, como na África (Perfect e Bicanic, 2015) e no Brasil. Além disso, quando usada em monoterapia, populações resistentes são comumente selecionadas (Akins, 2005).

Os azóis são substâncias fungistáticas que possuem o anel azólico composto por dois ou três átomos de nitrogênio e o anel benzeno halogenado. Eles são divididos em duas classes, os imidazóis (dois átomos de N)(cetoconazol, miconazol) e os triazóis (três átomos de N) (FLC, ITC, VRC, ravuconazol-RVC). A ação antifúngica dos azóis é por meio da interrupção da biossíntese de ergosterol com consequente efeito sobre a estabilidade da membrana plasmática. Essas drogas inibem a enzima lanosterol 14-α-demetilase (produto do gene *ERG11*) (Lupetti *et al.*, 2002; Zavrel e White, 2015), o que impede a conversão do lanosterol em 4,4-Dimethyl-8,14,24-trienol, interrompendo, assim, a produção do ergosterol (Akins, 2005). Apesar da inibição, não há acumulo de lanosterol no citoplasma da célula, pois acredita-se que o grupo 14-metil do lanosterol esteja envolvido na metilação de substâncias tóxicas que se acumulam na membrana plasmática, como o 14-metilergosta-8,24(28)-dien-3,6-diol (Akins, 2005). Estes metilesteróis, por não possuírem as mesmas propriedades físicas do ergosterol, levam à formação de uma membrana com estrutura e função alterada, o que resulta em alteração da permeabilidade da célula fúngica e interrupção do crescimento (Kelly *et al.*, 1997; Ghannoum e Rice, 1999; Heimark *et al.*, 2003; Casalinuovo *et al.*, 2004; Berkow e Lockhart, 2017). Alguns azóis, como o ITC, além de atuarem na inibição da síntese do ergosterol, podem induzir estresse oxidativo na célula e contribuírem para o seu efeito antifúngico (Ferreira *et al.*, 2013).

Ao contrário da ANB e da 5-FC, os azóis, especialmente o FLC, ocasionam menos efeitos colaterais (Zavrel e White, 2015). Além disso, o FLC tem adequada penetração em vários tecidos, incluindo o sistema nervoso central e excelente biodisponibilidade e tolerabilidade pelos pacientes, por isso é mais frequentemente prescrito na clínica (Allen *et al.*, 2015). Entretanto, é crescente o isolamento de linhagens “menos susceptíveis” e “resistentes/tolerantes” a esse antifúngico (Smith *et al.*, 2015; Berkow e Lockhart, 2017) .

1.3.3.1 – Resistência/Tolerância aos antifúngicos

O processo de resistência aos fármacos antifúngicos pode ser definido de modo distinto a partir de abordagens microbiológicas e clínicas. Do ponto de vista microbiológico, a resistência à droga é definido pela presença de um mecanismo de

resistência adquirido a drogas que estão sendo testadas e depende diretamente do micro-organismo. Essa resistência pode ser dividida em: (i) resistência primária ou inata, quando o micro-organismo é naturalmente resistente a droga, sem prévia exposição, e (ii) resistência secundária, quando populações resistentes são selecionadas após a exposição ao antifúngico (Perea e Patterson, 2002; Alcazar-Fuoli e Mellado, 2014). Já a resistência clínica consiste na falha de uma terapia apropriada para o tratamento de uma infecção ou recidiva da mesma devido à insuficiência da droga no soro ou no tecido. Esse tipo de resistência pode ser influenciado por fatores fúngicos, que estão relacionados à espécie, aos tipos celulares, à população de organismos; por fatores ligados ao agente antifúngico, que estão relacionados às classes (triazóis, polienos), à concentração inibitória mínima (CIM) da droga; à dosagem utilizada e à farmacocinética; e por fatores do hospedeiro, como o estado imune (Yang e Lo, 2001; Loeffler e Stevens, 2003). A resistência clínica pode não estar associada à resistência *in vitro* (Loeffler e Stevens, 2003).

Outra forma de resistência é denominada como resistência cruzada, que ocorre quando um mecanismo de resistência referente a uma droga também confere resistência a outras moléculas de diferentes grupos químicos ou não (Snelders *et al.*, 2012; Faria-Ramos *et al.*, 2014ab; Rocha *et al.*, 2016; Rossi *et al.*, 2016). O problema da resistência bacteriana na pecuária relacionado com a saúde humana é destacado pelo processo da resistência cruzada decorrente da semelhança entre os antimicrobianos utilizados em seres humanos com os utilizados nos animais (Korb *et al*, 2011).

Outro tipo de resistência, envolvendo *C. neoformans* e *C. gattii*, tem chamado atenção da comunidade científica nos últimos anos (Ferreira e Santos, 2017). Mondon e colaboradores (1999) demonstraram que linhagens de *Cryptococcus* isoladas de pacientes infectados ou não pelo HIV, com altas recidivas de neurocriptococose, tinham um padrão não usual de resistência ao FLC e ao VRC (Mondon *et al.*, 1999). Eles denominaram esse tipo de resistência de heterorresistência, que atualmente é definida como a capacidade de uma subpopulação em se adaptar a altas concentrações da droga, resultando em populações homogêneas mais resistentes. No entanto, as linhagens heterorresistentes retornam ao fenótipo inicial (susceptível) quando o estímulo com a droga é retirado (Sionov *et al.*, 2009; Ferreira *et al.*, 2015; Ben-Ami *et al.*, 2016; Ferreira e Santos, 2017). Em *Cryptococcus*, o nível de heterorresistência ao FLC é inato ao micro-organismo e pode ser variável entre as diversas linhagens (Sionov *et al.*, 2009; Varma e Kwon-Chung, 2010).

Não existem *breakpoints* específicos determinados pelos manuais de testes de sensibilidade a drogas antifúngicas para antifúngicos usados contra espécies de *Cryptococcus* (CLSI, 2012). Entretanto, alguns trabalhos têm utilizado os *breakpoints* determinados para *Candida* em documento emitido pelo *Clinical and Laboratory Standards Institute* em 2008 (CLSI, 2008; Chen *et al.*, 2015; Smith *et al.*, 2015). Dessa forma, usando os *breakpoints* antigos para *Candida*, isolados com CIM ≤ 8,0 são considerados susceptíveis, com CIM = 16,0 ou 32,0 suceptíveis dose-dependente e com CIM ≥ 64,0 são considerados resistentes ao FLC. Para anfotericina B os isolados são sensíveis quando sua CIM ≤ 1,0 e resistentes quando a CIM é ≥2,0 (CLSI, 2008).

Enquanto alguns extrapolam os *breakpoints* de *Candida* para *Cryptococcuss* spp. e desconsideram fatores específicos da criptococose, outros trabalhos utilizam o termo "mais ou menos tolerante" para descrever linhagens com alta ou baixa CIM, respectivamente.

Classicamente, tolerância é usado com outro sentido, para descrever uma população de células que sobrevive transientemente a exposição à drogas, sem ter a sua CIM alterada. Normalmente isso ocorre devido ao retardo do crescimento dessas células que passam a se manter viáveis na presença de concentrações maiores da droga que as células consideradas susceptíveis se manteriam. Esse termo, ainda, se difere de outros como persistência, uma vez que tolerância se refere a inteira população de células e a persistência é um fenômeno tempo e dose-dependente que é usado para descrever subpopulações de células, presentes em uma população considerada susceptível, que têm a capacidade de se manterem viáveis na presença de altas drogas microbianas (Ferreira e Santos, 2017).

Apesar da resistência aos antifúngicos não ser um problema reconhecidamente característico de espécies de *Cryptococcus* (Perfect *et al.*, 2010; Chen *et al.*, 2014), nos últimos anos tem aumentado os relatos de isolamento de linhagens mais tolerantes aos fármacos utilizados para o tratamento da criptococose. Recentemente, dois estudos demonstraram que nos últimos anos houve aumento no isolamento de linhagens de *C. neoformans* "menos sensíveis" e até "resistentes" (MIC ≥64) ao FLC em Uganda (Smith *et al.*, 2015) e em Taiwan (Chen *et al.*, 2015)(considerando os *breakpoints* para *Candida* sp.(CLSI, 2008)) . Além disso, as linhagens de *C. gattii* isoladas dos surtos no Noroeste do Pacífico dos EUA têm se mostrado mais tolerantes aos azóis que outras cepas de *C. gattii* e *C. neoformans* (Gast *et al.*, 2013). Dessa forma, cresce a

preocupação em relação a essas linhagens e sobre os mecanismos pelos quais esses micro-organismos tornam-se tolerantes a essas drogas.

1.3.3.2-Mecanismos de resistência/tolerância aos antifúngicos

Os mecanismos de resistência aos antifúngicos são melhor caracterizados em espécies de *Candida*, principalmente em *C. albicans* (Berkow e Lockhart, 2017). A partir do conhecimento sobre os mecanismos nesse patógeno, associações e/ou novos estudos buscam entender como outros fungos também se tornam resistentes.

Comumente, a resistência aos polienos, alilaminas, e equinocandinas é pouco observada (Ellis, 2002; Cannon *et al.*, 2009; Zavrel e White, 2015). Apesar da ocorrência incomum, a resistência aos polienos pode ser devida à redução dos níveis de ergosterol na membrana plasmática (Cannon *et al.*, 2009; Santos *et al.*, 2012) ou uma mudança desse alvo lipídico, o que determina a diminuição da ligação da ANB na célula fúngica (Ellis, 2002; Lupetti *et al*., 2002). Dessa forma, algumas células contendo mutação na via de síntese de ergosterol (mutação no gene *ERG2, ERG3* e *ERG11*, por exemplo) não produzem ergosterol, mas sim, compostos semelhantes a esse lipídeo que tem menor afinidade pela ligação com ANB (Geber *et al.*, 1995; Ellis, 2002; Akins, 2005).

A resistência microbiana aos azóis, principalmente ao FLC, tem sido mais relatada do que a resistência a outros grupos de antifúngicos. *Candida* spp podem ser intrinsecamente resistentes ao FLC (*C. krusei*) (Orozco *et al.*, 1998; Guinea *et al.*, 2006) ou podem obter resistência secundária (Berkow e Lockhart, 2017). Os mecanismos normalmente envolvidos nesse processo são: (i) aumento da expressão e (ii) mutação do gene *ERG11*, (iii) diminuição da atividade de ERG3p e outras enzimas da via de síntese do ergosterol e (iv): superexpressão de bombas de efluxo (Akins, 2005; Cannon *et al.*, 2009; Zavrel e White, 2015, Berkow e Lockhart, 2017).

O aumento da expressão de ERG11p, que pode ocorrer devido a mutação e constante expressão de seu regulador *Upc2* em *Candida*, aumenta a quantidade de moléculas alvo dos azóis, e, com isso, uma maior concentração da droga será necessária para inibir o crescimento fúngico. Além de superexpessar *ERG11*, *Candida* spp. podem produzir uma enzima com menor afinidade pelos azóis, devido a mutações no referido gene, resultando em células menos suceptíveis a essas drogas (Akins, 2005; Cannon *et*

al., 2009; Zavrel e White, 2015). Já a diminuição da atividade da enzima ERG3p pode conferir resistência aos antifúngicos porque essa enzima é responsável pela produção de metabólitos metilados tóxicos quando as células fúngicas estão na presença de drogas. Acredita-se que esses metabólitos sejam os principais responsáveis pela ação fungistática dos azóis ao se acumularem na membrana plasmática no lugar do ergosterol (Akins, 2005; Berkow e Lockhart, 2017). Em mutantes *ERG3*, a diminuição da atividade de ERG3p na presença de compostos azólicos favorece a via de produção de metilfecosterol por ERG24p, que, ao contrário dos metilesteróis, sustenta a integridade da membrana plasmática e o crescimento fúngico (Lupetti *et al.*, 2002; Berkow e Lockhart, 2017). Por fim, as bombas de efluxo podem bombear drogas do citoplasma para o ambiente extracelular, prevenindo que essas drogas encontrem seus alvos. A superexpressão de bombas de efluxo é o mecanismo de resistência antifúngica mais comum entre os fungos patogênicos, e em *Candida* ela ocorre devido à ativação constitutiva dos reguladores *Mrr1* e *Tac1* (Cannon *et al.*, 2009; Zavrel e White, 2015; Berkow e Lockhart, 2017).

Existem poucos estudos que relatam os mecanismos de resistência a drogas em *Cryptococcus* (Chen *et al.*, 2014). Rodero e colaboradores (2003) e Sionov e colaboradores (2012) demonstraram que substituições de aminoácidos na enzima ERG11p podem ser responsáveis pelo "fenótipo resistente" em algumas linhagens de *C. neoformans* (Rodero *et al.*, 2003; Sionov *et al.*, 2012). Entretanto, Gast e colaboradores (2013), comparando a susceptibilidade aos azólicos de 25 linhagens de *C. gattii* isoladas dos surtos do Noroeste do Pacífico dos EUA, 34 linhagens de *C. gattii* isoladas de outras localidades e 24 linhagens de *C. neoformans*, demonstraram que as linhagens isoladas dos surtos são mais tolerantes aos azóis do que as outras linhagens e que essa tolerância não está associada ao aumento da expressão de *ERG11* e nem a mutações nesse gene. Eles postularam, portanto, que a produção de bombas de efluxo poderia explicar essa alteração de tolerância (Gast *et al.*, 2013).

Basso e colaboradores (2015), por sua vez, ao compararem as mesmas linhagens de *C. gattii* isoladas dos surtos com outras linhagens dessa mesma espécie isoladas de outros locais e *C. neoformans*, mostraram que a concentração intracelular de FLC marcado radiotivamente era inversamente proporcional a CIM para o FLC, de forma significativa, apenas no grupo das linhagens de *C. gattii* do surto. Logo, eles demonstraram que as linhagens do surto provavelmente são mais tolerantes porque menos FLC é acumulado dentro das células. Em seguida, eles clonaram em

Saccharomyces cerevisiae os genes de *C. gattii* que eram ortólogos aos genes que codificam as bombas *AFR1*, *AFR2* e *MDR1* em *C. neoformans*, assim como os próprios genes de *C. neoformans,*. Os seis clones de *S. cerevisiae* capazes de expressar os genes para as possíveis bombas foram mais tolerantes ao FLC, VRC, ITC e PSC que a linhagem selvagem. Dessa forma, os pesquisadores mostraram que expressão dos genes *AFR1*, *AFR2* e *MDR1* de *C. neoformans* e *C. gattii* contribui para a tolerabilidade aos azóis. Entretanto, eles não conseguiram demonstrar que linhagens de *Cryptococcus* spp. mais tolerantes superexpressão essas bombas (Basso *et al.*, 2015).

Para tentar desvendar o papel do produto dos genes *MDR1* e *PDR1* (também chamado de *AFR1* ou Cg*AFR1*) na tolerabilidade aos azóis em *C. gattii*, Yang e colaboradores (2016) deletaram os referidos genes em uma linhagem de *C. gattii* que apresentava "elevada CIM para FLC" (CIM=32,0 µg/mL). O mutante para *PDR11* se tornou 512X (CIM= 0,0625 µg/mL) menos tolerante ao FLC do que a linhagem selvagem, ao passo que a CIM para Δ *mdr1* não se alterou. Δ *pdr1* também foi menos tolerante ao ITC, VRC e PSC, mas não a ANB e 5-FC. Assim, apesar de Basso e colaboradores (2015) demonstrarem que ambos os genes, *AFR1* e *MDR1*, possam estar envolvidos na tolerabilidade aos azóis, Yang e colaboradores (2016) comprovaram que apenas *PDR11* (*AFR1*) está envolvido nesse processo em *C. gattii* (Yang *et al.*, 2016).

Em relação ao mecanismo de heterorresistência, Sionov e colaboradores (2010) comprovaram que a tolerância momentânea ao FLC (só na presença da droga) observada em *C. neoformans* H99 era acompanhada por duplicações cromossômicas (dissomias). Inicialmente, o cromossomo 1 duplicava, acompanhado pelos genes *ERG11* e *AFR1*, e, em seguida, outros cromossomos também sofriam duplicações com aumento da concentração da droga (cromossomos 3, 4, 10 e 14) (Sionov *et al.*, 2010). É interessante observar que o cromossomo 4 de *C. neoformans* possui dois genes para transportadores ABC (*ATP-Binding Cassette*) e um gene para um trasportador *Pleiotropic Drug Resistance* (*PDR16*). Desses três genes, apenas a deleção de *PDR16* afetou a tolerância ao FLC dos clones heterorresistentes, deixando-os menos tolerantes (Ngamskulrungroj *et al.*, 2012). Entretanto, essas duplicações não parecem estar relacionadas a resistência secundária dessa mesma espécie ao FLC (Santos *et al.*, 2014). Logo, ainda não se sabe se *C. gattii* também é capaz de duplicar seus cromossomos em resposta ao estresse por drogas antimicrobianas (Chen *et al.*, 2014).

A exposição aos antifúngicos clínicos não é o único indutor de superexpressão de bombas de efluxo e de resistência em fungos. De modo recente, descobriu-se que

agroquímicos, especificamente substâncias antifúngicas usadas na agricultura, são capazes de induzir aumento da expressão de bombas de efluxo em *Candida parapsilosis* (Rocha *et al*., 2016) e seleção de *Aspergillus fumigatus* com mutação em *ERG11* (Ren *et al*., 2017). Em ambos os casos, os agroquímicos demontraram selecionar populações com resistência cruzada aos antifúngicos clínicos.

1.3.3.3- Agroquímicos e Resistência Cruzada

O controle químico de doenças fitopatogênicas é, na maioria dos casos, a única medida eficiente e economicamente viável de garantir as altas produtividade e qualidade de produção na agricultura (Chatterjee *et al.*, 2013; Wu e Ma, 2015). O mercado brasileiro de agrotóxicos expandiu rapidamente na última década (190%), em um ritmo de crescimento maior que o dobro do apresentado pelo mercado global (93%), o que coloca o Brasil em primeiro lugar no ranking mundial, desde 2008 (Rigotto *et al.*, 2014). No entanto, esse uso indiscriminado de agroquímicos adquiriu, nos últimos anos, uma grande importância devido ao impacto da contaminação do meio ambiente (Sehnem *et al.*, 2010; Pinto *et al.*, 2012; Chatterjee e Gupta, 2013; Chatterjee *et al.*, 2013) .

As evidências de que muitos pesticidas causam graves problemas de saúde humana estão crescendo (Rigotto *et al.*, 2014). Não está claro, no entanto, (i) quais são os efeitos dos agroquímicos sobre patógenos humanos que vivem no meio ambiente e (ii) quais são os impactos indiretos desses efeitos na saúde humana e animal. Uma possível consequência do uso desses compostos no ambiente seria a seleção de subpopulações fúngicas resistentes aos biocidas e que poderiam apresentar resistência cruzada com antifúngicos clínicos. Esse fenômeno vem sendo pouco estudado nos últimos anos, mas trabalhos já apontam que a exposição a antifúngicos ambientais como tebuconazol, procloraz e tetraconazol selecionam subpopulações de *Colletotrichum graminicola*, *Aspergillus fumigatus* e *Candida parapsilosis*, respectivamente, menos sensíveis a drogas clínicas (Serfling *et al.*, 2007; Snelders *et al.*, 2012; Faria-Ramos *et al.*, 2014; Faria-Ramos *et al.*, 2014). Outro trabalho confirmou a existência de resistência cruzada entre azóis usados na clínica e os usados na agricultura em espécies clínicas e ambientais de *Candida* (Müller *et al*., 2007).

Os trabalhos envolvendo resistência cruzada têm focado bastante na resistência entre azóis clínicos e ambientais e não entre classes diferentes de antifúngicos. Entretanto, nosso grupo demonstrou que a exposição a concentrações crescentes de um fungicida não-azólico, proibido em diversos países, denominado benomil (age sobre o citoesqueleto da célula), selecionou subpopulações de *C. gattii* mais tolerantes *in vitro* (Carneiro, 2015) e *in vivo* ao FLC (Dados não publicados). As células expostas ao agroquímico também se tornaram mais virulentas do que as células não expostas em modelo experimental murino (Dados não publicados). Embora esse trabalho mostre que antifúngicos ambientais selecionam populações de *C. gattii* mais tolerantes ao FLC, ainda não se sabe: (i) se outros agroquímicos, especialmente os que são vastamente utilizados, ocasionariam os mesmos resultados; (ii) se os mesmos efeitos ocorrem com *C. neoformans*; (iii) se a temperatura pode influenciar o fenômeno de resistência cruzada; (iv) quais os mecanismos moleculares envolvidos no processo de resistência cruzada e (v) se a característica de aumento de virulência ocorre independentemente do antifúngico ambiental utilizado.

Por isso, no atual trabalho nós utilizamos três antifúngicos usados na agricultura: tebuconazol (TBZ), piraclostrobina (PCT) e metalaxil (MTX). O TBZ é um azol (triazol) reconhecido como novo fungicida na agricultura, tem amplo espectro de ação e é utilizado para tratar ou prevenir doenças em frutas, nozes, cereais e hortaliças. Ele, assim como outros azóis, age sobre os fungos inibindo a síntese de ergosterol e induzindo o acúmulo de compostos metilados tóxicos na célula (Kwork *et al.*, 1993; Strickland *et al.*, 2004). PCT é um fungicida da classe das estrobilurinas, pertencente ao grupo das Q*uinone Outside Inhibitor* (QoI). Fungicidas *QOI* inibem a respiração mitocondrial por ligação ao sítio Qo do citocromo b, o que impede a transferência de elétrons entre o citocromo b e o citocromo c1. Isso tem como resultado uma interrupção na produção de energia da célula fúngica, levando a morte celular (Bartlett *et al.*, 2002). Já o MTX é um fungicida do grupo das acilalaninas e inibe a atividade da RNA polimerase I, afetando a síntese de RNA ribossômico. (Davidse *et al.*, 1983 (Wollgiehn *et al.*, 1984). A FRAC (*Fungicide Resistance Action Committee*) classifica como alto o risco de PCT e MTX selecionarem populações de fungos com resistência cruzada com outros antifúngicos ambientais (FRAC, 2016).

Especula-se que alguns mecanismos de resistência envolvendo esses agroquímicos são semelhantes aos mecanismos que tornam fungos resistentes/tolerantes aos antifúngicos clínicos, principalmente o aumento da expressão de bombas de efluxo,

que poderiam bombear tanto o agroquímico quanto a droga clínica (Rocha *et al.*, 2016). Dessa forma, é preocupante o uso indiscriminado dessas substâncias que além de terem efeitos diretos sobre a saúde humana (Rigotto *et al.*, 2014), poderiam afetá-la indiretamente, selecionando fungos patogênicos resistentes a drogas (Faria-Ramos *et al.*, 2014; Faria-Ramos *et al.*, 2014) e/ou linhagens mais virulentas. Por isso, faz-se necessário investigar quais fungicidas poderiam causar esses efeitos e os mecanismos moleculares envolvidos nesse fenômeno, no intuito de criar medidas que evitem o surgimento de linhagens mais tolerantes e hipervirulentas.

2 - JUSTIFICATIVA

Cryptococcus neoformans e *C. gattii* são os agentes etiológicos da criptococose oportunista e primária, respectivamente. *Cryptococcus* spp infectam o homem e outros animais por meio da inalação de leveduras dessecadas e/ou esporos do ambiente. Tais formas são normalmente encontradas associadas ao material vegetal e podem sofrer pressões ambientais nesses locais que levem a alterações na expressão de seus fatores de virulência. Contudo, ainda não se sabe se o ambiente ou ação antrópica, como o uso de agroquímicos, poderia alterar a tolerabilidade dessas espécies a drogas antifúngicas usadas para tratar a criptococose.

O Brasil é o país onde mais se faz uso de agroquímicos no mundo, sendo que o mercado envolvendo a venda desses compostos aumentou 190% nos últimos anos, mais do que o dobro do resto do mundo. Essas substâncias podem causar prejuízos à saúde humana diretamente ou indiretamente, como, por exemplo, contaminando água e solos e indisponibilizando-os para o uso humano.

Recentemente, alguns trabalhos mostraram que a exposição a antifúngicos da classe dos azóis utilizados na agricultura pode selecionar subpopulações de *Candida parapsilosis* e *Aspergillus fumigatus* resistentes aos antifúngicos utilizados na medicina humana e animal (resistência cruzada). Entretanto, pouco se sabe se esses compostos também possuem os mesmo efeitos sobre linhagens de *Cryptococcus* spp e se a exposição a essas substâncias altera a virulência fúngica.

Dessa forma, nesse trabalho nós estudamos os efeitos de três antifúngicos usados na agricultura, sendo dois não-azólicos (piraclostrobina e metalaxil) e um azólico (tebuconazol), sobre a morfo-fisiologia, perfil de tolerabilidade a antifúngicos clínicos *in vitro* e *in vivo* e virulência de *C. neoformans* e *C. gattii* e os mecanimos moleculares envolvidos nesse processo. Além de sua importância dentro do entendimento da biologia básica dos fungos em questão, esse trabalho é de grande relevância para enfatizar a criação de medidas e estratégias que visem minimizar o surgimento de linhagens com resistência cruzada no ambiente, o que pode causar grandes perdas e transtornos para a saúde pública.

3 - OBJETIVO

3.1 - Objetivo Geral

Estudar a influência de antifúngicos ambientais sobre o perfil de tolerabilidade a antifúngicos clínicos, a morfo-fisiologia e virulência em *Cryptococcus gattii* e em *C. neoformans*.

4 - CAPÍTULOS

4.1 - Capítulo I: "Environmental Triazole Induces Cross Resistance to Clinical Drugs and Affects Morphophysiology and Virulence of *Cryptococcus gattii* and *C. neoformans*"

Bastos RW, Carneiro HCS, Oliveira LVN, Rocha KM, Freitas GJC, Costa MC, Magalhães TFF, Carvalho VSD, Rocha CE, Ferreira GF, Paixão TA, Moyrand F, Janbon G, Santos DA. 2018. Environmental triazole induces crossresistance to clinical drugs and affects morphophysiology and virulence of *Cryptococcus gattii* and *C. neoformans*. **Antimicrob Agents Chemother** 62:e01179-17.

Objetivos Específicos

- Selecionar subpopulações de *C. gattii* e de *C. neoformans* mais tolerantes ao antifúngico ambiental tebuconazol (TBZ), avaliando a estabilidade desse fenômeno e possibilidade de resistência cruzada temporária ou permanete com antifúngicos clínicos como fluconazol, anfotericina B, itraconazol e ravuconazol, e o efeito da temperatura sobre esse processo;
- Estudar a influência da exposição ao TBZ na morfo-fisiologia de *Cryptococcus* spp.;
- Comparar a virulência das colônias de *C. gattii* e *C. neoformans* expostas e não expostas previamente ao TBZ em modelo experimental murino;
- Testar a eficiência do tratamento com fluconazol sobre camundongos infectados com colônias de *C. gattii* e *C. neoformans* expostas e não expostas previamente ao TBZ.
- Investigar os mecanismos moleculares clássicos associados às alterações da tolerabilidade aos azóis em colônias de *C. gattii* e *C. neoformans* expostas e não expostas previamente ao TBZ.

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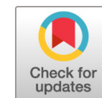
SUSCEPTIBILITY



Environmental Triazole Induces Cross-Resistance to Clinical Drugs and Affects Morphophysiology and Virulence of *Cryptococcus gattii* and *C. neoformans*

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ABSTRACT *Cryptococcus gattii* and *Cryptococcus neoformans* are environmental fungi that cause cryptococcosis, which is usually treated with amphotericin B and fluconazole. However, therapeutic failure is increasing because of the emergence of resistant strains. Because these species are constantly isolated from vegetal materials and the usage of agrochemicals is growing, we postulate that pesticides could be responsible for the altered susceptibility of these fungi to clinical drugs. Therefore, we evaluated the influence of the pesticide tebuconazole on the susceptibility to clinical drugs, morphophysiology, and virulence of *C. gattii* and *C. neoformans* strains. The results showed that tebuconazole exposure caused *in vitro* cross-resistance (CR) between the agrochemical and clinical azoles (fluconazole, itraconazole, and ravuconazole) but not with amphotericin B. In some strains, CR was observed even after the exposure ceased. Further, tebuconazole exposure changed the morphology, including formation of pseudohyphae in *C. neoformans* H99, and the surface charge of the cells. Although the virulence of both species previously exposed to tebuconazole was decreased in mice, the tebuconazole-exposed colonies recovered from the lungs were more resistant to azole drugs than the nonexposed cells. This *in vivo* CR was confirmed when fluconazole was not able to reduce the fungal burden in the lungs of mice. The tolerance to azoles could be due to increased expression of the *ERG11* gene in both species and of efflux pump genes (*AFR1* and *MDR1*) in *C. neoformans*. Our study data support the idea that agrochemical usage can significantly affect human pathogens present in the environment by affecting their resistance to clinical drugs.

KEYWORDS agrochemical, antifungal cross-resistance, fluconazole, pseudohyphae, tebuconazole

Losses of crops due to pests represent a major problem that must be faced by agriculture to achieve increased food production (1). One of the most frequent strategies to avoid these losses is the use of pesticides, which has grown in recent years. The use of agrochemicals increased by an average of 93% worldwide in recent years, and in Brazil, pesticide use has increased by 190% (2).

Tebuconazole (TBZ), an agrochemical triazole, has a broad spectrum of action and is used to treat or prevent diseases in fruits, cereals, and vegetables. Tebuconazole

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inhibits fungal sterol-(lanosterol)-14-α demethylase, an enzyme that participates in ergosterol synthesis (3).

Evidence clearly shows that pesticides can cause problems for human health (2, 4, 5). It is unclear, however, (i) what the effects of agrochemicals are on human pathogens present in the environment and (ii) what the indirect effects are on human and animal health. Some studies have shown that exposure to environmental antifungals selects subpopulations of medically important pathogens that are less susceptible to clinical drugs (6–8). However, these studies did not describe the effects of pesticides on the virulence of these pathogens after exposure or indicate whether resistance is maintained *in vivo*.

Cryptococcus gattii and *Cryptococcus neoformans*, which are commonly isolated from vegetal materials, are medically important pathogens as the main etiological agents of cryptococcosis. These pathogens infect humans and other animals through inhalation of desiccated environmental yeasts and/or spores from the environment, causing pneumonia and severe meningoencephalitis (9). It is estimated that cryptococcosis affects 1,000,000 people annually, with 650,000 deaths (10).

Cryptococcosis is treated with amphotericin B combined with fluconazole (FLC) and/or 5-flucytosine. Other azole drugs, such as itraconazole (ITC), are also used in some cases (11). Although antibiotic therapy is frequently effective, there are important drawbacks associated with its use. Amphotericin B and 5-flucytosine are nephrotoxic and hepatotoxic, respectively, and they are not available in all countries (12). Regarding azole drugs, especially fluconazole, the isolation of *Cryptococcus* species strains with increased tolerance of these drugs is increasing (13, 14).

In recent years, it has been suggested that environmental pressures affect the virulence of *Cryptococcus* spp. and their susceptibility to clinical drugs (15, 16). However, no study has yet confirmed this hypothesis. Therefore, the main goal of our work was to study the effects of exposure to the agrochemical tebuconazole on the susceptibility to clinical drugs and virulence of *C. gattii* and *C. neoformans*.

RESULTS

Antifungal drug susceptibility testing and screening of subpopulations more tolerant of tebuconazole (tebuconazole adaptation). As expected, all *C. gattii* and *C. neoformans* strains were inhibited by the drugs tested (fluconazole, amphotericin B, and tebuconazole) at temperatures of 30 and 35°C (data not shown). All strains were also sensitive to tebuconazole when the MIC was determined in solid medium (MIC^{solid}) (Table 1).

Further, we determined whether the strains were capable of growing in higher concentrations of tebuconazole in a stepwise manner, and we studied whether the temperature would affect this adaptation. Tables 1 and 2 show the maximum concentration achieved (MCA) of tebuconazole in the tebuconazole adaptation test and the MCA/sub-MIC^{solid} ratios (sub-MIC, MIC/2) at 30 and 35°C (the higher the ratio, the more passages through tebuconazole-containing media occurred). When the adaptation was performed at 30°C, 38% ($n = 5$) of the *C. gattii* strains were able to grow in a concentration 10 times higher than before the adaptation (MCA/sub-$MIC^{solid} = 10.0$) and the geometric mean of the ratio was 5.53 (Table 1). However, when the tests were carried out at 35°C, the strains grew in a lower concentration of tebuconazole (geometric mean = 2.78), demonstrating that temperature affected the adaptation process (Table 1). The same phenomenon was observed for *C. neoformans* strains, with a geometric mean of MCA/sub-MIC^{solid} ratio at 30°C almost 3-fold higher than that seen when the test was performed at 35°C (Table 1).

Tebuconazole-adapted colonies present cross-resistance (CR) with fluconazole and other azole drugs. Tables 2 and 3 show the MIC in liquid medium (MIC^{broth}) of fluconazole and tebuconazole for nonadapted (NA) and tebuconazole-adapted (A) colonies of *C. gattii* and *C. neoformans*, respectively, when the adaptation was performed at 30°C. Despite adaptation, not all *C. gattii* strains exhibited alterations of at least 2 dilutions of the MIC of tebuconazole compared to the MIC for NA colonies

TABLE 1 Screening of *C. gattii* and *C. neoformans* subpopulations with increased tolerance of tebuconazole[a]

Strain	MIC^{solid} (μg/ml) (geometric mean)		MCA (μg/ml) (geometric mean)		MCA/sub-MIC^{solid} (geometric mean)	
C. gattii	30°C	35°C	30°C	35°C	30°C	35°C
R265 (C)	2.0	1.0	7.5	2.0	7.5	4.0
ATCC 24065 (R)	1.0	2.0	5.0	2.0	10.0	2.0
ATCC 320608 (R)	2.0	2.0	10.0	2.0	10.0	2.0
547/OTTI/94-PI-10 (E)	2.0	2.0	10.0	2.0	10.0	2.0
ICB 181 (E)	1.0	1.0	2.0	2.0	4.0	4.0
L24/01 (C)	2.0	2.0	10.0	2.0	10.0	2.0
L27/01 (C)	1.0	2.0	2.0	2.0	4.0	2.0
L28/02 (C)	2.0	1.0	10.0	1.0	10.0	2.0
1913/ER (C)	1.0	0.5	1.5	0.75	3.0	3.0
196L/03 (C)	2.0	4.0	4.0	16.0	4.0	8.0
LMM 818 (C)	1.0	2.0	3.5	3.0	7.0	3.0
23/10893 (C)	1.0	0.5	0.75	0.5	1.5	2.0
29/10893 (C)	1.0	0.5	1.5	1.0	3.0	4.0
Range	1.0–2.0 (1.37)	0.5–4.0 (1.30)	0.75–10.0 (3.80)	0.5–16.0 (1.81)	1.5–10.0 (5.53)	0.5–8.0 (2.78)
C. neoformans	30°C	35°C	30°C	35°C	30°C	35°C
H99 (C)	1.0	1.0	2.0	2.0	4.0	4.0
ATCC 24067 (R)	0.5	2.0	2.0	2.0	8.0	2.0
ATCC 28957 (R)	1.0	2.0	10.0	2.0	20.0	2.0
ATCC 62066 (R)	1.0	2.0	2.0	2.0	4.0	2.0
Range	0.5–1.0 (0.84)	1.0–2.0 (1.68)	2.0–10.0 (2.99)	2.0 (2.0)	4.0–20.0 (7.11)	2.0–4.0 (2.38)

[a]MIC^{solid}, MIC in solid medium for tebuconazole (TBZ) before the adaptation process; MCA, maximum concentration of tebuconazole achieved in the TBZ adaptation test; C, clinical strain; R, reference strain; E, environmental strain.

(Tables 2 and 3). Overall, 61.5% ($n = 8$) of *C. gattii* (Table 2) and 100% ($n = 4$) of *C. neoformans* (Table 3) tebuconazole-adapted cells became more resistant to the environmental antifungal than the NA colonies when the tests were performed at the same adaptation temperature. In contrast, when MIC assays were carried out at 35°C using cells adapted at 30°C, 38% ($n = 5$) of *C. gattii* (Table 2) and 50% ($n = 2$) of *C. neoformans* (Table 3) tebuconazole-adapted colonies became more resistant to tebuconazole.

We then tested whether tebuconazole adaptation can also decrease susceptibility to fluconazole. The geometric means of the drug MICs for colonies subjected to tebuconazole adaptation at 30°C and 35°C increased almost 3-fold and 2-fold for *C. gattii* and 5-fold and 3-fold for *C. neoformans*, respectively (Tables 2 and 3). Additionally, the adaptation resulted in selection of populations with cross-resistance (CR). A total of 38% ($n = 5$) of *C. gattii* tebuconazole-adapted colonies and a total of 100% ($n = 4$) of *C. neoformans* tebuconazole-adapted colonies presented higher MICs of fluconazole, as well as of tebuconazole (Tables 2 to 4). Four strains of *C. gattii* (R265, ATCC 32608, L27/01, and 196L/03) and three strains of *C. neoformans* (H99, ATCC 24067, and ATCC 62066) returned to the original phenotype when grown in medium without the agrochemical for 10 passages (10p) (Tables 2 and 3) (referred to here as "temporary CR"). This phenomenon occurred in a manner dependent on the temperature (ATCC 32608, L27/01, and ATCC 62066) or not dependent on the temperature (R265, 196L/03, H99, and ATCC 24067) for different strains (Tables 2 to 4). The other strain of *C. gattii* (ATCC 24065) and the other strain of *C. neoformans* (ATCC 28957), which showed CR with fluconazole, did not return to the original susceptibility phenotype even after growth in tebuconazole-free medium, demonstrating "permanent CR" (Tables 3 to 5). This profile also appears to be temperature dependent (Tables 2 to 4).

We also evaluated whether CR with fluconazole in tebuconazole-adapted strains at 30°C would occur for other azoles, such as itraconazole and ravuconazole. As shown in Table 5, *C. gattii* and *C. neoformans* strains became less susceptible to ravuconazole after adaptation to tebuconazole, but only *C. gattii* R265 and *C. neoformans* H99 and ATCC 28957 showed CR with itraconazole. *C. gattii* ATCC 32608 and *C. neoformans* ATCC 28957 and ATCC 62066 exhibited altered susceptibility to ravuconazole only when the test was performed at 30°C (Table 5), confirming the importance of temperature in the resistance process.

TABLE 2 MICs of fluconazole and tebuconazole for non-TBZ-adapted *C. gattii* colonies, *C. gattii* colonies subjected to TBZ adaptation at 30°C, and TBZ-adapted *C. gattii* colonies subcultured 10 times in agrochemical-free medium[a]

	MIC (μg/ml) at indicated temp (geometric mean)[b]											
	Fluconazole						Tebuconazole					
	30°C			35°C			30°C			35°C		
Strain	NA	A	10p	NA	A	10p	NA	A	10p	NA	A	10p
R265	**8.0**	**64.0 [8×]**	16.0	**8.0**	**64.0 [8×]**	16,0	**0.5**	**4.0 [8×]**	1.0	**1.0**	**4.0 [4×]**	2.0
ATCC 24065	**4.0**	**32.0 [8×]**	**32.0 [8×]**	4.0	8.0	ND	**0.5**	**4.0 [8×]**	**4.0 [8×]**	0.5	1.0	ND
ATCC 32608	**16.0**	**64.0 [4×]**	32.0	8.0	16.0	ND	**0.5**	**2.0 [4×]**	1.0	1.0	1.0	ND
547/OTTI/94-PI-10	16.0	32.0	ND	8.0	16.0	ND	2.0	2.0	ND	2.0	2.0	ND
ICB 181	16.0	32.0	ND	8.0	16.0	ND	**1.0**	**4.0 [4×]**	1.0	**0.25**	**2.0 [8×]**	0.25
L24/01	**16.0**	**64.0 [4×]**	16.0	8.0	16.0	ND	4.0	4.0	ND	1.0	2.0	ND
L27/01	**16.0**	**64.0 [4×]**	16.0	32.0	32.0	ND	**0.5**	**4.0 [8×]**	0.5	2.0	2.0	ND
L28/02	32.0	64.0	ND	16.0	16.0	ND	1.0	1.0	ND	1.0	2.0	ND
1913R	16.0	16.0	ND	16.0	16.0	ND	**0.125**	**0.5 [4×]**	0.125	**0.125**	**0.5 [4×]**	0.125
196L/03	**16.0**	**128.0 [8×]**	16.0	**16.0**	**128.0 [8×]**	16.0	**1.0**	**8.0 [8×]**	1.0	**1.0**	**8.0 [8×]**	1.0
LMM 818	16.0	8.0	ND	16.0	8.0	ND	0.25	0.5	ND	0.25	0.5	ND
23/10893	8.0	16.0	ND	8.0	16.0	ND	**0.125**	**1.0 [8×]**	0.25	**0.125**	**1.0 [8×]**	0.25
29/10933	8.0	16.0	ND	4.0	4.0	ND	0.25	0.25	ND	0.25	0.25	ND
Range	4.0–32.0 (12.92)	8.0–128.0 (35.60)	ND (ND)	4.0–32.0 (9.90)	4.0–128.0 (17.80)	ND (ND)	0.125–4.0 (0.56)	0.25–8.0 (1.79)	ND (ND)	0.125–2.0 (0.56)	0.25–8.0 (1.37)	ND (ND)

[a]Tests were performed at 30°C and 35°C.

[b]MIC values represent endpoint values (MIC at 50% of growth inhibition). Numbers in square brackets indicate how many times (×) higher the drug MIC value for the TBZ-adapted colonies (A) or colonies subjected to 10 passages (10p) was (≥4×) than the drug MIC value for the non-TBZ-adapted (NA) colonies. Values highlighted in bold indicate MIC values that were at least 4× higher than those seen with the NA colonies. ND, not determined.

TABLE 3 MICs of fluconazole and tebuconazole for non-TBZ-adapted *C. neoformans* colonies, *C. neoformans* colonies subjected to TBZ adaptation at 30°C, and TBZ-adapted *C. neoformans* colonies subcultured 10 times in agrochemical-free medium[a]

	MIC (μg/ml) at indicated temp (geometric mean)[b]											
	Fluconazole						Tebuconazole					
	30°C			35°C			30°C			35°C		
Strain	NA	A	10p	NA	A	10p	NA	A	10p	NA	A	10p
H99	**16.0**	**128.0 [8×]**	32.0	**8.0**	**64.0 [8×]**	8.0	**1.0**	**4.0 [4×]**	1.0	**0.25**	**2.0 [8×]**	0.25
ATCC 24067	**16.0**	**64.0 [4×]**	16.0	**4.0**	**32.0 [8×]**	4.0	**0.5**	**2.0 [4×]**	0.5	**0.25**	**1.0 [4×]**	0.25
ATCC 28957	**4.0**	**16.0 [4×]**	**32.0 [8×]**	2.0	4.0	ND	**0.5**	**4.0 [8×]**	**2.0 [4×]**	0.5	0.5	ND
ATCC 62066	**4.0**	**16.0 [4×]**	4.0	4.0	4.0	ND	**0.25**	**1.0 [4×]**	0.5	0.5	0.5	ND
Range	4.0–16.0 (8.0)	16.0–128.0 (38.05)	ND (ND)	2.0–8.0 (4.0)	4.0–64.0 (13.45)	ND (ND)	0.25–1.0 (0.5)	1.0–4.0 (2.38)	ND (ND)	0.25–0.5 (0.35)	0.5–2.0 (0.84)	ND (ND)

[a]Tests were performed at 30°C and 35°C.
[b]MIC values represent endpoint values (MIC at 50% of growth inhibition). Numbers in square brackets indicate how many times (×) higher the drug MIC value for the TBZ-adapted colonies (A) or colonies subjected to 10 passages (10p) was (≥4×) than the drug MIC value for the non-TBZ-adapted (NA) colonies. Values highlighted in bold indicate MIC values that were at least 4× higher than those seen with the NA colonies. ND, not determined.

We also performed tebuconazole adaptation at 35°C using the same procedure (Tables 6 and 7). Overall, 42% ($n = 6$) of *C. gattii* strains exhibited an increased tebuconazole MIC after tebuconazole adaptation (Table 6) and 23% ($n = 3$) presented CR with fluconazole. For two strains (R265 and 23/10893), the phenotype reverted after subcloning was performed several times on nonselective medium. For one strain (196L/03), the phenotype was stable even after several subcultures on agrochemical-free medium (Tables 4 and 6). However, only adapted cells from *C. neoformans* H99 exhibited an increased tebuconazole MIC and CR with fluconazole (Tables 4 and 7).

The *Cryptococcus* species strains adapted at 35°C behaved similarly to those adapted at the lower temperature regarding susceptibility to itraconazole and ravuconazole (Table 8). Adapted cells of *C. gattii* R265 and 196L/03 became more resistant to ravuconazole, whereas cells coming from *C. neoformans* H99 were more resistant to itraconazole and ravuconazole (Table 8). In contrast, cells adapted from *C. gattii* 23/10893 did not show altered susceptibility (Table 8).

Two strains, one from each species (one isolated from *C. gattii* L24/01 adapted at 30°C and one isolated from *C. neoformans* ATCC 62066 adapted at 35°C), exhibited an increased MIC only of fluconazole (Tables 2 and 7). This phenomenon has also been observed when an environmental nonazole antifungal agent has been used (unpublished data).

Overall, our data demonstrated that exposing *Cryptococcus* spp. to tebuconazole can induce CR with other azole derivatives commonly used in the clinical setting. Interestingly, CR was not observed with amphotericin B (data not shown).

Tebuconazole adaptation induced morphophysiological changes. Tebuconazole adaptation caused morphological changes in *Cryptococcus* spp. The adapted cells of *C. gattii* R265 exhibited a significantly increased diameter ($P < 0.05$) (Fig. 1A) and a decreased surface/volume ratio (Fig. 1B) compared to NA cells ($P < 0.05$), but the capsule size was maintained (Fig. 1C). Strikingly, 70% to 90% tebuconazole-adapted

TABLE 4 Percentage of cross-resistance between TBZ and FLC seen with *C. gattii* and *C. neoformans* strains after TBZ adaptation at 30 and 35°C[a]

	% CR at indicated temp			
	C. gattii		*C. neoformans*	
CR category	30°C	35°C	30°C	35°C
Total	39	23	100	25
Temporary	31	15	75	25
Permanent	8	8	25	0

[a]CR, cross-resistance.

TABLE 5 MICs of itraconazole and ravuconazole for non-TBZ-adapted *C. gattii* and *C. neoformans* colonies, *C. gattii* and *C. neoformans* colonies subjected to TBZ adaptation at 30°C, and TBZ-adapted *C. gattii* and *C. neoformans* colonies subcultured 10 times in agrochemical-free medium[a]

Strain	MIC at indicated temp[b]											
	Itraconazole						Ravuconazole					
	30°C			35°C			30°C			35°C		
	NA	A	10p	NA	A	10p	NA	A	10p	NA	A	10p
C. gattii												
R265	**0.25**	**1.0 [4×]**	ND	**0.25**	**1.0 [4×]**	ND	**0.125**	**2.0 [16×]**	ND	**0.03**	**0.5 [16×]**	ND
ATCC 24065	0.5	1.0	1.0	0.25	0.5	1.0	**0.06**	**2.0 [32×]**	**2.0 [32×]**	**0.03**	**0.125 [4×]**	**0.125 [4×]**
ATCC 32608	0.5	1.0	ND	0.5	0.5	ND	**0.125**	**0.5 [4×]**	ND	0.125	0.125	ND
L27/02	0.5	1.0	ND	0.25	0.5	ND	**0.125**	**1.0 [8×]**	ND	**0.03**	**0.125 [4×]**	ND
196L/03	0.5	1.0	ND	0.5	1.0	ND	**0.25**	**2.0 [8×]**	ND	**0.125**	**2.0 [16×]**	ND
C. neoformans												
H99	**0.125**	**1.0 [8×]**	ND	**0.125**	**1.0 [8×]**	ND	**0.06**	**1.0 [16×]**	ND	**0.06**	**0.5 [8×]**	ND
ATCC 24067	0.5	1.0	ND	0.25	0.5	ND	**0.125**	**1.0 [8×]**	ND	**0.015**	**0.25 [16×]**	ND
ATCC 28957	**0.25**	**1.0 [4×]**	0.25	**0.125**	**0.5 [4×]**	ND	**0.06**	**0.5 [8×]**	**0.5 [8×]**	0.015	0.03	ND
ATCC 62066	0.5	1.0	ND	0.25	0.25	ND	**0.03**	**0.5 [16×]**	ND	0.03	0.03	ND

[a]Tests were performed at 30°C and 35°C.
[b]MIC values represent endpoint values (MIC at 50% of growth inhibition). Numbers in square brackets indicate how many times (×) higher the drug MIC value for the TBZ-adapted colonies (A) or colonies subjected to 10 passages (10p) was (≥4×) than the drug MIC value for the non-TBZ-adapted (NA) colonies. Values highlighted in bold indicate MIC values that were at least 4× higher than those seen with the NA colonies. ND, not determined.

cells of *C. neoformans* H99 presented elongated, irregular shapes, characterizing the formation of pseudohyphae, while the NA and 10p cells presented only yeast forms (Fig. 1D). These *C. neoformans* H99-derived elongated cells showed a significant ($P <$ 0.05) increase in their surface electronegativity (Fig. 1F), but this was not observed for *C. gattii* R265 (Fig. 1E).

In contrast, adaptation and the morphological changes did not affect the growth rate in Sabouraud dextrose agar (SDA) (data not shown).

Tebuconazole adaptation decreased the virulence of *C. gattii* R265 and *C. neoformans* H99 in C57BL/6 mice. Mice infected with NA cells of *C. gattii* R265 and *C. neoformans* H99 succumbed significantly ($P < 0.05$) earlier than those infected with

TABLE 6 MICs of fluconazole and tebuconazole for non-TBZ-adapted *C. gattii* colonies, *C. gattii* colonies subjected to TBZ adaptation at 35°C, and TBZ-adapted *C. gattii* colonies subcultured 10 times in agrochemical-free medium[a]

Strain	MIC (μg/ml) (geometric mean)[b]					
	Fluconazole			Tebuconazole		
	NA	A	10p	NA	A	10p
R265	**8.0**	**32.0 [4×]**	16.0	**1.0**	**4.0 [4×]**	1.0
ATCC 24065	4.0	8.0	ND	0.5	0.5	ND
ATCC 32608	8.0	16.0	ND	1.0	2.0	ND
547/OTTI/94-PI-10	8.0	16.0	ND	2.0	4.0	ND
ICB 181	8.0	16.0	ND	**0.25**	**1.0 [4×]**	**1.0 [4×]**
L24/01	8.0	4.0	ND	1.0	2.0	1.0
L27/01	32.0	16.0	ND	2.0	1.0	ND
L28/02	16.0	16.0	ND	1.0	2.0	ND
1913R	16.0	16.0	ND	**0.125**	**1.0 [8×]**	0.25
196L/03	**16.0**	**256.0 [16×]**	**256.0 [16×]**	**1.0**	**8.0 [8×]**	**16.0 [16×]**
LMM 818	16.0	16.0	ND	0.25	0.25	ND
23/10893	**8.0**	**32.0 [4×]**	4.0	**0.125**	**2.0 [16×]**	0.125
29/10933	4.0	8.0	ND	**0.25**	**1.0 [4×]**	0.5
Range	4.0–32.0 (9.90)	4.0–256.0 (17.80)	ND (ND)	0.125–2.0 (0.56)	0.25–8.0 (1.53)	ND (ND)

[a]Tests were performed at 35°C.
[b]MIC values represent endpoint values (MIC at 50% of growth inhibition). Numbers in square brackets indicate how many times (×) higher the drug MIC value for the TBZ-adapted colonies (A) or colonies subjected to 10 passages (10p) was (≥4×) than the drug MIC value for the non-TBZ-adapted (NA) colonies. Values highlighted in bold indicate MIC values that were at least 4× higher than those seen with the NA colonies. ND, not determined.

TABLE 7 MICs of fluconazole and tebuconazole for non-TBZ-adapted *C. neoformans* colonies, *C. neoformans* colonies subjected to TBZ adaptation at 35°C, and TBZ-adapted *C. neoformans* colonies subcultured 10 times in agrochemical-free medium[a]

	MIC (μg/ml) (geometric mean)[b]					
	Fluconazole			Tebuconazole		
Strain	NA	A	10p	NA	A	10p
H99	**8.0**	**32.0 [4×]**	16.0	**0.25**	**1.0 [4×]**	**2.0 [8×]**
ATCC 24067	8.0	8.0	ND	0.5	1.0	ND
ATCC 28957	4.0	8.0	ND	0.5	1.0	ND
ATCC 62066	**4.0**	**32.0 [8×]**	4.0	1.0	2.0	ND
Range	4.0–8.0 (5.65)	8.0–32.0 (16.00)	ND (ND)	0.25–1.0 (0.50)	1.0–2.0 (1.19)	ND (ND)

[a]Tests were performed at 35°C.
[b]MIC values represent endpoint values (MIC at 50% of growth inhibition). Numbers in square brackets indicate how many times (×) higher the drug MIC value for the TBZ-adapted colonies (A) or colonies subjected to 10 passages (10p) was (≥4×) than the drug MIC value for the non-TBZ-adapted (NA) colonies. Values highlighted in bold indicate MIC values that were at least 4× higher than those seen with the NA colonies. ND, not determined.

tebuconazole-adapted cells of the same strain (Fig. 2). Interestingly, R265-adapted cells were not able to kill the animals, even after 80 days (Fig. 2A).

Additionally, animals were infected for a better characterization of the disease characteristics caused by the different cells. The fungal burden in the lungs (Fig. 3A and E) and bronchoalveolar lavage fluid (BALF) (Fig. 3B and F) was significantly higher ($P < 0.05$) in mice infected with NA cells in both strains. *C. gattii* R265 cells were not detected in the brain of any infected animal; however, NA cells of *C. neoformans* H99, unlike the tebuconazole-adapted cells, disseminated into the brain in 40% of infected animals (Fig. 3G).

Histopathology analysis confirmed the decreased virulence of tebuconazole-adapted colonies. Mice infected with nonadapted cells of *C. gattii* R265 presented a moderate to accentuated amount of extracellular yeasts diffusely distributed in the alveolar and bronchial lumen (Fig. 3C). This was associated to discrete perivascular inflammatory infiltrate with a predominance of neutrophils and multifocal alveolar thickening due to discrete mononuclear inflammatory infiltrate (Fig. 3C). Mice infected with nonadapted *C. neoformans* H99 demonstrated an accentuated amount of yeasts diffusely distributed in the pulmonary parenchyma (Fig. 3H). We also observed accentuated perivascular inflammatory infiltrate with neutrophils, macrophages, and lymphocytes and an accentuated inflammatory infiltrate with predominance of macro-

TABLE 8 MICs of itraconazole and ravuconazole for non-TBZ-adapted *C. gattii* and *C. neoformans* colonies, *C. gattii* and *C. neoformans* colonies subjected to TBZ adaptation at 35°C, and TBZ-adapted *C. gattii* and *C. neoformans* colonies subcultured 10 times in agrochemical-free medium[a]

	MIC (μg/ml) (geometric mean)[b]					
	Itraconazole			Ravuconazole		
Strain	NA	A	10p	NA	A	10p
C. gattii						
R265	0.25	0.25	0.25	**0.03**	**0.5 [16×]**	ND
196L/03	0.5	1.0	1.0	**0.125**	**1.0 [8×]**	**1.0 [8×]**
23/10893	0.5	0.5	ND	0.03	0.06	ND
C. neoformans						
H99	**0.25**	**2.0 [8×]**	ND	**0.03**	**0.5 [16×]**	ND

[a]Tests were performed at 35°C.
[b]MIC values represent endpoint values (MIC at 50% of growth inhibition). Numbers in square brackets indicate how many times (×) higher the drug MIC value for the TBZ-adapted colonies (A) or colonies subjected to 10 passages (10p) was (≥4×) than the drug MIC value for the non-TBZ-adapted (NA) colonies. Values highlighted in bold indicate MIC values that were at least 4× higher than those seen with the NA colonies. ND, not determined.

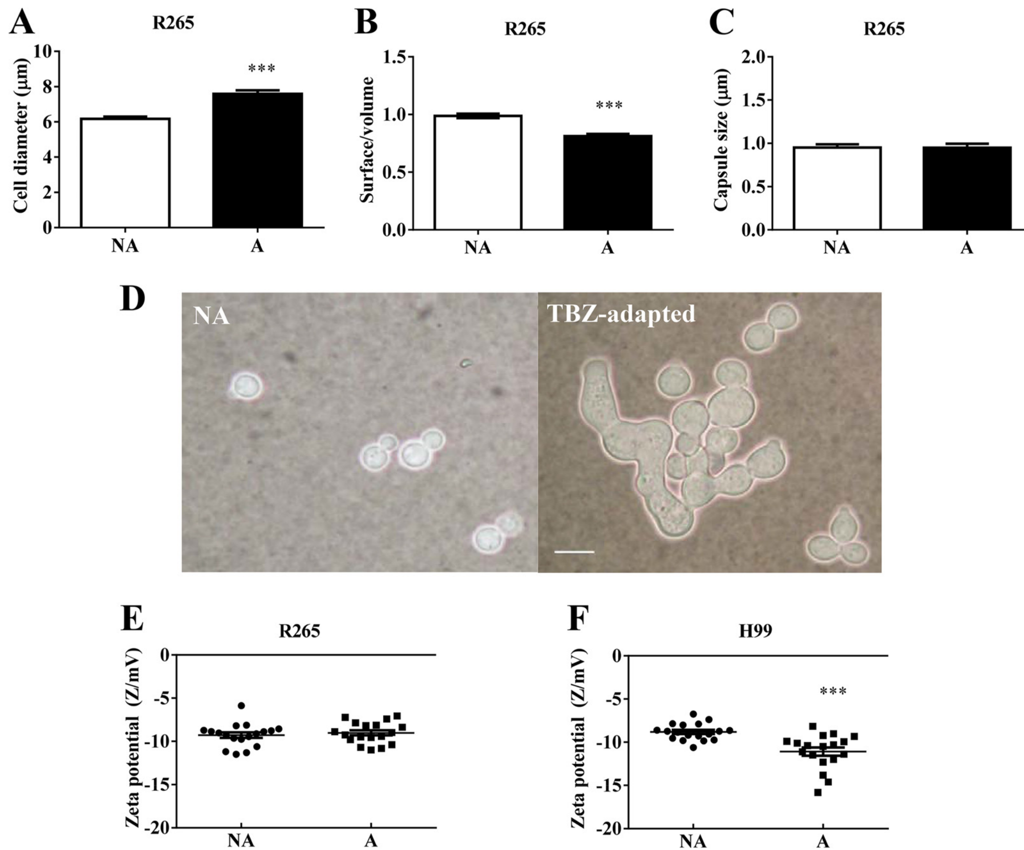

FIG 1 TBZ exposure causes morphophysiological changes in *Cryptococcus gattii* R265 and *C. neoformans* H99. (A to C) TBZ-adapted cells of *C. gattii* R265 exhibited increased cell diameter (A) and decreased surface/volume ratio (B) but unaltered capsule thickness (C) compared to nonadapted (NA) cells. (D) *C. neoformans* H99 presented the pseudohyphal form after TBZ adaptation. An India ink suspension was used. Bar, 10 μm. (E and F) The electronegativity of the cellular surface was not altered in *C. gattii* R265 (E); however, it was increased in TBZ-adapted cells of *C. neoformans* H99 (F). NA, nonadapted; A, TBZ adapted; **, $P < 0.01$; ***, $P < 0.001$.

phages and multinucleated giant cells in the alveolar space with multifocal to diffuse distribution (Fig. 3H). However, the animals infected with adapted cells exhibited a reduced amount of yeasts in lung parenchyma and, consequently, a significant reduction in associated inflammation (Fig. 3D and I). No change was observed in the lungs of control group mice (noninfected mice).

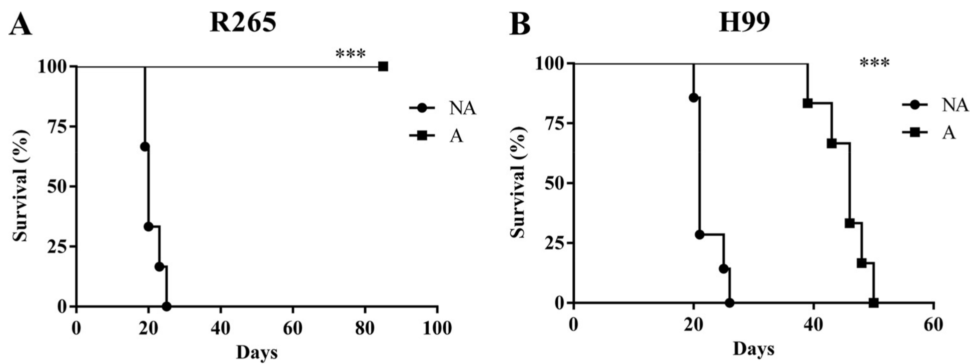

FIG 2 TBZ exposure decreases virulence in *Cryptococcus gattii* R265 and *C. neoformans* H99. C57BL/6 mice were infected by the intratracheal route with 1×10^5 CFU of nonadapted (NA) and TBZ-adapted (A) cells. The survival curve showed that animals infected with TBZ-adapted cells of *C. gattii* R265 (A) and *C. neoformans* H99 (B) survived longer than those infected with NA cells.

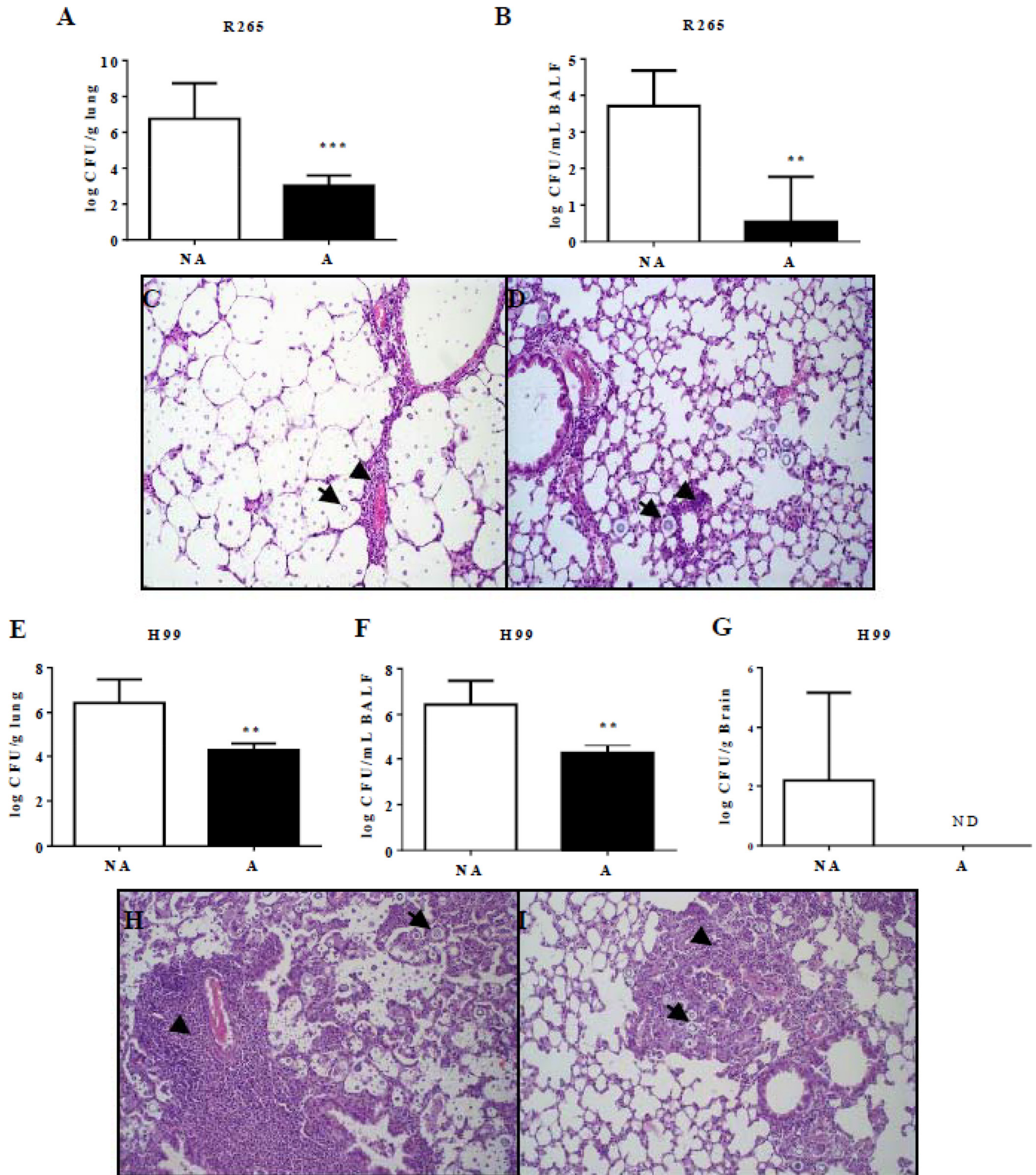

FIG 3 Animals infected with TBZ-adapted cells exhibit lower fungal load in the lungs, BALF, and brain and decreased lung inflammation. C57BL/6 mice were infected by the intratracheal route with 1×10^5 CFU of nonadapted (NA) and TBZ-adapted (A) cells for the determination of the CFU level per gram and for histopathology analysis. (A and B) After 15 days of infection, higher fungal burden was observed in the lungs (A) and bronchoalveolar lavage fluid (BALF) (B) of animals infected with NA cells of *C. gattii* R265. (C and D) Lung histopathology analysis showed that NA cells caused more inflammation (C) than A cells (D). (E to I) Animals infected with NA cells of *C. neoformans* H99 also exhibited a higher fungal load in the lungs (E), BALF (F), and brain (G) and more-intense inflammation in the lungs (H) than animals infected with A cells (I). **, $P < 0.01$; ***, $P < 0.001$; ND, not detected; NA, nonadapted; A, TBZ adapted. Arrows indicate yeast in the lungs, and arrowheads indicate the inflammatory infiltrate.

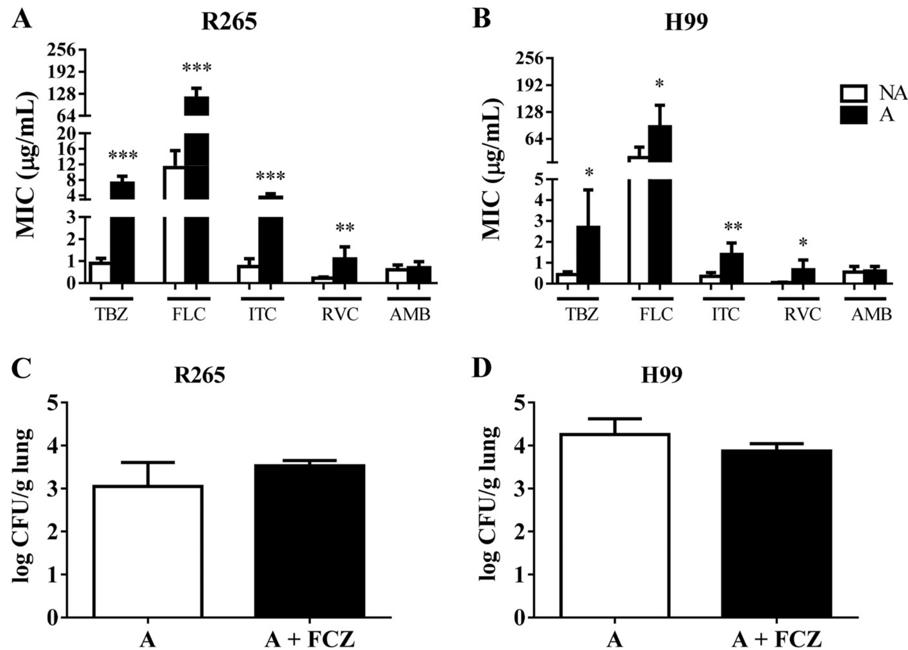

FIG 4 TBZ exposure causes antifungal resistance *in vivo*. The MICs of tebuconazole (TBZ), fluconazole (FLC), itraconazole (ITC), ravuconazole (RVC), and amphotericin B (AMB) were determined for colonies recovered from lungs of animals that had been infected with nonadapted (NA) and TBZ-adapted (A) cells of *C. gattii* R265 (A) or *C. neoformans* H99 (B) and treated (A + FCZ) or not treated (A) with FLC (10 mg/kg). After 15 days, the animals were euthanized and the lungs were collected for determination of the CFU level per gram. There were no statistical significant differences between the fungal loads in the lungs of animals that were infected with TBZ-adapted cells of *C. gattii* R265 (C) or *C. neoformans* H99 and (D) and treated or not treated with FLC. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

These results demonstrated that although *C. neoformans* was able to induce a greater inflammatory response in the lungs than *C. gattii*, the colonization and inflammatory response in mice infected with both species were reduced after tebuconazole adaptation.

Tebuconazole adaptation caused antifungal cross-resistance *in vivo*. The MIC of all azole drugs in colonies recovered from the lungs of animals infected with tebuconazole-adapted cells was significantly ($P < 0.05$) higher than that of drugs recovered from animals infected with NA cells (Fig. 4A and B). Furthermore, fluconazole did not reduce the fungal burden in lungs ($P > 0.05$) from mice (Fig. 4C and D), as was also observed in histology analyses (data not shown).

Tebuconazole adaptation induced different mechanisms of resistance in *C. gattii* and *C. neoformans*. We investigated the mechanism involved in the increased MIC of azole drugs for the adapted versus nonadapted *C. gattii* R265 and *C. neoformans* H99 strains. Figure 5 shows that the expression levels of the *ERG11* gene, but not those of the efflux pump *PDR11* and *MDR1* genes (Fig. 5A to C), were significantly ($P < 0.05$) higher in adapted *C. gattii* R265 cells than in the nonadapted cells. However, in *C. neoformans* H99, all genes (*ERG11*, *AFR1*, and *MDR1*) were expressed at a higher level ($P < 0.05$) in tebuconazole-exposed cells (Fig. 5D to F).

DISCUSSION

In this study, we showed that exposing *C. gattii* and *C. neoformans* to the triazole agrochemical tebuconazole resulted in greater tolerance, *in vitro* and *in vivo*, of clinical drugs (fluconazole and itraconazole) and less virulence in a murine model than were seen with cells not exposed to the agrochemical. We also tested an azole drug currently in clinical trials (ravuconazole) (17) to study whether there would be CR with drugs that are not yet commercially available, and such CR was confirmed.

First, we showed that previous exposure to tebuconazole selects cells with perma-

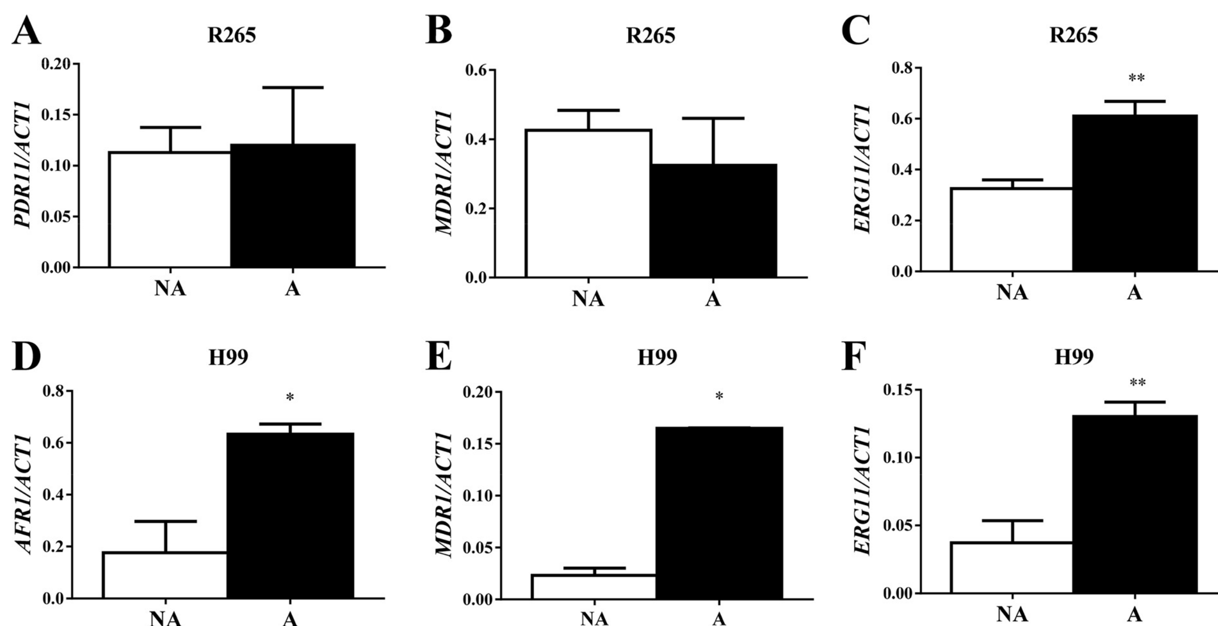

FIG 5 TBZ exposure changes in the expression of efflux pumps and *ERG11* genes. Expression of *PDR1* (A) and *MDR1* (B), but not that of *ERG11* (C), was altered by tebuconazole exposure in *C. gattii* R265. In *C. neoformans* H99, the levels of expression of *AFR1* (D), *MDR1* (E), and *ERG11* (F) were increased in adapted cells versus nonadapted cells. NA, nonadapted; A, TBZ adapted; *, $P < 0.05$; **, $P < 0.01$.

nent or temporary CR with fluconazole. The major mechanism of resistance to azole drugs that has been demonstrated for *Cryptococcus* spp. is the overexpression of efflux pump genes (*AFR1*, *AFR2*, *PDR11*, and *MDR1*) (18, 19) and, in some cases, overexpression of the target of these drugs, ERG11p (20). Here, we showed that the mechanisms of azole tolerance in *C. gattii* and *C. neoformans* can be different in strains that presented temporary CR, such as H99 and R265. We observed increased expression of the efflux pump and *ERG11* genes in *C. neoformans* H99 but observed increased expression only of *ERG11* in *C. gattii* R265. These results demonstrated that although the adapted cells of both species exhibited increased MICs of all the same azole drugs, the mechanisms were different for each species, and possibly for each strain. Another study confirmed that resistance mechanisms can be strain dependent, demonstrating that one azole-heteroresistant strain of *C. gattii* expressed more *PDR11* and *ERG11* than the original cells (20). Rocha and colleagues reported that in *Candida parapsilosis*, exposure to the agrochemical tetraconazole selected cells more resistant to azole drugs because of overexpression of efflux pumps, but they did not observe altered *ERG11* expression (7).

Tebuconazole exposure also caused changes in azole susceptibility that were observed even after 10 passages on agrochemical-free medium. We called this phenomenon "permanent CR," and it can indicate that a mutation could have occurred to confer resistance to the cells. Moreover, a study with *Aspergillus fumigatus* exposed to environmental antifungals, including tebuconazole, showed that *ERG11* mutation causes resistance to fluconazole and other azoles, except for itraconazole (8). Other studies also showed that CR between fluconazole and itraconazole is not common (21, 22). These phenomena may occur because itraconazole, in addition to inhibiting ERG11p, also inhibits NADH-dependent 3-ketosteroid reductase (an enzyme that catalyzes one of the last reactions of ergosterol synthesis) (21, 23), and the mechanism of resistance can be different from that seen with fluconazole (24). More studies should be performed to determine the mechanisms involved in the permanent CR caused by agrochemical exposure.

Temperature is a critical factor that the human-pathogenic fungus must overcome to cause illness (25). To test whether temperature can also affect the process of acquiring resistance, the strains were exposed to tebuconazole and incubated at 30 or 35°C. When adaptation was carried out at the lower temperature, the strains supported

higher concentrations of the drug and more strains became tolerant of the pesticide than at 35°C. This indicates that the temperature of 30°C is more favorable for the fungus to develop resistance, probably because the optimal temperature of growth of *Cryptococcus* is around 25°C (26). In addition to the importance of temperature during the adaptation process, we showed that it is relevant for determining MIC (incubation temperature). The tebuconazole-adapted colonies grown at 30°C were more tolerant to the drugs than colonies grown at 35°C, indicating that resistance in the environment may not occur *in vivo* because of the body temperature of endothermic animals. These data may also explain, at least in part, why *Cryptococcus* spp. are not considered to be a major problem in antimicrobial resistance in clinical practice (12).

The morphological characteristics of *Cryptococcus* cells influence their virulence (24). Usually, cells with a larger diameter and a smaller capsule are less virulent (27–29). Here, we observed that tebuconazole-adapted cells, which exhibited larger diameters than the NA cells, were less virulent in both species. Morphological analysis also showed that tebuconazole-adapted cells of *C. neoformans* H99 exhibited formation of pseudohyphae. Pseudofilamentation happens when budding cells do not fully separate, resulting in formation of a chain of bound cells (30). This process is common in *Candida* (30) but is rare and little studied in *Cryptococcus*. Pseudofilamentation seems to be a response to overcome environmental stresses (31), but during this process, the cells become less virulent (32), as our results demonstrated. This decreased virulence may occur because cell surface molecules become differentially presented and because they cannot be phagocytosed by macrophages (33) and do not reach the central nervous system (30, 32). The zeta potential data confirmed that formation of pseudohyphae can cause changes in cell surface molecules that influence the electronegativity of the cell surface.

To better characterize the influence of tebuconazole exposure on virulence, we analyzed the microscopic changes and the fungal quantity in the lungs. The higher fungal burden in the lungs and BALF of animals infected with NA cells of both species agreed with the survival curve data. Further, we detected fungus in the brain only of the animals infected with NA cells of *C. neoformans* H99. These results support those of previous studies indicating that pseudohyphae do not reach the central nervous system (30, 32), which explains why these cells are less virulent than NA cells.

Other researchers have reported that strains with secondary resistance to fluconazole are less virulent than susceptible strains (28, 34) and that strains that are heteroresistant to itraconazole and fluconazole are more virulent (27, 35). In most of these cases, morphological changes are crucial for altered virulence (27, 28, 34). In itraconazole-heteroresistant cells, increased virulence attributable to decreased cell size was observed (27), the inverse of what was observed in the cross-resistant cells in this study (cells were bigger and less virulent). Our study reinforced the idea of the importance of morphology for the virulence in *Cryptococcus* spp.

Although cells exposed to tebuconazole were less virulent than NA cells, they presented antifungal tolerance *in vivo*, as confirmed by the higher MIC values of azoles for the colonies recovered from mice and by the inability of fluconazole to reduce the fungal burden in mouse lungs.

In conclusion, exposure to tebuconazole selected cells with cross-resistance with clinical azole drugs *in vivo* and *in vitro* but not with amphotericin B. Tebuconazole exposure also altered fungal morphology and decreased the virulence of *C. gattii* and *C. neoformans*. To the best of our knowledge, this work is the first to demonstrate the implications of exposure to agrochemicals for the virulence and *in vivo* resistance of *Cryptococcus* spp.

MATERIALS AND METHODS

Microorganisms and study design. We used 13 strains of *C. gattii* (9 clinical and 2 environmental isolates, all from the culture collection of the Laboratório de Micologia da Universidade Federal de Minas Gerais, Minas Gerais, Brazil, and 2 reference strains from the culture collection of the University of Georgia, Atlanta, GA) (Table 1) (36). We also used four strains of *C. neoformans* (one clinical strain and three reference strains) (Table 1) (37). All isolates were maintained on Sabouraud dextrose broth at −80°C.

Antifungal susceptibility, tebuconazole adaptation, and cross-resistance tests were performed for all strains. The *C. gattii* R265 and *C. neoformans* H99 strains were chosen for further tests (i.e., morphophysiological, virulence change, *in vivo* antifungal resistance, and RT-PCR analyses).

Antifungal drug susceptibility testing. The MICs of fluconazole (FLC) (Sigma-Aldrich, St. Louis, MO), amphotericin B (AMB) (Sigma-Aldrich), and the agricultural fungicide tebuconazole (TBZ) (Alterne) were determined using the microdilution method proposed by the Clinical and Laboratory Standards Institute (CLSI) (M27-A3 method) (MIC^{broth}) (38). The MIC of tebuconazole was also determined by spot tests on Sabouraud dextrose agar (SDA) supplemented with different concentrations of the pesticide (MIC^{solid}) (27). For the spot tests, cell suspensions containing 1 to 5 $\times$ 10^4 cells were plated onto SDA plates containing different concentrations of tebuconazole (from 0.125 to 256.0 μg/ml). The growth pattern was determined after 72 h of incubation. The MIC^{broth} and MIC^{solid} tests were performed at two different incubation temperatures: 30 and 35°C. All tests were performed in duplicate for each strain, and the tests were repeated at least twice to confirm the results.

Tebuconazole adaptation (screening for subpopulations more tolerant of tebuconazole). After susceptibility testing on solid medium (MIC^{solid}) was performed, the strains were grown on SDA with increasing concentrations of the pesticide. Initially, all strains were grown on medium supplemented with tebuconazole at the MIC/2 (sub-MIC). After 1 week, an inoculum using at least five colonies was prepared in sterile saline solution, and the transmittance (530 nm) of the suspensions was adjusted to a range of 75% to 77% (1 $\times$ 10^6 to 5 $\times$ 10^6 fungal cells). Subsequently, 10 μl of this suspension was inoculated on a medium containing tebuconazole at the MIC. After 1 week, the process was repeated and the strains were grown, in a stepwise manner, at increasing concentrations of tebuconazole until the concentration where the growth ceased was reached. These tests were performed at both 30°C and 35°C. The colonies that were exposed to tebuconazole were named tebuconazole-adapted (A) colonies, and the original colonies (no exposure) were named nonadapted (NA) colonies.

The highest concentration of tebuconazole that the fungus was capable of growing in after the tebuconazole adaptation tests was called the maximum concentration achieved (MCA). We also quantified the ability of the microorganisms to grow in the presence of the agrochemical by determining the MCA-to-sub-MIC ratio (MCA/sub-MIC).

Cross-resistance tests (CR). The MIC^{broth} of fluconazole, amphotericin B, and tebuconazole was determined for nonadapted and tebuconazole-adapted colonies. The test was performed at 30 and 35°C for the colonies adapted at 30°C and was performed at 35°C for the colonies adapted at 35°C. A strain was considered cross-resistant when it presented decreased susceptibility to both tebuconazole and clinical drugs.

To test the stability of the cross-resistance to fluconazole and tebuconazole, at least five colonies of each adapted strain that showed an increased drug MIC^{broth} (increase of at least four times) were mixed and then subcultured every 48 h on SDA plates without tebuconazole for 10 passages (10p colonies) (27). Next, we determined the MIC^{broth} for 10p colonies.

We also tested the CR between tebuconazole and itraconazole (ITC) (Sigma-Aldrich) and ravuconazole (RVC) (Sigma-Aldrich) (an azole in phase II trials) (17) for the tebuconazole-adapted and 10p colonies that showed CR with fluconazole.

Morphometric and zeta potential analysis. Nonadapted and tebuconazole-adapted colonies were grown on SDA and on SDA supplemented with tebuconazole at the MCA, respectively, for 72 h at 30°C. Subsequently, the cells were visualized in a suspension in India ink with an optical microscope (Axioplan; Carl Zeiss) and the slides were photographed using a Coolpix 4500 (Nikon) digital camera. The capsule and diameter of at least 50 cells with regular form were measured using ImageJ 1.40 g software (http://rsb.info.nih.gov/ij/; National Institutes of Health, NIH, Bethesda, MD). In addition, the surface-to-volume ratio (S/V) was calculated using the formula 3/*r*, where *r* is the radius (27). For cells with irregular form, qualitative analyses were performed. The zeta potentials of the NA and A yeast cells were calculated using a zeta potential analyzer (Zetasizer NanoZS90; Malvern, United Kingdom) as described previously (39).

Ethics statement, virulence, and cross-resistance *in vivo*. C57BL/6 male mice, 6 to 8 weeks of age, were used for animal experiments. All experimental procedures were carried out according to the standards of the Brazilian Society of Laboratory Animal Science/Brazilian College for Animal Experimentation (available at http://www.sbcal.org.br). The study was approved by the Ethics Committee in Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA/UFMG; protocol 306/2015).

The animals (six per group) were anesthetized by intraperitoneal (i.p.) injection with ketamine hydrochloride (60 mg/kg of body weight) and xylazine (10 mg/kg) in sterile saline solution. Next, each animal received 30 μl of 1 $\times$ 10^5 cells of *C. gattii* R265 or *C. neoformans* H99 by the intratracheal route. The mice were monitored daily for survival (27).

Other groups of animals were infected and euthanized under anesthesia 15 days postinoculation to obtain lungs, bronchoalveolar lavage fluid (BALF), and brain tissue. The organ homogenates and BALF were plated onto SDA to determine the fungal burden, expressed as CFU per gram or per milliliter (28). We also determined the MIC^{broth} of tebuconazole, fluconazole, itraconazole, ravuconazole, and amphotericin B for the colonies recovered from the lungs. Moreover, lungs were collected, fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE) for histopathological analysis. Histopathology was evaluated in two aspects: presence of yeast and inflammation in the lung parenchyma. The amount (discrete, moderate, or accentuated) and distribution (multifocal or diffuse) of yeasts and the type of inflammatory cells, as well as the location (perivascular or parenchyma), intensity (discrete, moderate, or accentuated), and distribution (multifocal or diffuse) of inflammation, were

evaluated. Change described as "multifocal to diffuse" means that the lesion distribution varied in this way in the mice of this group.

To test the antifungal cross-resistance *in vivo*, mice infected with tebuconazole-adapted *C. gattii* R265 and *C. neoformans* H99 received 10 mg/kg of fluconazole daily by the intraperitoneal route. At 15 days postinoculation, the animals were euthanized and the lungs collected for determination of the CFU levels per gram.

RNA extraction and RT-PCR analysis. Nonadapted and adapted cells of *C. gattii* R265 and *C. neoformans* H99 were grown on SDA and SDA plus tebuconazole plates, respectively, at 30°C. After 72 h, the colonies were collected and the RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Total RNA (5 μg) was subjected to DNase I treatment (Roche), and then 1 μg of the DNase I-treated RNA was used for reverse transcription (RT) using a QuantiTect reverse transcription (Qiagen) kit. Subsequently, the cDNAs were subjected to PCR amplification in the presence of dCTP (α33P) (PerkinElmer) with the primers for the following genes: *ACT1*, *AFR1*, and *MDR1* for *C. neoformans* and *ACT1*, *MDR1* (18), and *PDR11* (20) for *C. gattii*. PCR products were resolved on a 7.5% polyacrylamide gel and quantified using a Typhoon 9200 imager and ImageQuant 5.2 software (Molecular Dynamics) (40).

Statistical analyses. All statistical analyses were performed using GraphPad Prism, version 6.00, for Windows (GraphPad Software, San Diego, CA, USA), with P values of <0.05 considered significant. The results of antifungal experiments (performed *in vitro* and *in vivo*), morphometric analysis, zeta potential determinations, quantification of CFU levels per gram of organs, BALF analysis, and RT-PCR were analyzed by the use of Student's *t* test. Survival curves were plotted by the use of Kaplan-Meier analysis, and results were analyzed using the log rank test. All tests, including animal experiments, were repeated at least twice.

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We declare that we have no conflicts of interest.

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4.2 – Capítulo II: Non-azole Agrochemical Pyraclostrobin Increases Clinical Antifungal Tolerance and Changes the Virulence of *Cryptococcus gattii* and *C. neoformans*

Objetivos Específicos:

- Selecionar subpopulações de *C. gattii* e de *C. neoformans* mais tolerantes ao antifúngico ambiental piraclostrobina (PCT) e metalaxil (MTX), avaliando a estabilidade desse fenômeno e possibilidade de resistência cruzada temporária ou permanete com antifúngicos clínicos como fluconazol, anfotericina B, itraconazol e ravuconazol, e o efeito da temperatura sobre esse processo;
- Estudar a influência da exposição ao PCT na morfo-fisiologia de *Cryptococcus gattii* R265;
-Realizar o ensaio de fagocitose com as colônias de *C. gattii* R265 não expostas a PCT e aquelas expostas e subcultivadas em meio sem o agroquímico;
- Comparar a virulência em modelo experimental murino das colônias de *C. gattii* R265 não expostas a PCT e aquelas expostas e subcultivadas em meio sem o agroquímico;
- Testar a eficiência do tratamento com fluconazol em camundongos infectados com colônias de *C. gattii* não expostas a PCT e aquelas expostas e subcultivadas em meio sem o agroquímico.
- Realizar o transcriptoma das colônias de *C. gattii* R265 não expostas a PCT e aquelas expostas e subcultivadas em meio sem o agroquímico, ambas cultivadas em meio sem agroquímico.

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Non-azole agrochemical pyraclostrobin increases clinical antifungal tolerance and changes the virulence of *Cryptococcus gattii*

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ABSTRACT

The treatment of cryptococcosis is performed mainly with amphotericin B and fluconazole, but there are cases of emergence of fluconazole-tolerant strains. Humans get ill when inhale infectious propagules of *Cryptococcus gattii* and *C. neoformans* from the environment, where they are currently found associtated with plants. In recent years, the hypothesis that pesticides used to treat plants deseases could induce changes in drug tolerance and virulence of *Cryptococcus* has emerged. In this study, we tested two non-azole agrochemical, pyraclosotrobin and metalaxyl. We show that only pyraclostrobin exposure induces both temporary and permanent cross-resistance (CR) with clinical azoles (fluconazole, itraconazole and ravuconazole), but not with amphotericin B, in *C. gattii* and *C. neoformans*. Besides, pyraclostrobin exposure caused pseudohyphae formation in one strain of *C. gattii*. We also evaluated the virulence of *C. gattii* R265 non-adapted (NA) cells and also cells that had been exposed to the agrochemical and then cultivated in a medium without the substance for 10 passages (culture called 10p), which presented permanent CR with all clinical azoles. Although the 10p cells were less virulent than the NA ones, these cells presented CR *in vivo* when there was no significant difference ($p>0.05$) in survival between mice infected with 10p cells treated or not with fluconazole, which was observed for those animals infected with NA cells. The trascriptomic analysis showed that the 10p cells expressed more efflux pumps genes, including *AFR1* and *MDR1*, and the pyraclostrobin target than the NA ones. In addition, genes related to ion metabolism, which could be involved in virulence, were downregulated in the 10p cells, which could explain the lower virulence. In general, our data showed that pyraclostrobin can induce CR with clinical azole drugs in *C. gattii* and *C. neoformans*, and it can also cause changes in morphology and virulence of *C. gattii*.

Key-words: pseudohyphae, antifungal cross-resistance, efflux pumps, metalaxyl, temperature

INTRODUCTION

Cryptococcus gattii and *C. neoformans* are the main ethiologic agents of cryptococcosis, which affects more than 223,100 people per year worldwide, with a mortality rate higher than 81% (Williamson *et al*., 2017; Rajasingham *et al*., 2017). The infection occurs by inhalation of spores and/or dissecated yeasts from the environment. This infection may present itself as assyntomatic or as a primary pneumonia. In more severe cases, yeasts can translocate to other organs, including the central nervous system (CNS), causing most problematic symptoms (Kronstad *et al*., 2011, Williamson *et al*., 2017).

The treatment of cryptococcosis is performed with antifungal drugs, like amphotericin B, 5-flucytosine and azoles, e.g. fluconazole and itraconazole (Perfect *et al*., 2010). Despite the growing cases of azole resistance (Smith *et al*., 2015; Chen *et al*., 2016), the resistance mechanism in *Cryptococcus*, especially in *C.gattii*, is still poorly understood. According to previous studies, it happens due to mutations in the gene *ERG11*, which encodes the protein-target to the drug (ERG11p), and also to the overexpression of efflux pumps, such as *AFR1* (*PDR-11*), *AFR2* and *MDR11* (Basso *et al*., 2015; Yang *et al*., 2016). Furthermore, *Cryptococcus* is able to develop heteroresistance to fluconazole and itraconazole (Sionov *et al*., 2012; Ferreira *et al*., 2015), which is related to the duplication of chromosomes (disomy) that carry important genes to azole resistance (Sionov *et al*., 2012, Ngamskulrungroj *et al*., 2012).

Cryptococcus gattii and *C. neoformans* are often associated with vegetal materials, being *C. gattii* found in more than 50 tree species (Chatuverdi and Chatuverdi, 2011). Recently, it was sugested that the primary niche of *C. neoformans* may also be related to plants, and not to birds' feces, as it was previously thougth (Cogliati *et al*., 2016). However, it is unclear (i) how the environment and the interaction with plants influence the biology of these fungi; and (Xue *et al*., 2007) (ii) whether the human practice, such as the use of agrochemicals (Del Poeta and Casadevall, 2012), could affect clinical drugs tolerance, as it has been shown for *Aspergillus fulmigatus* (Snelders *et al*., 2012; Ren *et al*., 2017). Previously, we showed that tebuconazole, a triazole agrochemical, causes permanent and temporary cross-resistance (CR) with clinical azole drugs in *Cryptococcus* spp., both *in vitro* and *in vivo* (Bastos *et al*., 2018). Nevertheless, it is unknown whether non-azole agrochemicals, such as pyraclostrobin and metalaxyl, can also cause CR.

The enviromental antifungal pyraclostrobin (PCT), i.e. a strobilurin fungicide, belongs to the group of quinone outside inhibitors (QoI). QoI inhibit mitochondrial respiration by binding to the Qo site of the cytochrome b and then blocking the electron transference between cytochrome b and cytochrome c_1, which results in the disruption of the energy cycle. Because of their broad-spectrum activity against major groups of plant pathogenic fungi, QoIs have become one of the most important groups of fungicides in agriculture (Bartlett *et al.*, 2002). Metalaxyl is a fungicide of the acylalanine group and inhibits the activity of RNA polymerase I, affecting the synthesis of ribosomal RNA. The use of this type of fungicide has a high risk of selecting resistant populations, and the CR between this fungicide and other agrochemicals in oomycetes is already well-documented (FRAC, 2016).

The goal of this work is to study whether the exposure to non-azole agrochemicals could affect the susceptibility to clinical drugs, the morpho-phisiology and the virulence of *C. gattii* and *C. neoformans*.

MATERIALS AND METHODS

Microorganisms and study design

We used 13 strains of *C. gattii* (nine clinical and two environmental isolates, all from the culture collection of the Laboratório de Micologia da Universidade Federal de Minas Gerais, Minas Gerais, Brazil, and two reference strains from the culture collection of the University of Georgia, Atlanta, GA) (Table 1) (Santos *et al.*, 2012). We also used four strains of *C. neoformans* (one clinical and three reference strains) (Table 1) (Magalhães *et al.*, 2013). All isolates were maintained in Sabouraud Dextrose Broth at -80°C.

Antifungal susceptibility, pyraclostrobin- and metalaxyl-adaptation and cross-resistance tests were performed for all strains. *C. gattii* R265 strain was chosen for further tests (morphophysiological, virulence changes, *in vivo* antifungal resistance and molecular analysis).

Antifungal drug susceptibility testing

The minimum inhibitory concentration (MIC) of fluconazole (FLC) (Sigma-Aldrich, St. Louis, MO), amphotericin B (AMB) (Sigma-Aldrich) and the enviromental

antifungal pyraclostrobin (PCT) (COMET®) and metalaxyl (MTX) (RIDOMIL GOLD®) were determined by the microdilution method proposed by the Clinical and Laboratory Standards Institute (CLSI) M27-A3 method (MIC^{fluid}) (CLSI, 2012). The MIC of pyraclostrobin and metalaxyl were also determined by spot tests on Sabouraud Dextrose Agar (SDA) supplemented with different concentrations of the pesticides (MIC^{solid}) as described by Bastos and colleagues (Bastos *et al.*, 2018). The MIC^{fluid} and MIC^{solid} tests were performed at two different temperatures of incubation, 30°C and 35°C. All tests were performed in duplicate for each strain, and the tests were repeated at least twice to confirm the results.

Agrochemical adaptation and cross-resistance tests (CR)

After performing the susceptibility tests on solid medium (MIC^{solid}), the strains were grown on SDA medium with increasing concentrations of the pesticide. Initially, all strains were grown in medium supplemented with pyraclostrobin or metalaxyl at a concentration of MIC/2 (sub-MIC). After the colonies have grown, an inoculum of 1−5 $\times 10^4$ fungal cells was prepared using at least five colonies and incoulated in a medium containing the agrochemicals at the MIC^{solid}. After one week, the process was repeated, and the strains were grown, in a stepwise manner, at increasing concentrations of the pesticides, up to the concentration in which the growth ceased or the value reached the limit of 256 µg/mL. Both tests were performed at 30 and 35°C (Bastos *et al.*, 2018). The highest concentration of agrochemicals in which the fungus was capable of growing after the adaptation tests was called Maximum Concentration Achieved (MCA). We also quantified the ability of the microorganisms to grow in the presence of the agrochemical by determining the ratio between the MCA and the sub-MIC (MCA/sub-MIC). The colonies that were exposed to the agrochemicals were named pyraclostrobin- or metalaxyl-adapted (A) colonies, and the original colonies (no exposure) were named non-adapted (NA) colonies.

Subsequently the adapatation process, the MIC^{broth} of fluconazole, amphotericin B, and the agrochemicals were determined for non-adapted and agrochemical-adapted colonies. The test was performed at 30 and 35°C (incubation temperature MIC^{broth}) for the colonies adapted at 30°C and at 35°C for the colonies adapted at 35°C. The strain was considered cross-resistant when it was tolerant to the agrochemicals and to the clinical drugs at the same time. The CR was also classified as temporary when the agrochemical-adapted colonies returned the original tolerance after cultivation in

agrochemical-free medium for 10 passages (10p colonies), and as permanent when the 10p colonies remained more tolerant to the drugs (Bastos *et al*., 2018).

We also tested the CR between the agrochemicals and itraconazole (ITC) (Sigma-Aldrich) and ravuconazole (RVC) (Sigma-Aldrich) (an azole in Phase II trials) (Alle *et al*., 2015) for the agrochemicals-adapted and 10p colonies that showed CR with fluconazole.

Morphological analysis

All the non-adapted, agrochemical-exposure and 10p cells, after 72h of cultivation in SDA or SDA plus agrochemical, were observed in optical microscope (Axioplan;Carl Zeiss). The cells that presented major change in morphology were observed in optical and Scanning electron microscopy (SEM). For optical microscopy, the cells were observed in suspension in India ink, with an optical microscope, and the slides were photographed using Coolpix 4500 (Nikon) digital camera (Ferreira *et al*, 2015). Experiments and analyses involving electron microscopy were performed in the Center of Microscopy at the Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil. For the SEM, 4 cycle log cells were applied on a coverall with poly-L-lysine. Subsequently, the coverslips were treated with fixative (2.5% glutaraldehyde and 0.1M sodium cacodylate) for at least 1 hour at room temperature. The fixative was then removed by washing the samples (1x) with buffer solution containing 0.1M cacodylate, 0.2M sucrose and 2mM MgCl. The samples were visualized in FEG - Quanta 200 FEI scanning electron microscope operated at 15 Kv (Van Duin *et al*., 2004).

Morphometric analysis (capsule and cell diameters and surface-volume ratio) for NA and 10p (30°C) cells of *C. gatii* R265 and zeta potential were also carried out as described by Ferreira and colleagues (2015).

Ethics statement, phagocytosis, killing assay, reactive oxygen species and peroxynitrite production by macrophages

C57BL/6 male mice, 6-8 weeks-old, were used for recovery of bone marrow-derived macrophages (BMDM). All experimental procedures were carried out according to the standards of the Brazilian Society of Laboratory Animal Science/Brazilian College for Animal Experimentation (available at http://www.sbcal.org.br). The study was approved by the Ethics Committee in Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA/UFMG, protocol n° 306 / 2015).

NA and 10p cells of *C. gattii* R265 were cultivated in SDA medium during 72h at 30°C. After this time, the yeasts were inoculated with BMDM (5 cells: 1 yeast) to determine phagocytosis; killing assay, detection of reactive oxygen species (ROS) and peroxynitrite (PRN) were also performed. BMDMs were isolated as previously described (Ribeiro *et al.*, 2017). After that, BMDM cells recovered from mice femurs and tibias were counted using a Neubauer chamber, and the concentration was adjusted to 2×10^6 cells/mL for incubation in BMDM medium (RPMI supplemented with 30% L929 growth conditioning media, 20% bovine fetal serum [Gibco], 2mM glutamine [Sigma-Aldrich], 25 mM HEPES pH 7.2, 100 units/mL of penicillin-streptomycin [Life Technologies]). Fresh media were added every 48h. BMDMs were collected on day 7 and used for subsequent experiments. Then, 2×10^5 BMDM/mL were plated into 24-well plates for determining phagocytosis and killing assay, and into 96-well plates for detecting ROS and PRN, which were then incubated overnight in RPMI and supplemented with 10% fetal bovine serum at 37 ºC under 5% CO_2.

Three hours post-infection, the phagocytic index was calculated as the percentage of cells with internalized *C. gattii* by optical microscopy. For the killing assay, cell culture supernatants were removed, and noninternalized and adherent yeast cells were removed by two washings with PBS. BMDMs were then lysed with 200 µL of sterile distilled water for 30 min at 37°C, and 50 µL of this suspension were plated on SDA for CFU determination (Ribeiro *et al.*, 2017). We also determined the MIC^{broth} of pyraclostrobin, fluconazole, itraconazole, ravuconazole and amphotericin B for the colonies recovered from the plates after the killing assay. For the ROS and the PRN determination, the 96 well-plates were added of 2′,7′-dichlorofluoresceindiacetate (DCFHDA; Invitrogen, Life Technologies, Carlsbad, CA, USA) (ROS) or dihydrorhodamine-123 (DHR-123; Invitrogen) (PRN). Fluorescence was assessed 3 hours post-inoculation of the yeasts by using a fluorometer (Synergy 2 SL Luminescence Microplate Reader; Biotek) with excitation and emission wavelengths of 485/530 nm. The data were expressed as arbitrary units of fluorescence (Ferreira *et al.*, 2013).

Virulence and cross-resistance *in vivo*

C57BL/6 male mice (n=6), 6-8 weeks-old, were divided into the following four groups: i and ii were infected with non-adapted or 10p (30°C) cells of *C. gattii* R265, respectively, and they were not treated with fluconazole; the other groups (iii and iv)

were infected with the same yeasts, respectively, but received 20 mg/Kg of fluconazole daily through intraperitoneal route. For infection, mice were anesthetized through intraperitoneal injection with ketamine hydrochloride (60 mg/kg) and xylazine (10 mg/kg) in sterile saline solution. After that, each animal received 30 µL of $1x10^5$ cells through intratracheal route. The mice were monitored daily to keep their survival (Ferreira *et al*., 2015).

RNA extraction and sequencing

NA and 10p cells of *C. gattii* R265 were grown in YPD medium (1% yeast extract, 1% peptone and 2% dextrose) without drugs at 30°C for total RNA extraction following the previously described protocol (Moyrand *et al*., 2008). We performed each extraction experiment in independent triplicates. For high-throughput sequencing, strand-specific, paired-end cDNA libraries were prepared from 10 µg of total RNA using the Illumina mRNA-Seq-Sample Prep Kit according to manufacturer's instructions. cDNA fragments of ~400 bp were purified from each library and confirmed for quality by Bioanalyzer (Agilent). Then, 100 bp were sequenced from both ends using an Illumina HiSeq2000 instrument, according to the manufacturer's instructions (Illumina). Differential expression was investigated using DESeq1 v1.1659, DESeq2 v1.4.160, and edgeR v3.6.161 with default settings and false discovery rate (FDR) cutoff at 0.05. Only genes with > 10 mapped fragments in at least one library were considered. A gene was considered to be significantly differentially expressed when it passed the FDR cutoff in at least 1.30. The fold change output from DESeq1 was considered to be the final fold change.

Statistical analyses

All statistical analyses, except the transcriptome one, were performed using GraphPad Prism, version 6.00, for Windows (GraphPad Software, San Diego, CA, USA), with $p<0.05$ significance. The results of morphometric analysis, zeta potential, phagocytosis, killing assays and MIC^{fluid} for yeasts recovered from BMDMs were analyzed by the Student's t test.; ROS and PRN production were analyzed by Analysis of Variance (ANOVA), followed by the Tukey's test. The survival curve was plotted by Kaplan-Meier analysis, and the results were analyzed using the log rank test. All tests were repeated at least twice.

RESULTS

Adaptation process increases the tolerance to the agrochemicals

Initially we determined the MIC of clinical (fluconazole and amphotericin B) and enviromental (pyraclostrobin and metalaxyl) drugs for all strains of *C.gattii* and *C. neoformans* at 30 and 35°C. All strains were inhibited by the drugs when the MIC was performed in fluid medium (MIC^{broth}), for both clinical and enviromental antifungals (Data not shown), and also in solid medium (MIC^{solid}), when we used pyraclostrobin (Table 1) and metalaxyl (Supplementary Material Table S1).

After the passage in medium with the agrochecamicals in a stepwise manner, 100% of the *C. gattii* and the *C. neoformans* strains were capable of tolerating higher concentrations of pyraclostrobin added to the solid medium, when the adaptation test was performed at 30°C (Table 1). *C. gattii* L27/02 was the strain that tolerated more agrochemical in the adaptation test, being able to grow on a medium with 2048-fold more pyraclostrobin (Table 1).

When the adaptation was carried out at 35°C, 77% of the *C. gattii* and 100% of the *C. neoformans* strains tolerated more pyraclostrobin in the solid medium (Table 1). Regarding metalaxyl, the temperature was also important for the tolerability of the agrochemical. Colonies adapted at 30°C of both species tolerated more metalaxyl than those adapted at the higher temperature (Table S1).

The importance of the temperature in the adaptation can be confirmed when we observe that the geometric means of the ratio MCA/Sub-MIC at 30°C are higher than at 35°C for both pesticides (Tables 1 and S1).

Table 1. Screening of subpopulations of *C. gattii* and *C. neoformans* strains less susceptible to pyraclostrobin (PCT-adaptation).

Strain or parameter	**MIC^{solid} (µg/mL)**		**MCA (µg/mL)**		**MCA/Sub-MIC^{solid}**	
C. gattii	**30°C**	**35°C**	**30°C**	**35°C**	**30°C**	**35°C**
R265 (C)	1.0	1.0	200.0	1.0	400.0	2.0
ATCC 24065 (R)	1.0	0.5	256.0	1.0	512.0	4.0
ATCC 320608 (R)	1.0	0.5	10.0	1.0	20.0	4.0
547/OTTI/94-PI-10 (E)	1.0	0.5	1.0	1.0	2.0	4.0
ICB 181 (E)	1.0	1.0	10.0	0.5	20.0	2.0
L24/01 (C)	1.0	1.0	1.0	0.5	2.0	2.0
L27/01 (C)	0.25	0.25	256.0	0.5	2048.0	4.0
L28/02 (C)	0.25	1.0	0.5	0.5	4.0	1.0
1913/ER (C)	1.0	2.0	256.0	256.0	512.0	256.0
196L/03	1.0	2.0	265.0	256.0	512.0	256.0
LMM 818 (C)	2.0	2.0	256.0	1.0	256.0	1.0
23/10893 (C)	1.0	2.0	12.0	1.0	24.0	1.0
29/10893 (C)	1.0	2.0	256.0	175.0	512.0	175.0
Range	0.25 – 2.0	0.25 – 2.0	1.0 – 256.0	1.0 – 256.0	2.0 – 2048.0	1.0– 256.0
Geometric mean	0.85	1.0	31.86	2.82	64.6	6.27
C. neoformans	**30°C**	**35°C**	**30°C**	**35°C**	**30°C**	**35°C**
H99 (C)	1.0	1.0	2.0	2.0	4.0	4.0
ATCC 24067 (R)	0.5	2.0	2.0	2.0	8.0	2.0
ATCC 28957 (R)	1.0	2.0	10.0	2.0	20.0	2.0
ATCC 62066 (R)	1.0	2.0	2.0	2.0	4.0	2.0
Range	0.5 – 1.0	1.0 – 2.0	2.0 – 10.0	2.0	4.0 – 20.0	2.0 – 4.0
Geometric mean	0.84	1.68	2.99	2.0	7.11	2.38

MIC^{solid}: Minimum Inhibitory Concentration of pyraclostrobin in solid medium before the adaptation process. MCA: Maximum Concentration Achieved of pyraclostrobin in the PCT-adaptation test. C. clinical strain; R. reference strain; E. environmental strain.

Pyraclostrobin, but not metalaxyl, exposure causes both temporary and permanent cross-resistance with azole drugs in a temperature dependent manner

After the adaptation process, we determined if the MIC^{broth} of the agrochemicals changed. The MIC of pyraclostrobin increased (≥4fold) after the adaptation process at 30°C for 61,5% (n=8) of the *C. gattii* and 100% (n=4) of the *C. neoformans* strains (Tables 2 and 3). When MIC^{broth} determination tests were performed at 35°C, only six strains of *C. gattii* demonstrated to be more tolerant to the pesticide. In *C. neoformans*, all pyraclostrobin-adapted at 30°C colonies behaved similarly in both incubation temperatures (Table 3).

Furthermore, we tested if pyraclostrobin exposure could affect the tolerance to fluconazole and amphotericin B. Tables 2 and 3 show that the geometric means of MIC^{broth} of fluconazole for pyraclostrobin-adapted cells of *C. gattii* increased almost 2.5 times when the tests were carried out at the lower temperature. However, when the MIC^{broth} determination was performed at 35°C for *C. gattii*, and at both temperatures for *C. neoformans*, the geometric means of adapted cells were not quite different than those of non-adapted (NA) cells (Tables 2 and 3).

We also analysed cross-resistance (CR) between pyraclostrobin and fluconazole, and 46% (n=6) of the *C. gattii* and 25% (n=1) of the *C. neoformans* strains presented CR when the adapation test and the incubation temperature was 30°C (Tables 2-4). Five (38%) *C. gattii* strains (R265, ATCC 24065, L24/01, L27/01 and 196L/03) presented a permanent CR (even after 10 passages on agrochemical-free media, the increasing tolerance was maintained) (Table 2). *C. gattii* 547/OTTI/94-PI-10 and *C. neoformans* ATCC 62066, however, presented only a temporary CR, once the adapted cells returned to the initial tolerance to fluconazole after 10 passages on medium without pyraclostrobin (Tables 2 to 4). The permanent and temporary CR presented by *C. gattii* R265 and 547/OTTI/94-PI-10, respectively, were independent of the temperature, since the cells presented CR at both 30 and 35°C (Table 2).

Table 2. MICs (µg/mL) of fluconazole and pyraclostrobin for non-PCT-adapted *C. gattii* colonies, *C. gattii* colonies subjected to PCT adaptation at 30°C, and PCT-adapted *C. gattii* colonies subcultured 10 times in agrochemical-free medium[a].

Strain or Parameter	**Fluconazole[b]**						**Pyraclostrobin[c]**					
	Temperature 30°C			**Temperature 35°C**			**Temperature 30°C**			**Temperature 35°C**		
	NA	**A**	**10p**	**NA**	**A**	**10p**	**NA**	**A**	**10p**	**NA**	**A**	**10p**
R265	**8.0**	**128.0 (16X)**	**128.0 (16X)**	**8.0**	**32.0 (4X)**	**32.0 (4X)**	**1.0**	**16.0 (16X)**	**8.0 (8X)**	**0.5**	**2.0 (4X)**	**2.0 (4X)**
ATCC 24065	**4.0**	**16.0 (4X)**	**16.0 (4X)**	4.0	4.0	ND	**0.125**	**1.0 (8X)**	**1.0 (8X)**	**0.125**	**1.0 (8X)**	**1.0 (8X)**
ATCC 32608	16.0	32.0	ND	8.0	16.0	ND	2.0	4.0	ND	1.0	2.0	ND
547/OTTI/94-PI-10	**16.0**	**128.0 (8X)**	16.0	**8.0**	**32.0 (4X)**	8.0	**1.0**	**8.0 (8X)**	1.0	**1.0**	**4.0 (4X)**	1.0
ICB 181	16.0	16.0	ND	8.0	16.0	ND	**0.5**	**4.0 (8X)**	**4.0 (8X)**	**0.25**	**1.0 (4X)**	**2.0 (8X)**
L24/01	**16.0**	**64.0 (4X)**	**64.0 (4X)**	8.0	16.0	ND	**0.25**	**1.0 (4X)**	**2.0 (8X)**	**0.25**	**1.0 (4X)**	**2.0 (8X)**
L27/01	**16.0**	**64.0 (4X)**	**64.0 (4X)**	32.0	16.0	ND	**1.0**	**128.0 (128X)**	**4.0 (4X)**	2.0	1.0	ND
L28/02	32.0	64.0	ND	16.0	32.0	ND	1.0	1.0	ND	0.5	1.0	ND
1913R	16.0	32.0	ND	16.0	8.0	ND	8.0	8.0	ND	1.0	2.0	ND
196L/03	**16.0**	**128 (8X)**	**128.0 (8X)**	16.0	32.0	ND	**0.5**	**8.0 (16X)**	**2.0 (4X)**	**0.5**	**4.0 (8X)**	**2.0 (4X)**
LMM 818	16.0	16.0	ND	16.0	8.0	ND	8.0	8.0	ND	2.0	4.0	ND
23/10893	8.0	4.0	ND	8.0	4.0	ND	4.0	4.0	ND	4.0	4.0	ND
29/10933	8.0	16.0	ND	4.0	4.0	ND	**4.0**	**128.0 (32X)**	**128.0 (32X)**	4.0	4.0	ND
MIC range	4.0 – 32.0	4.0 – 128.0	ND	4.0 – 32.0	4.0 – 32.0	ND	0.125 – 8.0	1.0 – 128.0	ND	0.125 – 4.0	1.0 – 4.0	ND
Geometric mean	12.92	35.6	ND	9.90	12.92	ND	1.23	6.81	ND	0.81	2.0	ND

a:Tests were performed at 30 and 35°C; b: MIC endpoint considering 50% of growth inhibition; c: MIC endpoint considering 100% of growth inhibition. The number in parentheses shows how many times (X) the MIC value of the PCT-adapted or of the10p colonies was higher (≥4X) than the MIC of the NA colonies. Values in bold indicate increased MIC by at least 4X more than NA colonies. ND = not determined.

Table 3. MICs (µg/mL) of fluconazole and pyraclostrobin for non-PCT-adapted *C. neoformans* colonies, *C. neoformans* colonies subjected to PCT adaptation at 30°C, and PCT-adapted *C. neoformans* colonies subcultured 10 times in agrochemical-free medium[a].

Strain or parameter	Fluconazole[b]						Pyraclostrobin[c]					
	Temperature 30°C			Temperature 35°C			Temperature 30°C			Temperature 35°C		
	NA	A	10p	NA	A	10p	NA	A	10p	NA	A	10p
H99	16.0	16.0	ND	8.0	8.0	ND	**0.5**	**128.0 (256X)**	**8.0 (16X)**	**0.5**	**2.0 (4X)**	**2.0 (4X)**
ATCC 24067	16.0	8.0	ND	4.0	2.0	ND	**2.0**	**128.0 (64X)**	**128.0 (64X)**	**1.0**	**128.0 (128X)**	**32.0 (32X)**
ATCC 28957	4.0	8.0	ND	2.0	2.0	ND	**0.5**	**8.0 (16X)**	**4.0 (8X)**	**0.25**	**1.0 (4X)**	**2.0 (8X)**
ATCC 62066	**4.0**	**16.0 (4X)**	8.0	4.0	4.0	ND	**1.0**	**8.0 (8X)**	2.0	**1.0**	**4.0 (4X)**	2.0
MIC range	4.0 - 16.0	8.0 – 16.0	ND	2.0 - 8.0	2.0 - 8.0	ND	0.25 - 1.0	8.0 - 128.0	ND	0.25 - 1.0	1.0 - 128.0	ND
Geometric mean	8.0	11.31	ND	4.0	3.36	ND	0.84	32.0	ND	0.59	5.65	ND

a:Tests were performed at 30 and 35°C; b: MIC endpoint considering 50% of growth inhibition; c: MIC endpoint considering 100% of growth inhibition. The number in parentheses shows how many times (X) the MIC value of the PCT-adapted or of the10p colonies was higher (≥4X) than the MIC of the NA colonies. Values in bold indicate increased MIC by at least 4X more than NA colonies. ND = not determined.

Table 4. Percentage (%) of Cross-resistance (CR) between PCT and FLC presented in *C. gattii* and *C. neoformans* strains after PCT-adaptation at 30 and 35°C.

Resistance	*C. gattii*		*C. neoformans*	
	30°C	35°C	30°C	35°C
Cross- Resistance (CR)	46	8	25	0
Temporary CR	8	0	25	0
Permanent CR	38	8	0	0

Execept for *C. gattii* 196L/03, the cells adapted to pyraclostrobin at 30°C that presented CR with fluconazole also presented CR with ravuconazole (Table 5), but not with amphotericin B (Data not shown). Regarding itraconazole, only *C. gattii* R265 presented CR with this drug (Table 5).

Table 5. MICs (µg/mL) of itraconazole and ravuconazole for non-PCT-adapted *C. gattii* and *C. neoformans* colonies, *C. gattii* and *C. neoformans* colonies subjected to PCT adaptation at 30°C, and PCT -adapted *C. gattii* and *C. neoformans* colonies subcultured 10 times in agrochemical-free medium[a].

Strain	Itraconazole[b]						Ravuconazole[b]					
	Temperature 30°C			Temperature 35°C			Temperature 30°C			Temperature 35°C		
C. gattii	**NA**	**A**	**10p**	**NA**	**A**	**10p**	**NA**	**A**	**10p**	**NA**	**A**	**10p**
R265	**0.5**	**2.0 (4X)**	**2.0 (4X)**	**0.25**	**1.0 (4X)**	**1.0 (4X)**	**0.125**	**2.0 (16X)**	**2.0 (16X)**	**0.03**	**0.125 (4X)**	**0.125 (4X)**
ATCC 24065	0.5	1.0	0.5	0.25	0.5	0.5	**0.06**	**0.25 (4X)**	0.125	**0.03**	**0.125 (4X)**	0.03
547/OTTI/94-PI-10	0.5	1.0	ND	0.25	0.5	ND	**0.25**	**1.0 (4X)**	ND	**0.06**	**0.25 (4X)**	ND
L24	1.0	1.0	1.0	0.5	1.0	1.0	**0.125**	**1.0 (8X)**	**1.0 (8X)**	**0.06**	**0.5 (8X)**	**0.25 (4X)**
L27	0.25	0.25	0.5	0.25	0.25	0.25	**0.125**	**0.5 (4X)**	**0.5 (4X)**	**0.03**	**0.25 (8X)**	**0.25 (8X)**
196L/03	0.5	0.5	1.0	ND	ND	ND	0.25	0.25	0.25	ND	ND	ND
C. neoformans	**NA**	**A**	**10p**	**NA**	**A**	**10p**	**NA**	**A**	**10p**	**NA**	**A**	**10p**
ATCC 62066	0.25	0.5	ND	0.25	0.5	ND	**0.03**	**0.5 (16X)**	ND	**0.031**	**0.12 (4X)**	ND

a: Tests were performed at 30 and 35 °C; b: MIC endpoint considering 50% of growth inhibition. The number in parentheses shows how many times (X) the MIC value of the PCT-adapted or of the10p colonies was higher (≥4X) than the MIC of the NA colonies. Values in bold indicate increased MIC by at least 4X more than NA colonies. ND = not determined.

Also, we showed that, when the adaptation test was performed at 35°C, the MIC[broth] of pyraclostrobin for three strains of *C. gattii* and one of *C. neoformans* increased (Tables 6). However, CR with fluconazole was observed only for *C. gattii* 196L/03 (8%) (Tables 4 and 6). This strain also presented CR with ravuconazole, but not with itraconazole (Table 7) and amphotericin B (Data not shown).

Regarging matalaxyl, the adaptation process performed at 30 and 35°C did not change the MIC[broth] of the agrochemical, fluconazole (Tables S2 to S4) and amphoterin B (Data not shown) in *Cryptococcus* strains.

Table 6. MICs (µg/mL) of fluconazole and pyraclostrobin for non-PCT-adapted *C. gattii* and *C. neoformans* colonies, *C. gattii* and *C. neoformans* colonies subjected to PCT adaptation at 30°C, and PCT -adapted *C. gattii* and *C. neoformans* colonies subcultured 10 times in agrochemical-free medium[a].

Strain or parameter	Fluconazole[b]			Pyraclostrobin[c]		
C. gattii	**NA**	**A**	**10p**	**NA**	**A**	**10p**
R265	8.0	8.0	ND	0.5	1.0	ND
ATCC 24065	4.0	4.0	ND	0.125	0.25	ND
ATCC 32608	8.0	8.0	ND	1.0	1.0	ND
547/OTTI/94-PI-10	8.0	16.0	ND	1.0	2.0	ND
ICB 181	8.0	8.0	ND	**0.25**	**1.0 (4X)**	0.5
L24/01	8.0	16.0	ND	0.25	0.25	ND
L27/01	32.0	16.0	ND	**2.0**	**8.0 (4X)**	**8.0 (4X)**
L28/02	16.0	16.0	ND	0.5	1.0	ND
1913R	16.0	16.0	ND	1.0	2.0	ND
196L/03	**16.0**	**64.0 (4X)**	**64.0 (4X)**	**0.5**	**2.0 (4X)**	**4.0 (8X)**
LMM 818	16.0	8.0	ND	2.0	4.0	ND
23/10893	8.0	4.0	ND	4.0	8.0	ND
29/10933	4.0	4.0	ND	4.0	2.0	ND
MIC range	4.0 – 32.0	4.0 – 16.0	ND	0.125 – 4.0	0.25 – 8.0	ND
Geometric mean	9.9	10.44	ND	0.75	1.35	ND
C. neoformans	**NA**	**A**	**10p**	**NA**	**A**	**10p**
H99	8.0	16.0	ND	0.5	0.5	ND
ATCC 24067	4.0	8.0	ND	**1.0**	**16.0 (16X)**	**4.0 (4X)**
ATCC 28957	2.0	4.0	ND	0.25	0.5	ND
ATCC 62066	4.0	4.0	ND	1.0	1.0	ND
MIC range	2.0 – 8.0	4.0 – 16.0	ND	0.25 – 1.0	0.5 – 16.0	ND
Geometric mean	4.0	6.72	ND	0.59	1.41	ND

a: Tests performed at 35°C. b: MIC endpoint considering 50% of growth inhibition; c: MIC endpoint considering 100% of growth inhibition. The number in parentheses shows how many times (X) the MIC value of the PCT-adapted or 10p colonies was higher (≥4X) than the MIC of the NA colonies. Values highlighted indicate increased MIC by at least 4X more than NA colonies. ND = not determined.

Table 7. MIC (µg/mL) of itraconazole and ravuconazole for non-PCT-adapted *C. gattii* colonies, *C. gattii* colonies subjected to PCT adaptation at 30°C, and PCT-adapted *C. gattii* colonies subcultured 10 times in agrochemical-free medium[a].

Strain	**Itraconazole**[b]			**Ravuconazole**[c]		
C. gattii	**NA**	**A**	**10p**	**NA**	**A**	**10p**
196L/03	0.5	0.5	0.5	**0.125**	**1.0 (8X)**	**1.0 (8X)**

a: Tests were performed at 35 °C; b: MIC endpoint considering 50% of growth inhibition. The number in parentheses shows how many times (X) the MIC value of the PCT-adapted or of the10p colonies was higher (≥4X) than the MIC of the NA colonies. Values in bold indicate increased MIC by at least 4X more than NA colonies. ND = not determined.

Pyraclostrobin exposure causes morphological changes

It was recently demonstrated that *C. neoformans* H99 forms pseudohyphae when exposed to the agrochemical tebuconazole (Bastos *et al*., 2018). Therefore, we observed cells that had been exposed to the two agrochemicals under optical microscope to verify if the same phenomenon happens with non-azole agrochemicals. Of all cultures analysed, the adapted cells at 35°C of *C. gattii* 196L/03 formed pseudohyphae (Figures 1A and B). The NA and the 10p cells, however, maintained the yeast form (Figures 1A and B).

We also tested whether lower concentrations of pyraclostrobin could cause pseudohyphae formation, and we observed pseudohyphal formation at 10, 50, 100 and 150 µg/mL of pyraclostrobin (Data not shown).

Morphometric analysis (cellular diameter, capsule size, S/V ratio) also were carried out for the non-adapted and the 10p cells of *C. gattii* R265. This strain was chosen for the further tests, because it presented a permanent CR, when the adaptation was performed at 30°C with fluconazole, itraconazole and ravuconazole (Table 2). The 10p cells were significantly ($p<0.05$) smaller (Figure 2A) and showed a higher S/V ratio than NA cells (Figure 2B). However, there were not statistical differences ($p>0.05$) between the capsule sizes (Figure 2C). Regarding the cellular surface charge, the 10p cells presented a surface charge lower than the NA ones (Figure 2D).

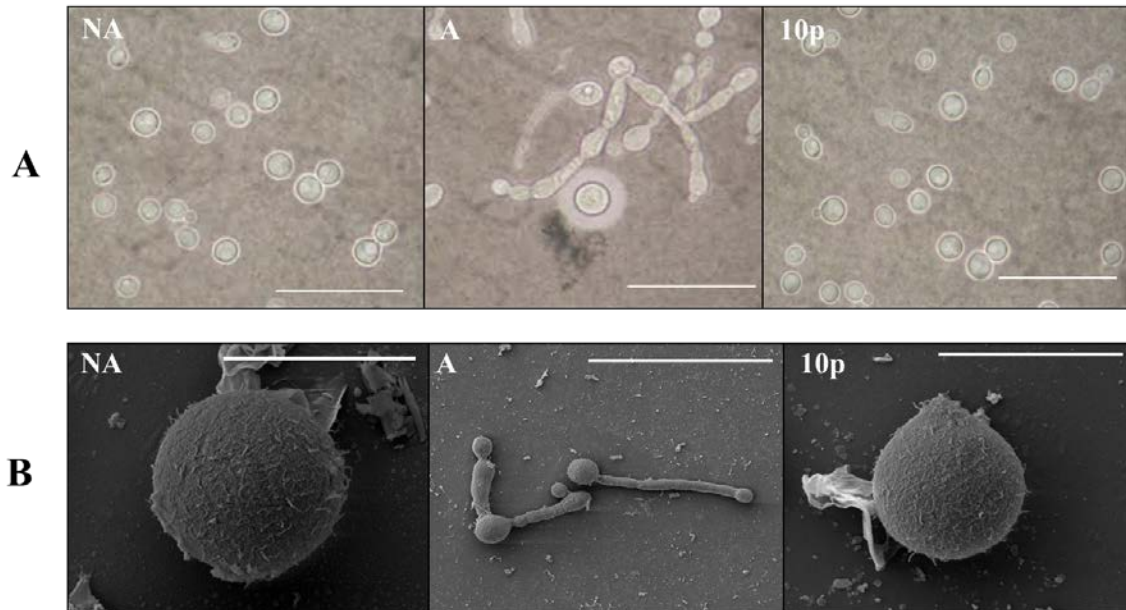

Figure 1. Cell morphology of non-adapted (NA), pyraclostrobin-adapted (A) and adapted cells subcultivated in medium without agrochemical in optical (India ink suspension) (A) and scanning electron microscopy (B)of *C. gattii* 196L/03. The cells of the adapted culture presented pseudohyphae, while the NA and 10p cultures presented cells with yeast form. Scale bars are equal to 10 µm (A), 5.0 µm, for NA and 10p, and 20 µm for adapted cells (B).

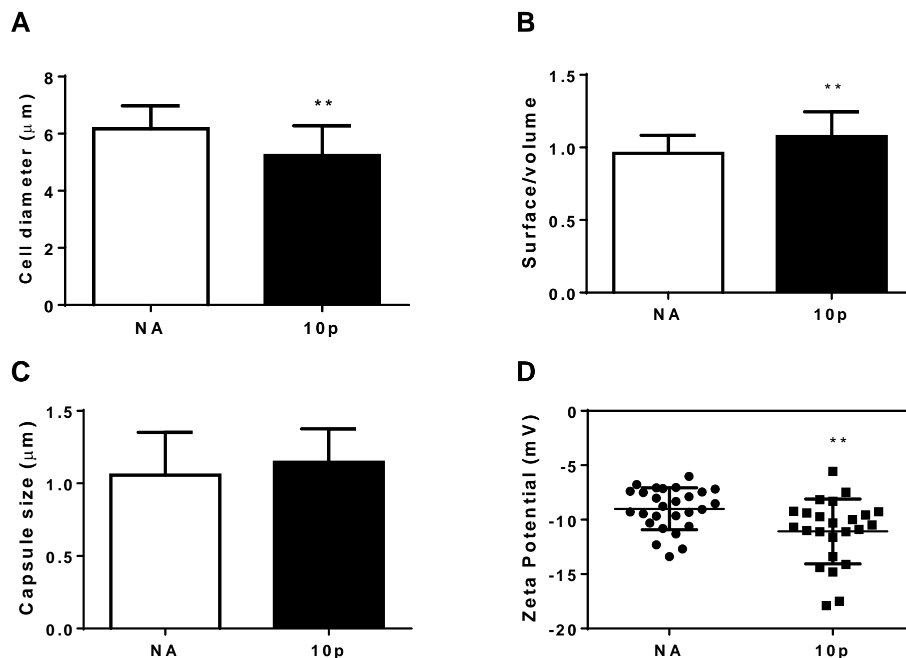

Figure 2. Morphometric and Zeta potential analysis of non-adapted (NA) and adapted cells subcultivated in medium without agrochemical (10p culture). The 10p cells are significantly ($p<0.05$) smaller (A) and have a higher S/V ratio (B) than the NA cells, but there is not significant ($p>0.05$) difference in capsule size (C). The cellular surface (D) of the 10p cells is also more electronegative than in the NA ones. ** $p<0.01$.

Interation between BMDMs and non-adapted and 10p cells of *C. gattii* R265

C. gatiii R265 10p cells were significantly ($p<0.05$) less phagocytosed than non-adapted cells (Figures 3A), but there were not significant difference ($p>0.05$) in the number of viable yeasts recovered from BMDMs (Figure 3B). Further, the 10p cells induced more ROS and PRN production ($p<0.05$) by the BMDMs than the NA ones (Figures 3C and D).

We also determined the MIC^{fluid} for the colonies recovered from the BMDMs. The figure 3E shows that the 10p cells were more tolerant to pyraclostrobin, fluconazole, itraconazole and ravuconazole, but not amphotericin B, than non-adapted cells. The same result, regarding the tolerance to the drugs, was obtained when we increased the time of interaction between the yeasts and the BMDMs for 24h (Data not shown).

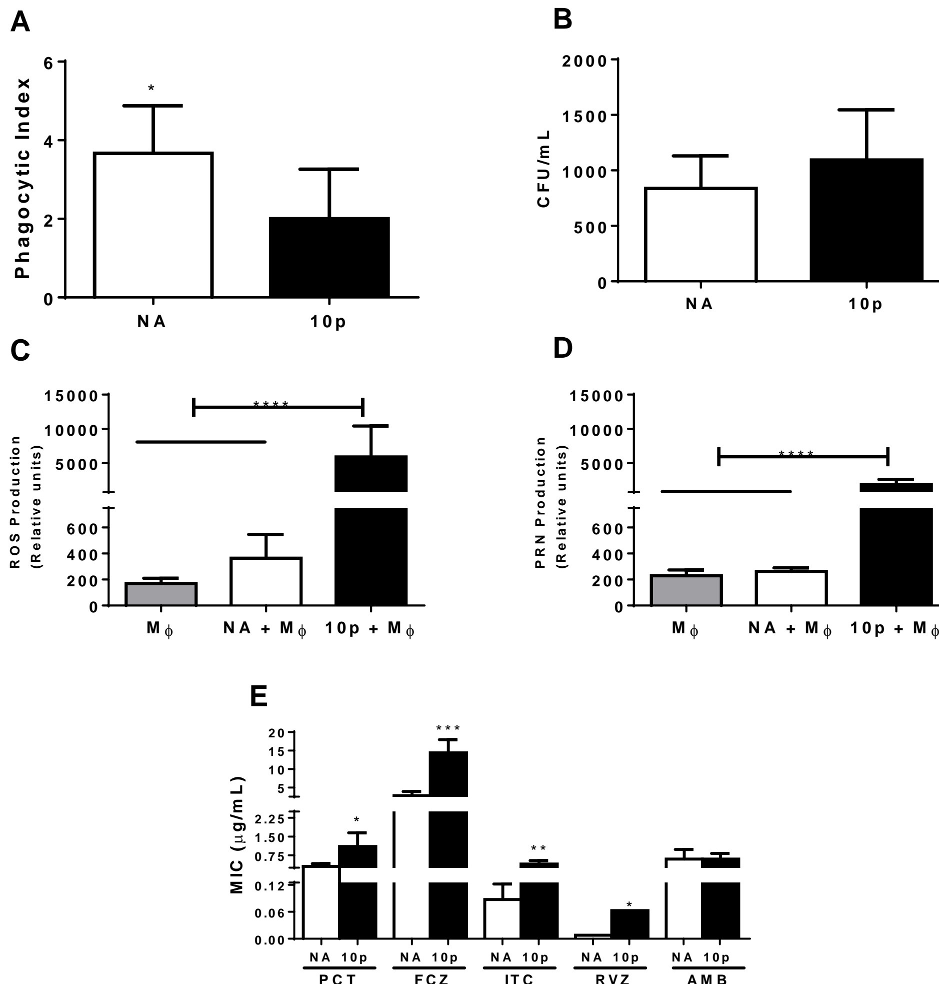

Figure 3: Phagocytosis assay. Non-adapted (NA) cells from *C. gattii* R265 were significantly ($p<0.05$) more phagocytosed (A) than adapted cells subcultivated in medium without agrochemical (10p). There were no significant difference in the killing assay (B) between NA

and 10p groups. 10p cells induced more ROS (C) and PRN (D) production by BMDMs and maintained significantvely ($p<0.05$) more tolerant (E) to pyraclostrobin (PCT), fluconazole (FCZ), itraconazole (ITC), ravuconazole (RVZ), but not to amphotericin B (AMB) than NA cells after recovered from BMDMs. * $p<0.05$; ** $p<0.01$; ***$p<0.001$, ****$p<0.0001$.

C. gattii R265 10p cells are less virulent and more tolerant *in vivo* to fluconazole than NA cells

We tested the virulence of NA and 10p cells of *C. gattii* R265 in mice. The 10p cells were significantly ($p<0.05$) less virulent than the NA cells, since 20% of the mice infected with the 10p cells were still alive 60 days post-infection (d.p.i), while all animals infected with the NA ones died (Figure 4).

When the infected mice were treated with fluconazole, all animals infected with NA yeasts remained alive 60 d.p.i, while only 50% of the ones infected with 10p cells survived (Figure 4). There was not significant difference between the groups infected with 10p cells treated and not treated with fluconazole, but the same did not happen between the groups infected with NA cells (Figure 4).

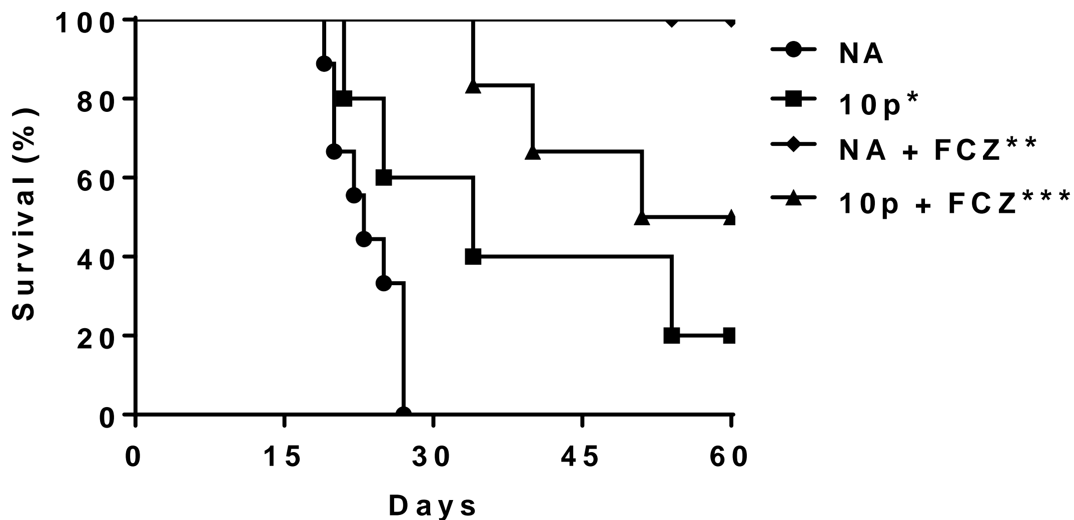

Figure 4: Virulence and Cross-resistance *in vivo* in non-adapted (NA) and adapted cells subcultivated in medium without agrochemical (10p culture). The 10p cells were significantly ($p<0.05$) less virulent than the NA cells. The treatment with fluconazole (FCZ) significantly ($p<0.05$) increased the survival of the mice infected with NA cells, but not of those infected with 10p cells ($p>0.05$). * $p<0.05$; ** $p<0.01$ compared to the NA group.

***C. gattii* R265 10p cells transcriptomic profile is different from NA cells**.

To investigate the permanent changes caused by pyraclostrobin exposure, we compared the RNA expression in NA and 10p cells of *C. gattii* R265 grown in medium without the agrochemical at 30°C. Three hundred fifty four genes were differentially expressed, being 151 genes up- and 203 genes down-regulared, when comparing 10p with NA cells. Then, we looked for the putative function of the genes and we found that, despite the fact that most of the genes encode hypothetical proteins, the genes related to the oxidation-reduction process, transportation (amino acid, drugs, nucleobase and other), ribosome biogenesis, metabolic process and amino acid, protein and cell wall synthesis were more expressed in the 10p than in the NA cells (Figure 5 and Table S5). Other genes related to sugar, amino acid, DNA and fatty acids metabolism, ion uptake, drug transport and protein synthesis were less expressed in 10p cells (Figure 5 and Table S6). Regarding ion uptake, three putative iron uptake genes (*ferric-chelate reductase 1 -FRE1-*, *FRE7* and *CNBG_9038*, homology to *FRE4* in *C. neoformans*) and two genes enconding copper transporter (*solute carrier family 31 (copper transporter), member 1- CTR4-* and *CNBG_1601* homology to *CTR2* in *C. neoformans*) were down-regulated in 10p cells (Table S6).

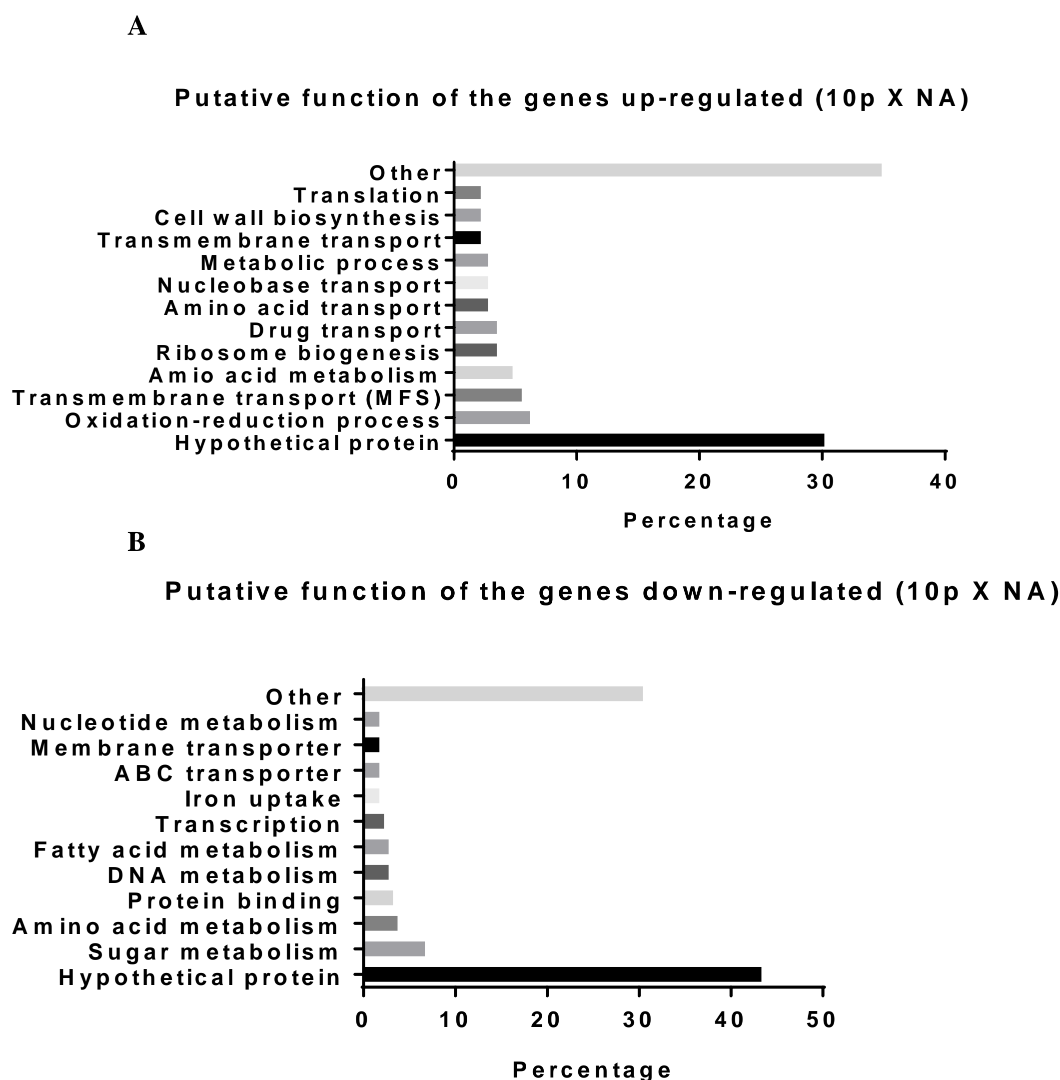

Figure 5: *C. gattii* R265 10p cells transcriptomic profile is different from that of NA cells. In comparison to the NA cells, in the 10p cells, 151 genes were up-regulated (A) and 203 were down-regulated (B).

Efflux pumps and genes encoding the pyraclostrobin target were up-regulated in 10p cells of *C. gattii* R265

We also searched in the transcriptome for genes that had already been described as being involved in antifungal resistance. We found that the genes for the two most studied and characterized efflux pumps in *Cryptococcus* spp., *ATP-binding cassette transporter* (*AFR1*) and *Putative ABC multidrug resistance transporter with similarity to Ste6* (*MDR11*), were more expressed (> 1.5 fold) in the 10p than in the NA cells (Table 8). Other possible efflux pump gene also up-regulated in the 10p cells was the *Pleiotropic drug resistance* gene, which has homology with *PDR16* (*CNAG_04984*) in

C. neoformans H99. In addition, gene encoding cytochrome b2 (*CNBG_4400*), the target for the agrochemical pyraclostrobin, was 2-fold more expressed in 10p cells (Table 8).

Although *AFR1*, *MDR1* and *PDR* were more expressed in the 10p cells, three other putative drug transporter genes were downregulated, *CNBG_4708* (1.6-fold), *CNBG_6088* (1.4-fold), *CNBG_9590* (1.5-fold) (Table S6).

Table 8. List of the genes up-regulated that could be involved in drug resistance in *C. gattii* R265 10p.

ORF	Genes Identification	Function	10p/NA Fold Change
CNBG_4400	*Cytochrome b2, mitochondrial* (*cytb*)	Respiration (target of Pyraclostrobin)	2.058
CNBG_1200	*ATP-binding cassette transporter* (*AFR1*)	Drug transport	1.566
CNBG_1138	*Putative abc multidrug resistance transporter with similarity to Ste6* (*MDR11*)	Drug transport	1.526
CNBG_4693	*Pleiotropic drug resistance* protein (*PDR*)	Drug transport	1.391

DISCUSSION

World population is growing fast, and it is estimated that in 2050 it will achieve 9.6 billion people, leading to an increasing necessity of food production. An alternative to food production is the use of agrochemicals to avoid losses of food due to plagues, like insects and microorganisms infections (Popp *et al.*, 2013). However, such substances may have harmful effects on human and animal health and the environment (Rigotto *et al.*, 2014). Recently, we showed that the environmental triazole, tebuconazole, causes temporary and permanent cross-resistance (CR) with clinical azoles in *C gattii* and *C. neoformans* (Bastos *et al.*, 2018). Nevertheless, it was unknown if other non-azoles agrochemicals could induce the same effect.

In this work, we showed that the agrochemical pyraclostrobin, which acts inhibiting the activity of cytochrome b and the electron transport chain in mitochondria,

is also able to select cells of *Cryptococcus* that are more tolerant to azole drugs (fluconazole, itraconazole and ravuconazole).

The rate of CR varied according to the temperature that the adaptation test was performed. When the test was carried out at 30°C, more strains of *C. gattii* and *C. neoformans* presented CR with fluconazole. In addition, the lower temperature influenced positively the concentration of pyraclostrobin supported on the medium by the fungi. Indeed, the temperature is an important factor that controls the growth of *Cryptococcus* and can be involved in various phenomena. It has been reported that *C. neoformans* can colonize birds, but normally without causing disease in these animals, due to the fact that the yeasts do not survive inside avian macrophages in the body temperature of the birds, 42°C, although they are able to grow at this elevated temperature (Johnston *et al*. 2017). The temperature also is important for generation of titan cells, hyphal growth, inheritance patterns of mitochondria, capsule size and virulence in general (Zaragoza and Casadevall, 2004; Bielska and May, 2015; Garcia-Rodas *et al*., 2014; Wang *et al*., 2015; Watkins *et al*., 2017). In this study, we added to this list the capacity of acquiring mechanisms related to the CR between agrochemicals and azoles clinical drugs. Besides, the incubation temperature in the MIC^{broth} tests was also relevant to the CR presented by the strains adapted at 30°C. While some strains presented CR with fluconazole independently of the incubation temperature, others showed increased tolerance only when the cells were incubated at 30°C. These results reinforce that even with increased tolerance in the environment at low temperatures, it may not manifest in animal infections due to mammal's body temperature (Bastos *et al*., 2018).

To investigate if the tolerance *in vitro* also manifests *in vivo*, we infected animals with NA and 10p cells of *C. gattii* R265 that presented CR with all azoles clinical tested, and then treated them or not with fluconazole. The results showed that the treatment with the drug did not change the survival of animals infected with the 10p cells, unlike what happened to the mice infected with the NA cells. The tolerance to the drugs was also presented by the 10p cells recovered from the BMDMs after 3 and 24h of interaction. In addition, the 10p cells induced more production of ROS and PRN by the BMDMs than the non-adapted cells, which indicates that these cells probably have a great system of detoxification, since there were not significant difference in recovery yeasts from BMDMs.

While the target of pyraclostrobin is the mitochondrial metabolism, azole drugs act in ergosterol synthesis. Then, the next question was why cells exposed to the non-

azole agrochemical became more tolerant to clinical azoles. In *Cryptococcus neoformans* the relationship between mitochondrial metabolism and multidrug resistance is less understood than in *S. cerevisiae* and *Candida* (Panepinto *et al*., 2010; Shingu-Vazquez e Traven, 2011). It has been reported that *C. neoformans*, when exposed to tetracycline, an antibiotic that interferes in the synthesis of bacterial and mitochondrial proteins, becomes more tolerant to fluconazole (Oliver *et al*., 2008). In addition, a mutant of *C. neoformans* (*Δvad1* - DEAD-box RNA helicase) with respiratory deficiency due to decreased expression of *TUF-4* (mitochondrial translation elongation factor) are more tolerant to fluconazole. Overexpression of *TUF-4* in *Δvad* cells, in its turn, not only restores "respiratory growth" (cells become able to grow in the presence of carbon sources, like the wild-type ones), as well as fluconazole tolerance (Panepinto *et al*., 2010). Thus, these data also indicate a relationship between mitochondrial metabolism deficiency and drug tolerance in *Cryptococcus* (Panepinto *et al*., 2010).

To try to understand why pyraclostrobin exposed cells become more tolerant to clinical azoles, we performed the transcriptome of NA and 10p cultures of *C. gattii* R265, which presented permanent CR with these drugs. The cells of 10p culture up-regulated the efflux pump genes *AFR1* and *MDR1*, which lead us to believe that, when these cells come into contact with antifungal drugs, they could pump these drugs out of the cell, which would justify the increase of azole tolerance in this culture, in comparison to the NA culture. In addition, the 10p cells up-regulated the *CNBG_4693* gene, which is homologous to the *PDR16* gene in *C. neoformans* H99, an efflux pump that is important for heteroresistance to fluconazole (Ngamskulrungroj *et al*., 2012). However, three genes (*CNBG_4708*, *CNBG_6088* and *CNBG_9590* (*pdr-5*)) that encode ABC transporters with putative drug pumping function were less expressed in the 10p cells than in the NA cells. Not all genes that codes efflux pumps appear to be important for fluconazole tolerance in *C. neoformans*. Ngamskulrungroj *et al*. (2012) and Ko *et al*. (2009) have shown that deletion of the genes *CNAG_07799* and *CNAG_05150* (Ngamskulrungroj *et al*., 2009) and *pdr5-2* and *pdr5-3* (Ko *et al*., 2009), which encode ABC-type transporters, does not alter the tolerance of *C. neoformans* H99 to fluconazole, whereas the deletion of *pdr-5* causes a slight decrease in tolerance (Ko *et al*., 2009). Thus, we speculate that genes encoding ABC transporters that are less expressed in 10p cells would not be important to the fluconazole tolerance process, because *CNBG_4708*, *CNBG_6088* and *CNBG_9590* are genes homologous to *CNAG_07799*, *pdr-5* and *pdr5-2* in *C. neoformans* H99, respectively.

It is still unknown whether pyraclostrobin can be pumped by the efflux pumps of *Cryptococcus*. The most common mechanism of resistance to pyraclostrobin in environmental fungi is mutation in the gene encoding the drug target *cytb* (cytochrome b) (YIN *et al*., 2012). In our work, 10p cells, which are more tolerant to pyraclostrobin, were able to express 2-fold more *cytb* than NA cells, which may be the cause of the less susceptible phenotype. It is important to note that both up-regulation of the efflux pumps and of the *cytb* genes were greater in the 10p culture cells than in the NA, in the absence of direct induction by pyraclostrobin, which makes us speculate that a mutation might have happen in the 10p cells. New studies are being done to determine the genomic changes in 10p cell in comparison to NA ones.

Although pyraclostrobin induced CR with fluconazole, metalaxyl, an agrochemical that acts in the RNA metabolism, did not. This result indicates that not always an agrochemical can induce an increase in tolerance to clinical drugs in *Cryptococcus* spp., which also is reported by Rocha and colleagues in *Candida glabrata* (Rocha *et al*., 2016).

Regarding morphology, the 10p cells of *C. gattii* R265 had a smaller cell diameter and a higher S/V ratio than the NA cells. Usually, smaller cells are more virulent (Ferreira *et al*., 2015; Bastos *et al*., 2018), so it was expected that 10p cells would be more virulent than NA, which did not occur. This result indicates that other mechanisms of virulence, besides morphological variation, could be responsible for the change in virulence in the 10p culture.

Cryptococcus spp. have several factors of virulence, being the classic ones: capsule production, ability to grow at 37°C, and production of enzymes, like phospholipase, urease, laccase, SOD, etc. (Bielska and May, 2015). Other important pathways of virulence have been studied in this genus, such as the ways of obtaining and using metals, like iron, copper and zinc. These ions act as cofactors of several enzymes and they are essential in processes like respiration. Because of the importance of these ions, microorganisms, including *Cryptococcus*, must be able to acquire them from the environment and from the host to ensure their growth (Silva *et al*., 2011).

In our work, transcriptome data demonstrated that five genes possibly involved in the uptake of iron and copper in the 10p culture were less expressed in the NA culture. They are: *FRE1*, *FRE7* and *CNBG_9038* (homologous to *FRE4* in *C. neoformans* H99), *CTR4* and *CNBG_1601* (homologous to *CTR2* in *C. neoformans* H99).

Saikia and colleagues (2014) studied the relationship between iron reductase and virulence in *C. neoformans* and found that *Δfre2* and *Δfre2Δfre4* mutants were less virulent than the wild-type strain. Hu and colleagues (2014) studied the transcriptome of *C. neoformans* strains trying to understand why certain strains, when they undergo successive passages in a murine infection model, become more virulent. They found that *FRE3* was more expressed in two strains that were more virulent than cells that were not passed successively in mice. The overexpression of *FRE3* in the two original environmental strains increased virulence whereas the silencing of this gene in *C. neoformans* H99 decreased virulence (Hu *et al.*, 2014). Regarding cooper uptake, a study showed that *Δctr4* is less virulent than the wild-type (Waterman *et al.*, 2012). Thus, our hypothesis is that the lower expression of the genes related to the obtention of iron and copper could explain, in parts, the decrease in virulence in the 10p culture in comparison to the NA one. Further studies need to be performed in order to better understand this relationship.

In conclusion, exposure to pyraclostrobin, but not to metalaxyl, selected cells with cross-resistance with clinical azole drugs both *in vivo* and *in vitro*, but not with amphotericin B. Pyraclostrobin exposure also altered fungal morphology and decreased the virulence of *C. gattii* R265 after the contact with the agrochemical ceased. To the best of our knowledge, this work is the first to demonstrate the permanent implications of the exposure to non-azole agrochemicals on the virulence and tolerance to drugs in fungi.

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Transparency declarations.

None to declare.

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SUPPLEMENTARY MATERIAL

Table S1. Screening of subpopulations of *C. gattii* and *C. neoformans* strains less susceptible to metalaxyl (MTX–adaptation).

Strain or parameter	MIC^{solid} (µg/mL)		MCA (µg/mL)		MCA/$SubMIC^{solid}$	
C. gattii	**30°C**	**35°C**	**30°C**	**35°C**	**30°C**	**35°C**
R265 (C)	4.0	0.5	40.0	0.25	20.0	1.0
ATCC 24065 (R)	1.0	0.5	5.0	0.25	2.0	1.0
ATCC 320608 (R)	1.0	0.25	1.0	0.25	2.0	2.0
547/OTTI/94–PI–10 (E)	2.0	1.0	256.0	0.5	256.0	1.0
ICB 181 (E)	1.0	0.25	0.5	0.25	1.0	2.0
L24/01 (C)	4.0	1.0	40.0	0.5	20.0	1.0
L27/01 (C)	2.0	1.0	5.0	0.5	5.0	1.0
L28/02 (C)	2.0	1.0	1.0	2.0	1.0	1.0
1913/ER (C)	4.0	4.0	4.5	2.0	2.25	1.0
196L/03	0.5	0.5	0.25	0.25	1.0	1.0
LMM 818 (C)	4.0	4.0	2.5	2.0	1.25	1.0
23/10893 (C)	4.0	4.0	2.5	2.0	1.25	1.0
29/10893 (C)	4.0	2.0	256.0	1.0	128.0	1.0
Range	1.0 – 4.0	0.25 – 2.0	1.0 – 256.0	0.25 – 2.0	1.0 – 256.0	1.0 – 2.0
Geometric mean	2.10	1.00	5.84	0.61	4.89	1.11
C. neoformans	**30°C**	**35°C**	**30°C**	**35°C**	**30°C**	**35°C**
H99 (C)	2.0	0.5	256.0	0.25	256.0	1.0
ATCC 24067 (R)	2.0	0.5	256.0	0.25	256.0	1.0
ATCC 28957 (R)	0.5	0.5	0.5	0.25	1.0	1.0
ATCC 62066 (R)	0.5	0.25	0.5	0.25	2.0	2.0
Range	0.5 – 2.0	0.25 – 0.5	0.5 – 256.0	0.25	1.0 – 256.0	1.0 – 2.0
Geometric mean	1.00	0.42	11.31	0.25	19.02	1.19

MIC^{solid}: Minimum Inhibitory Concentration in solid medium of metalaxyl before the adaptation process. MCA: Maximum Concentration Achieved of metalaxyl in the MTX–adaptation test. C. clinical strain; R. reference strain; E. environmental.

Table S2. MICs (µg/mL) of fluconazole and metalaxyl for non-MTX-adapted *C. gattii* colonies and *C. gattii* colonies subjected to MTX adaptation at 30°C[a].

Strain or parameter	Fluconazole[b]				Metalaxyl[c]			
	Temperature 30°C		Temperature 35°C		Temperature 30°C		Temperature 35°C	
	NA	A	NA	A	NA	A	NA	A
R265	8.0	16.0	8.0	8.0	0.5	1.0	0.25	0.25
ATCC 24065	4.0	4.0	4.0	4.0	0.25	0.25	0.25	0.25
ATCC 32608	16.0	32.0	8.0	8.0	0.5	0.5	0.25	0.25
547/OTTI/94–PI–10	16.0	32.0	8.0	8.0	0.5	0.5	0.25	0.5
ICB 181	16.0	16.0	8.0	8.0	0.25	0.25	0.06	0.125
L24/01	16.0	32.0	8.0	4.0	0.5	0.5	0.125	0.25
L27/01	16.0	8.0	32.0	16.0	0.5	1.0	0.125	0.25
L28/02	32.0	32.0	16.0	8.0	0.5	0.5	0.25	0.5
1913R	16.0	8.0	16.0	8.0	1.0	2.0	2.0	2.0
196L/03	16.0	16.0	16.0	16.0	0.5	0.5	0.5	0.5
LMM 818	16.0	16.0	16.0	8.0	1.0	2.0	2.0	2.0
23/10893	8.0	8.0	8.0	4.0	1.0	1.0	2.0	2.0
29/10933	8.0	4.0	4.0	2.0	2.0	4.0	2.0	2.0
MIC range	4.0 – 32.0	8.0 – 128.0	4.0 – 32.0	4.0 – 128.0	0.125 – 4.0	0.25 – 4.0	0.125 – 2.0	0.25 – 8.0
Geometric mean	12.92	13.60	9.90	6.82	0.59	0.76	0.40	0.53

a:Tests were performed at 30 and 35°C; b: MIC endpoint considering 50% of growth inhibition; c: MIC endpoint considering 100% of growth inhibition.

Table S3. MICs (µg/mL) of fluconazole and metalaxyl for non-MTX-adapted *C. neoformans* colonies and *C. neoformans* colonies subjected to MTX adaptation at 30°C[a].

Strain or parameter	Fluconazole[b]				Metalaxyl[c]			
	Temperature 30°C		Temperature 35°C		Temperature 30°C		Temperature 35°C	
	NA	A	NA	A	NA	A	NA	A
H99	16.0	16.0	8.0	4.0	1.0	2.0	0.5	0.5
ATCC 24067	16.0	8.0	4.0	8.0	2.0	4.0	0.5	1.0
ATCC 28957	4.0	4.0	2.0	2.0	0.5	0.25	0.25	0.25
ATCC 62066	4.0	8.0	4.0	4.0	0.25	0.5	0.25	0.25
MIC range	4.0 – 16.0	8.0 – 16.0	2.0 – 8.0	2.0 – 8.0	0.25 – 1.0	8.0 – 128.0	0.25 – 1.0	1.0 – 128.0
Geometric mean	8.0	8.0	4.0	4.0	0.7	1.0	0.35	0.42

a:Tests were performed at 30 and 35°C; b: MIC endpoint considering 50% of growth inhibition; c: MIC endpoint considering 100% of growth inhibition.

Table S4. MICs (µg/mL) of fluconazole and metalaxyl for non-MTX-adapted *C. gattii* and *C. neoformans* colonies and *C. gattii* and *C. neoformans* colonies subjected to MTX adaptation at 35°C[a].

Strain or parameter	**Fluconazole[b]**		**Metalaxyl[c]**	
C. gattii	**NA**	**A**	**NA**	**A**
R265	8.0	8.0	0.25	0.25
ATCC 24065	4.0	2.0	0.25	0.25
ATCC 32608	8.0	8.0	0.25	0.25
547/OTTI/94–PI–10	8.0	8.0	0.25	0.125
ICB 181	8.0	8.0	0.625	0.625
L24/01	8.0	4.0	0.125	0.125
L27/01	32.0	16.0	0.125	0.25
L28/02	16.0	8.0	0.25	0.125
1913R	16.0	8.0	2.0	1.0
196L/03	16.0	16.0	0.5	0.5
LMM 818	16.0	8.0	2.0	1.0
23/10893	8.0	4.0	2.0	1.0
29/10933	4.0	2.0	0.25	1.0
MIC range	4.0 – 32.0	4.0 – 16.0	0.125 – 2.0	0.06 – 1.0
Geometric mean	9.90	6.46	0.41	0.37
C. neoformans	**NA**	**A**	**NA**	**A**
H99	8.0	4.0	0.5	0.25
ATCC 24067	4.0	4.0	0.5	0.25
ATCC 28957	2.0	2.0	0.25	0.25
ATCC 62066	4.0	4.0	0.25	0.125
MIC range	2.0 – 8.0	1.0 – 4.0	0.25 – 1.0	0.125 – 0.25
Geometric mean	4.0	3.36	0.35	0.21

a:Tests were performed at 35°C; b: MIC endpoint considering 50% of growth inhibition; c: MIC endpoint considering 100% of growth inhibition.

Table S5. Genes up-regulated comparing 10p and NA cells from *C. gattii* R265.

ORF	Gene Identification	Function	10p/NA Fold Change
CNBG_0009	ATP-dependent RNA helicase HAS1	ATP binding	1.447
CNBG_0028	Pescadillo	Ribosome biogenesis	1.656
CNBG_0072	Hypothetical protein	Hypothetical protein	1.64
CNBG_0127	Ribosomal RNA methyltransferase Nop2	RNA metabolism	1.459
CNBG_0301	Sorbitol dehydrogenase	Oxidation-reduction process	3.714
CNBG_0302	Acetolactate synthase, mitochondrial	Amino acid biosynthesis	1.927
CNBG_0408	Peptidase	Proteolysis	1.363
CNBG_0417	Monooxygenase	Monooxygenase	1.897
CNBG_0520	Aldolase	Transmembrane transport MFS	1.548
CNBG_0523	Conserved hypothetical protein	Hypothetical protein	2.194
CNBG_0529	Hypothetical protein	Hypothetical protein	1.734
CNBG_0531	Adenosylmethionine decarboxylase	Spermine biosynthetic process	1.651
CNBG_0645	Neutral amino acid transporter	Amino acid transporter	1.908
CNBG_0649	Aldehyde dehydrogenase (NAD+)	Oxidation-reduction process	1.708
CNBG_0756	Hsp70-like protein	No function reported	1.359
CNBG_0798	Arginase	Amino acid metabolism	1.743
CNBG_0835	Hypothetical protein	Hypothetical protein	1.419
CNBG_0918	Hypothetical protein	Transmembrane transport MFS	1.802
CNBG_0953	Hypothetical protein	Hypothetical protein	1.615
CNBG_0954	Carboxylic acid transporter	Transmembrane transport	1.472
CNBG_0963	Periodic tryptophan protein 1	No function reported	1.459
CNBG_1020	Uracil permease	Nucleobase transport	2.144
CNBG_1022	Hypothetical protein	Hypothetical protein	1.579
CNBG_1042	Hypothetical protein	Hypothetical protein	1.331
CNBG_1063	RNA helicase	RNA metabolism	1.655
CNBG_1078	Drug transporter	Drug transporter	1.942
CNBG_1085	Hypothetical protein	Hypothetical protein	1.89
CNBG_1125	Anthranilate phosphoribosyltransferase	Tryptophan biosynthetic process	1.568
CNBG_1138	Putative abc multidrug resistance transporter with similarity to Ste6	Drug transporter	1.526
CNBG_1200	ATP-binding cassette transporter	Drug transporter	1.566
CNBG_1277	Putative plasma membrane fusion protein	No function reported	1.398
CNBG_1362	Hypothetical protein	Hypothetical protein	2.831
CNBG_1371	Amino acid transporter	Amino acid transporter	2.547
CNBG_1378	Hypothetical protein	Hypothetical protein	1.585
CNBG_1379	Membrane transporter	Transmembrane transport MFS	1.788
CNBG_1402	Nucleolar protein 53	No function reported	1.468
CNBG_1428	Conserved hypothetical protein	Metabolic process	1.366

CNBG_1433	Nucleolin	Nucleotide metabolism	1.404
CNBG_1437	Hypothetical protein	Hypothetical protein	2.948
CNBG_1618	Peptidyl-prolyl cis-trans isomerase D	Protein peptidyl-prolyl isomerization	1.427
CNBG_1640	Pyruvate carboxylase	Sugar metabolism	1.506
CNBG_1648	Pyrroline-5-carboxylate reductase	Amino acid metabolism	1.369
CNBG_1663	Importin beta-4 subunit	Intracellular transport	1.67
CNBG_1677	Hypothetical protein	Hypothetical protein	2.005
CNBG_1730	40S ribosomal protein S10	Ribosome biogenesis	1.382
CNBG_1816	Ketol-acid reductoisomerase, mitochondrial	Amino acid metabolism	1.571
CNBG_1822	Hypothetical protein	Hypothetical protein	1.394
CNBG_1868	Phosphatase	Phosphatase	1.602
CNBG_1897	Hypothetical protein	RNA metabolism	1.471
CNBG_1979	Hypothetical protein	Hypothetical protein	1.812
CNBG_2025	Hypothetical protein	Oxidation-reduction process	1.822
CNBG_2090	Transmembrane receptor	No function reported	1.416
CNBG_2144	Laccase	Melanization	1.671
CNBG_2152	Arginine-trna ligase	Translation	1.348
CNBG_2170	Hexose transporter	Sugar transporter	2.177
CNBG_2176	Endo alpha-1,4 polygalactosaminidase precursor	Catalytic activity	2.104
CNBG_2344	Sterol 3beta-glucosyltransferase	Sugar metabolism	1.402
CNBG_2360	Hypothetical protein	Hypothetical protein	3.409
CNBG_2387	Ribosomal protein S8.e	Translation	1.333
CNBG_2437	Rhomboid-like protein	Endopeptidase activity	1.337
CNBG_2518	Beta-glucosidase	Sugar metabolism	1.353
CNBG_2535	Tartrate dehydrogenase	Oxidation-reduction process	2.003
CNBG_2544	Hypothetical protein	Hypothetical protein	2.693
CNBG_2547	Glyceraldehyde-3-phosphate dehydrogenase, type I	Sugar metabolism	1.953
CNBG_2692	Mandelate racemase/muconate lactonizing enzyme	No function reported	1.502
CNBG_2696	Chaperone	Chaperone	1.762
CNBG_2742	Hypothetical protein	Hypothetical protein	1.471
CNBG_2810	Pyridoxal phosphate phosphatase phospho2	Phosphatase	1.595
CNBG_2823	Protein SDA1	No function reported	1.504
CNBG_2843	Hypothetical protein	Hypothetical protein	2.116
CNBG_2958	Hypothetical protein	Hypothetical protein	2.097
CNBG_2964	Two-component-like sensor kinase/putative phytochrome	RNA metabolism	1.431
CNBG_3007	Hypothetical protein	Hypothetical protein	1.906
CNBG_3064	GMP synthase [glutamine-hydrolyzing]	Nucleotide metabolism	1.443
CNBG_3091	Hypothetical protein	Hypothetical protein	1.952
CNBG_3220	MFS transporter	Transmembrane transport MFS	1.634
CNBG_3227	Cytosine permease	Nucleobase transport	1.944
CNBG_3297	Hypothetical protein	Hypothetical protein	1.57
CNBG_3304	Hypothetical protein	Hypothetical protein	1.429
CNBG_3331	Hypothetical protein	Hypothetical protein	2.191
CNBG_3433	Phosphate:H+ symporter	Phosphate transport	2.072

CNBG_3436	Dihydrodipicolinate synthetase	Metabolic process	1.673
CNBG_3503	Amidophosphoribosyltransferase	Nucleotide metabolism	1.368
CNBG_3551	Trna (guanine-N(1)-)-methyltransferase	Translation	1.366
CNBG_3699	Hypothetical protein	Hypothetical protein	1.741
CNBG_3841	Ribosome assembly protein RRB1	Ribosome biogenesis	1.521
CNBG_3883	Diploid state maintenance protein chpa	No function reported	1.409
CNBG_3937	Myo-inositol 2-dehydrogenase	Oxidation-reduction process	1.837
CNBG_4032	Hypothetical protein	Hypothetical protein	2.717
CNBG_4073	Transmembrane transporter Liz1p	Transmembrane transport MFS	1.667
CNBG_4173	Flavin-containing monooxygenase	No function reported	1.509
CNBG_4204	Hypothetical protein	Hypothetical protein	1.779
CNBG_4207	Hypothetical protein	N-acetyltransferase activity	1.939
CNBG_4219	Hypothetical protein	Hypothetical protein	1.551
CNBG_4235	Hypothetical protein	Transmembrane transport	3.608
CNBG_4278	Hypothetical protein	Hypothetical protein	1.579
CNBG_4326	Hypothetical protein	Hypothetical protein	2.042
CNBG_4400	Cytochrome b2, mitochondrial	Oxidation-reduction process	2.058
CNBG_4404	Pentatricopeptide repeat protein	No function reported	1.605
CNBG_4429	Asparagine synthase (glutamine-hydrolyzing)	Amino acid metabolism	1.718
CNBG_4470	Sugar transporter	Sugar transport	2.024
CNBG_4509	Hypothetical protein	Hypothetical protein	2.061
CNBG_4510	Hypothetical protein	Hypothetical protein	1.345
CNBG_4548	Carbonic anhydrase	Carbonate dehydratase activity	1.954
CNBG_4553	Glucan 1,3-beta-glucosidase	Cell wall biosynthesis	2.187
CNBG_4561	Hypothetical protein	Transmembrane transport MFS	2.946
CNBG_4620	Hypothetical protein	Hypothetical protein	2.017
CNBG_4637	Hypothetical protein	Hypothetical protein	2.203
CNBG_4647	Ferredoxin-NADP+ reductase	Oxidation-reduction process	1.328
CNBG_4693	Pleiotropic drug resistance protein	Drug transporter	1.391
CNBG_4695	Putative cytosine-purine permease	Nucleobase transport	1.684
CNBG_4737	Multidrug transporter	Drug transporter	1.582
CNBG_4742	Glycoprotein	Glycoprotein	2.057
CNBG_4785	Amino acid transporter	Amino acid transport	1.572
CNBG_4822	Carbamoyl-phosphate synthase subunit arginine-specific large	Nitrogen metabolism	1.569
CNBG_4882	Aconitate hydratase, mitochondrial	Metabolic process	1.346
CNBG_4946	Protein TIF31	No function reported	1.416
CNBG_4951	Uracil transporter furd	Nucleobase transport	2.142
CNBG_5083	Malate dehydrogenase (oxaloacetate-decarboxylating)(NADP+)	Malate metabolic proccess	2.774
CNBG_5092	Hypothetical protein	Hypothetical protein	1.525
CNBG_5099	Mitochondrion protein	Ubiquitin-like modifier activating enzyme activity	1.738
CNBG_5109	Hypothetical protein	Hypothetical protein	1.96

CNBG_5178	Hypothetical protein	Hypothetical protein	1.562
CNBG_5205	Hypothetical protein	Hypothetical protein	4.449
CNBG_5291	Hypothetical protein	Hypothetical protein	1.514
CNBG_5346	Nuclease	Endonuclease (mitochondrial)	1.563
CNBG_5357	Putative chitin synthase	Cell wall biosynthesis	1.676
CNBG_5383	Predicted protein	Oxidation-reduction process	2.339
CNBG_5408	Cytosolic large ribosomal subunit protein	Ribosome biogenesis	1.503
CNBG_5554	Hypothetical protein	Hypothetical protein	1.627
CNBG_5555	Membrane transporter	Transmembrane transport MFS	2.088
CNBG_5638	Ribosomal RNA-processing protein 9	Ribosome biogenesis	1.439
CNBG_5657	Deoxyhypusine synthase	Peptidyl-lysine modification to peptidyl-hypusine	1.464
CNBG_5682	Hypothetical protein	Hypothetical protein	1.55
CNBG_5742	Transposase subfamily	Oxidation-reduction process	1.823
CNBG_5757	Hypothetical protein	Hypothetical protein	1.93
CNBG_5758	Hypothetical protein	Hypothetical protein	1.678
CNBG_5818	Hypothetical protein	Hypothetical protein	1.88
CNBG_5832	Hypothetical protein	Hypothetical protein	2.134
CNBG_5863	Eukaryotic translation initiation factor 3 subunit B	Translation	1.362
CNBG_5977	Adenine phosphoribosyltransferase	Nucleoside metabolic process	1.804
CNBG_6026	Glucan 1,3-beta-glucosidase	Sugar metabolism	2.368
CNBG_6050	Urea transporter	Transmembrane transport	1.701
CNBG_6058	Hypothetical protein	Transmembrane transport MFS	2.986
CNBG_6120	Dethiobiotin synthase	Vitamin metabolism	1.341
CNBG_6142	Branched-chain-amino-acid transaminase	Amino acid biosynthesis	1.769
CNBG_9013	Efflux protein enct	Transmembrane transport MFS	1.817
CNBG_9057	Pyruvate dehydrogenase complex dihydrolipoamide acetyltransferase	Metabolic process	1.319
CNBG_9344	Chaperone dnaj	Chaperone	1.431
CNBG_9416	AAT family amino acid transporter	Amino acid transporter	1.721

Table S6. Genes down-regulated comparing 10p and NA cells from *C. gattii* R265

ORF	Gene Identification	Function	10p/NA Fold Change
CNBG_0031	CPA1 peptidyl-prolyl cis-trans isomerase	Thermotolerance	-1.40
CNBG_0069	Acyl-coa dehydrogenase	Fatty acid metabolism	-1.44
CNBG_0080	Hypothetical protein	Hypothetical protein	-1.70

CNBG_0082	Predicted protein	Hypothetical protein	-1.65
CNBG_0098	Hypothetical protein	Hypothetical protein	-1.70
CNBG_0116	Hypothetical protein	Hypothetical protein	-1.52
CNBG_0136	Hypothetical protein	Nucleic acid binding	-1.75
CNBG_0137	Hypothetical protein	Hypothetical protein	-1.42
CNBG_0147	Predicted protein	Hypothetical protein	-2.60
CNBG_0241	Hypothetical protein	Hypothetical protein	-1.35
CNBG_0242	Hypothetical conserved protein	Fatty acid metabolism	-1.91
CNBG_0286	Hypothetical protein	Hypothetical protein	-1.44
CNBG_0322	Saccharopine dehydrogenase	Amino acid metabolism	-1.73
CNBG_0348	Cystinosin	Amino acid metabolism	-1.63
CNBG_0400	Glycosyltransferase	Sugar metabolism	-1.32
CNBG_0463	Hypothetical protein	Hypothetical protein	-1.57
CNBG_0480	Protein PNS1	Membrane transporter	-1.29
CNBG_0560	CTR4 solute carrier family 31 (copper transporter). member 1 CTR4	Copper transport	-2.45
CNBG_0561	Hypothetical protein	Hypothetical protein	-1.41
CNBG_0575	Hypothetical protein	Hypothetical protein	-1.37
CNBG_0585	Glutaredoxin	Cell redox homeostasis	-1.48
CNBG_0611	Hypothetical protein	Hypothetical protein	-1.50
CNBG_0824	Hypothetical protein MATE family multidrug resistance protein	Drug transmembrane transport	-1.74
CNBG_0905	Mannose-6-phosphate isomerase	Sugar metabolism	-1.47
CNBG_0961	DNA clamp loader	DNA metabolism (replication)	-1.39
CNBG_0970	Stearoyl-coa desaturase (delta-9 desaturase)	Lipid metabolism	-1.47
CNBG_1030	Glycogen(starch) synthase	Sugar metabolism	-1.40
CNBG_1057	Hypothetical protein	Hypothetical protein	-1.70
CNBG_1069	Predicted protein	Hypothetical protein	-1.46
CNBG_1093	Predicted protein	Hypothetical protein	-2.05
CNBG_1099	Hypothetical protein	Transcription	-1.40
CNBG_1110	Pathway-specific nitrogen regulator	Transcription	-1.62
CNBG_1196	Succinate-semialdehyde dehydrogenase	Oxidation-reduction process	-1.72
CNBG_1217	Hypothetical protein	Hypothetical protein	-2.10
CNBG_1233	Alpha-mannosidase	Sugar metabolism	-1.30
CNBG_1248	Hypothetical protein	Transferase	-1.53
CNBG_1251	Hypothetical protein	Hypothetical protein	-1.81
CNBG_1340	Hypothetical protein	Hypothetical protein	-1.41
CNBG_1342	DNA ligase 1	DNA metabolism (replication)	-1.35
CNBG_1380	Conserved hypothetical protein	Transferase	-1.67
CNBG_1383	Hypothetical protein	Hypothetical protein	-1.39
CNBG_1386	Potassium/sodium efflux P-type atpase. fungal-type	Membrane potential	-1.36
CNBG_1440	Hypothetical protein	Hypothetical protein	-1.83
CNBG_1465	Predicted protein	Hypothetical protein	-1.72
CNBG_1466	Hypothetical protein	Membrane transporter	-1.47
CNBG_1486	Vacuolar sorting protein VPS24	Protein transport	-1.36
CNBG_1499	Glyoxal oxidase	Lignin oxidative cleavage	-1.57

CNBG_1601	Hypothetical putative copper ion transporter CTR2	Cooper transport	-1.49
CNBG_1674	Predicted protein	Hypothetical protein	-1.60
CNBG_1676	Conserved hypothetical protein	Sugar transport	-3.35
CNBG_1679	Hypothetical protein	Hypothetical protein	-1.51
CNBG_1689	Predicted protein	Hypothetical protein	-1.91
CNBG_1741	Glycoside hydrolase family 5 protein	Sugar metabolism	-1.31
CNBG_1839	TPM 1 tropomyosin	Cytoskeletal arrangement	-1.50
CNBG_1854	Hypothetical protein	Signal transduction	-1.71
CNBG_1876	Hypothetical protein	Hypothetical protein	-1.33
CNBG_1904	Hypothetical protein	Sugar metabolism	-1.95
CNBG_1943	Hypothetical protein	Hypothetical protein	-1.63
CNBG_1990	Protein serine/threonine kinase	Transferase	-1.60
CNBG_2002	Carnitine O-acetyltransferase	Fatty acid metabolism	-1.39
CNBG_2031	Hypothetical protein	Phosphatase	-1.91
CNBG_2054	Opsonin-1	Membrane component	-1.73
CNBG_2056	Hypothetical protein	Hypothetical protein	-1.57
CNBG_2059	Aspartate-trna(Asn) ligase	Protein synthesis	-1.40
CNBG_2110	Hypothetical protein	Hypothetical protein	-1.56
CNBG_2119	Hypothetical protein	Protein binding	-1.65
CNBG_2134	Hypothetical protein	Catalytic activity	-2.10
CNBG_2140	Glucan endo-1.3-alpha-glucosidase agn1	Sugar metabolism	-1.94
CNBG_2200	Hypothetical protein	Hypothetical protein	-1.48
CNBG_2210	Hypothetical protein	Hypothetical protein	-1.38
CNBG_2215	DNA supercoiling protein	DNA metabolism	-1.45
CNBG_2284	RDI 1 rho gdp-dissociation inhibitor	Cytoskeletal arrangement	-1.36
CNBG_2304	Hypothetical protein	Protein binding	-1.82
CNBG_2328	Hypothetical protein	Membrane component	-1.50
CNBG_2358	Lactamase	No function reported	-1.42
CNBG_2438	Alpha-amylase	Sugar metabolism	-1.53
CNBG_2441	Hypothetical protein	Response to stress	-1.57
CNBG_2469	Hypothetical protein	Hypothetical protein	-1.77
CNBG_2539	Enoyl-coa hydratase	Fatty acid metabolism	-1.46
CNBG_2586	Hypothetical protein	Hypothetical protein	-1.32
CNBG_2604	Predicted protein	DNA repair	-3.00
CNBG_2627	FRE1 ferric-chelate reductase	Iron uptake	-1.62
CNBG_2659	Hypothetical protein	Oxidation-reduction process	-1.75
CNBG_2662	Thioredoxin-like protein 4A	RNA metabolism	-1.67
CNBG_2690	Hypothetical protein	Cell wall component	5.76
CNBG_2693	Specific RNA polymerase II transcription factor	Transcription	-1.30
CNBG_2730	Hypothetical protein	Hypothetical protein	-1.81
CNBG_2760	Hypothetical protein	Electron transport chain	-1.39
CNBG_2780	Peptidase	Peptidase	-1.40
CNBG_2809	Hypothetical protein	Hypothetical protein	-1.91
CNBG_2835	Voltage-gated potassium channel beta-2 subunit	Potassium channel	-1.71
CNBG_2930	Hypothetical protein	Hypothetical protein	-1.56
CNBG_2946	Protein of unknown function	No function reported	-1.33
CNBG_2959	Hypothetical protein	Hypothetical protein	-1.38

CNBG_2983	Oligopeptide transporter	Oligopeptide transporter	-1.55
CNBG_3004	Predicted protein	Hypothetical protein	-1.96
CNBG_3094	Hypothetical protein	Hypothetical protein	-1.69
CNBG_3267	Hypothetical protein	Protein binding	-1.28
CNBG_3290	Hypothetical protein	Hypothetical protein	-1.44
CNBG_3374	Heparinase II/III family protein	Heparinase	-1.39
CNBG_3395	Monosaccharide transporter	Sugar transport	-1.79
CNBG_3416	Hypothetical protein	Hypothetical protein	-1.59
CNBG_3432	Hypothetical protein	Hypothetical protein	-1.77
CNBG_3456	TRX1 thioredoxin	Cell redox homeostasis	-1.49
CNBG_3480	Ubiquitin-like protein Nedd8	Proteolisis	-1.57
CNBG_3489	30S small subunit ribosomal protein S17	Ribosome biogenesis	-1.42
CNBG_3526	Hypothetical protein	Hypothetical protein	-1.50
CNBG_3530	Cytidine deaminase	Nucleotide metabolism	-1.43
CNBG_3586	Cytoplasmic protein	No function reported	-1.32
CNBG_3643	Hypothetical protein	Hypothetical protein	-1.31
CNBG_3648	Hypothetical protein	Hypothetical protein	-2.13
CNBG_3660	Long-chain-fatty-acid-coa ligase	Fatty acid metabolism	-1.40
CNBG_3681	Hypothetical protein	Hypothetical protein	-1.32
CNBG_3684	Hypothetical protein	Protein binding	-1.60
CNBG_3690	Hypothetical protein	Hypothetical protein	-1.55
CNBG_3711	Hypothetical protein (DNA ligase D. 3'-phosphoesterase domain-containing protein)	DNA metabolism	-1.49
CNBG_3733	Beta-fructofuranosidase	Sugar metabolism	-1.57
CNBG_3762	Membrane transporter	Membrane transporter	-1.48
CNBG_3775	Acetyl/propionyl coa carboxylase	Amino acid metabolism	-1.41
CNBG_3846	Glycine dehydrogenase	Amino acid metabolism	-1.74
CNBG_3851	Plasma membrane proteolipid 3	Abiotic stress response	-1.76
CNBG_3854	Hypothetical protein	Hypothetical protein	-1.75
CNBG_3855	Hydrolase	Hydrolase	-1.43
CNBG_3861	Hypothetical protein	mRNA metabolism	-1.40
CNBG_4048	Uracil phosphoribosyltransferase	Nucleotide metabolism	-1.45
CNBG_4117	reran-processing protein CGR1	Ribosome biogenesis	-1.37
CNBG_4148	Response regulator receiver protein	Two-component regulatory	-1.68
CNBG_4168	Phosphoric monoester hydrolase	Phosphatase	-1.29
CNBG_4274	Hypothetical protein	Hypothetical protein	-1.59
CNBG_4383	Hypothetical protein	Hypothetical protein	-1.32
CNBG_4516	Hypothetical protein	Protein binding	-1.44
CNBG_4527	Hypothetical protein	Hypothetical protein	-1.47
CNBG_4651	Succinyl-coa:3-ketoacid-coenzyme A transferase	Ketone body catabolic process	-1.35
CNBG_4708	ABC transporter	Abc transporter	-1.64
CNBG_4763	Aspartate transaminase	Amino acid metabolism	-1.30
CNBG_4764	Aryl-alcohol dehydrogenase	Oxidoreductases	-1.76
CNBG_4769	Allantoin permease	Allantoin transport	-2.03
CNBG_4788	Cytoplasmic protein	Oxidoreductases	-1.52
CNBG_4808	Glucosamine-6-phosphate isomerase	Sugar metabolism	-1.88
CNBG_4812	Mitochondrial ADP	Mitochondrial transporter	-1.90
CNBG_4814	Hypothetical protein	Hypothetical protein	-1.45

CNBG_4819	Hypothetical protein	Hypothetical protein	-1.45
CNBG_4911	Hypothetical protein	Hypothetical protein	-1.55
CNBG_4922	Conserved hypothetical protein	Hypothetical protein	-1.97
CNBG_4938	Glutathione-S-transferase	Xenobiotic metabolism	-1.77
CNBG_4968	Uridine permease	Nucleotide transporter	-2.14
CNBG_4973	Hypothetical protein	Hypothetical protein	-1.40
CNBG_5036	Claudin family protein	Hypothetical protein	-1.75
CNBG_5100	Hypothetical protein	Cofactor binding	-1.70
CNBG_5144	Hypothetical protein	Hypothetical protein	-1.74
CNBG_5145	Hypothetical protein	Hypothetical protein	-2.01
CNBG_5147	Hypothetical protein	Hypothetical protein	-2.86
CNBG_5193	Hypothetical protein	Hypothetical protein	-1.51
CNBG_5209	Predicted protein	Hypothetical protein	-1.43
CNBG_5267	Hypothetical protein	Hypothetical protein	-1.85
CNBG_5273	Hypothetical protein	Zinc ion binding	-1.50
CNBG_5278	Hypothetical protein	Hypothetical protein	-1.59
CNBG_5299	Hypothetical protein	Hypothetical protein	-1.38
CNBG_5314	Hypothetical protein	Protein binding	-1.80
CNBG_5365	Glucan 1.3-beta-glucosidase	Sugar metabolism	-3.07
CNBG_5392	Predicted protein	Hypothetical protein	-1.97
CNBG_5414	Endoribonuclease L-PSP	Deaminase activity	-1.58
CNBG_5509	Hypothetical protein	Hypothetical protein	-1.39
CNBG_5582	Hypothetical protein	Protein ubiquitination	-1.31
CNBG_5588	Hypothetical protein	Hypothetical protein	-1.61
CNBG_5726	Isocitrate lyase	Sugar metabolism	-1.98
CNBG_5733	Nipsnap family protein	Vesicular transport	-1.42
CNBG_5789	Glutamate decarboxylase	Amino acid metabolism	-1.68
CNBG_5828	Hypothetical protein	Hypothetical protein	-1.59
CNBG_5853	Hypothetical protein	Hypothetical protein	-1.48
CNBG_6010	Mannose-6-phosphate isomerase class I	Sugar metabolism	-1.46
CNBG_6052	Hypothetical protein	Hypothetical protein	-1.71
CNBG_6082	FRE7 ferric-chelate reductase	Iron uptake	-1.92
CNBG_6087	Hypothetical protein	Transcription	-1.38
CNBG_6088	ATP-binding cassette transporter	Abc transporter	-1.46
CNBG_6139	Hypothetical protein	Fatty acid biosynthesis	-1.96
CNBG_6175	Hypothetical protein	Hypothetical protein	-1.46
CNBG_9019	Hypothetical protein	Peptide processing	-1.55
CNBG_9037	Predicted protein	Hypothetical protein	-1.64
CNBG_9038	Ferric-chelate reductase	Iron uptake	-1.62
CNBG_9101	Hypothetical protein	Nucleotide metabolism	-1.53
CNBG_9112	Transmembrane receptor	Transmembrane receptor	-1.52
CNBG_9177	Hypothetical protein	Hypothetical protein	-1.48
CNBG_9282	Hypothetical protein	Hypothetical protein	-1.66
CNBG_9347	Hypothetical protein	Hypothetical protein	-1.81
CNBG_9348	Hypothetical protein	Hypothetical protein	-1.77
CNBG_9349	DASH complex subunit DAD4	Chromosome segregation	-1.55
CNBG_9362	Glycine cleavage system T protein	Amino acid metabolism	-1.29
CNBG_9365	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 7	Respiration	-1.35
CNBG_9372	Hypothetical protein	Hypothetical protein	-3.51

CNBG_9385	Quorum sensing-like molecule	Quorum sensing	-1.47
CNBG_9418	Hypothetical protein	Hypothetical protein	-1.64
CNBG_9424	L-serine ammonia-lyase	Amino acid metabolism	-2.96
CNBG_9428	Hypothetical protein	Hypothetical protein	-1.54
CNBG_9506	Hypothetical protein	Hypothetical protein	-2.49
CNBG_9525	Hypothetical protein	Spermine transporter	-1.58
CNBG_9590	ATP-binding cassette. subfamily G (WHITE). member 2	Abc transporter	-1.53
CNBG_9605	Hypothetical protein	Hypothetical protein	-1.44
CNBG_9636	Hypothetical protein	Hypothetical protein	-1.66
CNBG_9637	Hypothetical protein	Fatty acid metabolism	-1.71
CNBG_9638	Hypothetical protein	Metabolic process	-1.54
CNBG_9654	Membrane protein	Membrane protein	-1.35

5 - RESUMO DOS RESULTADOS

Capítulo I. Nesse trabalho avaliou-se os efeitos da exposição de linhagens de *Cryptococcus gattii* e *C. neoformans* ao antifúngico ambiental tebuconazol. Nossos dados mostraram que essa exposição pode causar resistência cruzada temporária ou permanente com antifúngicos clínicos, principalmente com o fluconazol, e que esse processo é influenciado pela temperatura. Além disso, nós mostramos que a exposição ao tebuconazol alterou a morfologia das células e as tornou menos virulentas em modelo experimental murino, embora a tolerabilidade ao fluconazol tenha sido mantida *in vivo*. Por fim, nós mostramos que o aumento da tolerância aos antifúngicos provavelmente ocorreu pois a exposição ao triazol ambiental induz aumento da expressão dos genes classicamente envolvidos no processo de resistência antifúngica: *ERG11*, *MDR11* e *PDR1* em *Cryptococcus* spp.

Capítulo II- Nesse trabalhou avaliou-se os efeitos de dois antifúngicos ambientais não-azólicos, piraclostrobina (PCT) e metalaxil (MTX). Apenas PCT induziu resistência cruzada com azólicos clínicos em *C. gattii* e *C. neoformans*. A temperatura de 30°C foi um importante fator na indução de tolerância, já que quando os testes foram feitos a 35°C menos linhagens apresentaram resistência cruzada. A linhagem *C. gattii* R265 apresentou resistência cruzada permanente a todos os azólicos testados (fluconazol, itraconazol e ravuconazol). Por isso, a cultura 10p dessa linhagem foi escolhida para mais testes. Ao se comparar a morfologia da cultura não-adaptada e 10p, observou-se que o diâmetro da célula 10p diminuiu e a razão S/V aumentou, assim como a superfície dessas células se tornou mais eletronegativa comparada a cultura NA. Em relação aos resultados em camundongos, 10p se mostrou menos virulenta, mas apresentou tolerância ao fluconazol *in vivo* e a todos os azólicos testados após ser recuperada de macrófagos desafiados com as leveduras. Dados do transcriptoma mostraram que a maior tolerância da cultura 10p pode ser atribuída a maior expressão de bombas de efluxo e do gene que codifica o alvo da PCT. Além disso, constatou-se que genes envolvidos na captação de cobre e ferro, que podem estar envolvidos na virulência, foram menos expressos em 10p. Por fim, a linhagem *C. gattii* 196L/03 apresentou formação de pseudohifas quando em contato com PCT.

6 - DISCUSSÃO

Tebuconazol (TBZ), piraclostrobina (PCT) e metalaxil (MTX) são antifúngicos ambientais sistêmicos utilizados pra tratar ou prevenir doenças principalmente na fruticultura (Kwork *et al.*, 1993; Bartlett *et al.*, 2002; Strickland *et al.*, 2004). No entanto, também sugere a utilização de TBZ e PCT para doenças que atacam eucaliptos, como é o caso da ferrugem (*Puccinia psidii*) (Zauza *et al.*, 2008). Nesse trabalho, nós mostramos que *C. gattii* e *C. neoformans*, que são patógenos humano encontrados associados a vegetais, também são inibidos por esses esses antifúngicos, tanto a 30 como a 35°C.

A adaptação a TBZ, PCT e MTX selecionou colônias de várias linhagens das duas espécies com capacidade de crescerem em meio sólido com altas concentrações desses agroquímicos. Algumas linhagens foram capazes de crescer em meio com até 256 µg/mL de PCT ou MTX, a maior concentração testada nesse estudo. No entanto, *Cryptococcus* não parece suportar concentrações maiores que 16 µg/mL de TBZ. Isso indica que o prévio contato com os agroquímicos em concentrações sub-inibitórias é capaz de selecionar subpopulações de *Cryptococcus* capazes de suportarem e crescerem na presença de concentrações maiores dessas drogas. O prévio e constante contato com concentrações sub-inibitórias de antifúngicos ambientais também permite a *Colletotrichum graminicola, Candida parapsilosis* e *Aspergillus fumigatus* suportarem concentrações elevadas de antimicrobianos ambientais (Serfling *et al.*, 2007; Faría-Ramos *et al.*, 2014; Rocha *et al.*, 2016). É interessante observar, também, a importância da temperatura no processo de adaptação. A temperatura de 30°C possibilitou um número maior de linhagens a crescerem em maiores concentrações dos agroquímicos.

Apesar de serem capazes de crescer na presença de maiores concentrações de TBZ, PCT e MTX por meio da exposição contínua e gradual, nem todas as colônias adaptadas tiveram seu valor de CIM aumentado, no teste em meio líquido, em pelo menos 2 diluições, comparado as colônias não adaptadas (NA). Isso indica que o tipo de meio pode influenciar a tolerância do micro-organismo às drogas. Tal fenômeno também foi observado por Carneiro (2015) com linhagens de *C. gattii* adaptadas ao fungicida benomil. Algumas culturas adaptadas não tinham aumento no valor da CIM após a exposição ao fungicida, mesmo sendo capazes de crescerem, em meio sólido na presença do agroquímico (Carneiro, 2015).

Nossa hipótese inicial era que a exposição prévia a agroquímicos poderia selecionar populações mais tolerantes a essas drogas e que possuíam resistência cruzada

com antifúngicos clínicos. Quando os testes foram feitos com TBZ e PCT, observou-se que um número considerado de linhagens apresentou resistência cruzada com fluconazol (FLC) e com outros azólicos, como o itraconazol (ITC) e ravuconazol (RVZ). No entanto, em MTX, nós não observamos resistência cruzada com o FLC. Esse resultado é intrigante uma vez que um dos motivos que o MTX foi escolhido para esse trabalho foi o fato de ser comprovado que ele causa resistência cruzada em oomicetos (FRAC 2016), mas, tudo indica que em *C. gattii* e em *C. neoformans*, não há resistência cruzada entre MTX e os antifúngicos clínicos testados.

É possível inferir que alguns mecanismos de resistência aos azóis ambientais poderiam funcionar também contra os azóis clínicos (Rocha *et al.*, 2016), o que justificaria a resistência cruzada entre TBZ e FLC, ITC e RVZ. Um resultado inesperado foi que as culturas adaptadas a TBZ e PCT que apresentaram resistência cruzada com FLC, também apresentaram, na maioria das vezes, com RVZ, mas não com ITC, salvo algumas exceções. A resistência cruzada entre FLC e ITC não é comum em *C. neoformans*, mesmo ambos sendo azóis (Trpković *et al.*, 2013; Espinel-Ingroff *et al.*, 2012). Postula-se que isso ocorra porque ITC, além de inibir ERG11p, inibe, também, 3-cetoesteroide redutase NADH-dependente (enzima que catalisa uma das últimas reações da síntese do ergosterol) (Vanden Bossche *et al.*, 1993; Espinel-Ingroff *et al.*, 2012) e induz a produção de ROS (Ferreira *et al.*, 2013). Além disso, sugere-se que a estrutura química do FLC é mais semelhante a estrutura do TBZ do que a estrutura química do ITZ, o que também justificaria a diferença na indução de resistência cruzada (Ren *et al.*, 2017). Por fim, diferenças nos mecanismos de resistência entre FLC e ITC poderiam explicar a baixa resistência cruzada ao ITC. Sionov e colaboradores (2012) mostraram que linhagens resistentes ao FLC, devido à mutação no gene *ERG11*, também apresentaram resistência ao voriconazol (VRC), mas eram menos tolerantes ao ITC (Sionov *et al.*, 2012). De forma semelhante, Ren e colaboradores (2017) reveleram que linhagens de *A. fumigatus* com mutação em *ERG11* são resistentes ao FLC e VRC, mas não ao ITC (Ren *et al.*, 2017).

Algumas linhagens que apresentaram resistência cruzada retornaram ao fenótipo original de tolerância ao FLC após serem cultivadas em meio sem o agroquímico, processo que nós denominamos de resistência cruzada temporária. Nós acreditamos que a diminuição da tolerância às drogas clínicas das culturas adaptadas seja devido à variabilidade fenotípica que os fungos possuem, especialmente *Cryptococcus*, e processos como *Splicing* alternativo e processos epigenéticos, como duplicação

cromossômica, poderiam estar envolvidos, assim como estão envolvidos nos processos de heterorresistência ao FLC (Sionov *et al.*, 2010).

Outras linhagens, ao serem adaptadas em TBZ e PCT, e, posteriormente, cultivadas na ausência do mesmo (cultura 10p), não retornaram ao fenótipo de tolerância inicial (resistência cruzada permanente). Nesse caso, nós postulamos que alterações genotípicas permanentes, como mutações, poderiam ter ocorrido ao decorrer da seleção ou ter acontecido a seleção de colônias que naturalmente já apresentavam alguma modificação genética que possibilita a alteração da tolerabilidade. Contudo, alterações epigenéticas não podem ser descartadas, já que em *C. neoformans* elas podem permanecer durante dezenas de repiques (Sionov *et al.*, 2010).

Santos e colaboradores (2014), usando metodologia semelhante de adaptação, demonstraram que o contato prévio com concentrações crescentes de FLC selecionou uma linhagem (*C. gattii* L27/01) mais tolerante a esse antifúngico. E, nesse caso, as alterações de tolerância, morfológicas e de virulência se mantinham mesmo após centenas de repiques em meio sem a droga (Santos *et al.*, 2014). Nosso trabalho é pioneiro em mostrar tanto a resistência cruzada permanente como a temporária entre agroquímicos e antifúngicos clínicos. Na literatura, os trabalhos que estudam esse assunto, focam apenas na alteração da tolerância/sensibilidade das culturas adaptadas e não adaptadas, não pesquisando o que acontece com o fungo após o contato com o agroquímico ter cessado (Serfling *et al.*, 2007; Faría-Ramos *et al.*, 2014; Rocha *et al.*, 2016; Ren *et al.*, 2017).

A importância da temperatura no processo de tolerância antifúngica fica clara, mais uma vez, quando se analisa a resistência cruzada. Nesse caso, nós observamos que a adaptação a 30°C possibilita um número maior de linhagens a sobreviverem a uma maior concentração dos antifúngicos clínicos e ambientais do que a adaptação a 35°C. Parte disso pode ser explicada pelo fato de que o processo de adaptação em si é também influenciado pela temperatura, como já discutido. Porém, fica visível o papel desse fator quando nós analisamos as colônias adaptadas a 30°C, mas submetidas a teste de CIM a 35°C. Nessa condição, foi observado menos alteração da CIM e, consequentemente, menos resistência cruzada. No geral, a temperatura provavelmente influencia todos esses fatores, pois *Cryptococcus*, assim como grande parte dos fungos ambientais, cresce melhor em temperaturas próximas a 25°C (Howard, 1961).

A menor tolerância aos antifúngicos na temperatura de 35°C indica que mesmo que esses processos de resistência cruzada ocorram no ambiente, não é certo que o fungo vai apresentar tolerância antifúngica também no hospedeiro e pode explicar, em

parte, o porquê que *Cryptococcus* não é considerado um grande problema em relação à resistência a antimicrobianos na prática clínica (Perfect *et al*., 2010; Chen *et al*., 2014).

Cryptococcus normalmente se torna mais tolerante aos azóis por três mecanismos: (i) aumento da expressão e (ii) mutação do gene *ERG11* e (iii) superexpressão de bombas de efluxo (Rodero *et al*., 2003; Sionov *et al*., 2010; Santos *et al*., 2014; Basso *et al*., 2015, Yang *et al*., 2016). Dessa forma, nós analisamos a expressão de *ERG11* e das bombas de efluxo *PDR11* e *MDR1* em culturas de *C. gattii* R265 e *C. neoformans* H99 expostas e não expostas a TBZ. Os resultados mostraram que *ERG11* foi mais expresso nas células adaptadas de ambas as espécies, comparado as células NA. No entanto, apenas em células adaptadas de *C. neoformans* H99 foi observado maior expressão dos genes para bombas de efluxo (*MDR1* e *PDR11*).

O mecanismo molecular de como os azóis induzem o aumento da expressão de bombas e de *ERG11* ainda não foi totalmente elucidado em *Cryptococcus* spp. O que se tem relatado é que células heterorresistentes de *C. neoformans* sofrem duplicações cromossômicas, principalmente em cromossomos que carregam os genes *ERG11* e *AFR1*. Coincidentemente, o nível desses genes nas células heterrosistentes duplica em relação as células "susceptíveis" (Sionov *et al*., 2010). Nesse trabalho, não foi determinado se o mesmo mecanismo seria responsável pelo aumento da expressão dos genes previamente descritos. Contudo, o DNA genômico das colônias de *C. gattii* R265 e *C. neoformans* H99 expostas e não expostas a TBZ foi sequenciado para se tentar observar, em trabalhos futuros, se alterações cromossômicas também estariam envolvidas no processo de tolerância a azóis.

A resistência cruzada entre azóis ambientais e clínicos em fungos patogênicos já tem sido relatada por outros trabalhos, especialmente em *A. fumigatus* (Snelders *et al*., 2012; Faria-Ramos *et al*., 2014; Ren *et al*., 2016). Nesse organismo, fica claro que a exposição a azóis ambientais leva a seleção de mutantes para *ERG11* (Ren *et al*., 2016). Porém, poucos estudos demonstram resistência cruzada entre antifúngicos ambientais não-azóis e azóis clínicos, e os mecanismos de indução de resistência por essas substâncias.

No nosso estudo, PCT também demonstrou resistência cruzada com FLC, ITC e RVZ mesmo tendo mecanismo de ação diferente dos azóis, agindo sobre o metabolismo mitocondrial. Brun e colaboradores (2003 e 2004) demonstraram que existe uma relação entre metabolismo mitocondrial e sensibilidade ao FLC ao observar que colônias de *Candida glabrata* resistentes ao FLC tinham disfunção respiratória e que os blastoconídios dos mutantes não apresentavam mitocôndrias (Brun *et al*., 2003).

A resistência ao azóis em *Candida* e em *S. cerevisiae* com disfunção mitocondrial pode ser justificada pelo aumento da expressão de bombas de efluxo (Brun *et al*., 2004; Shingu-Vazquez e Traven, 2011). O porquê fungos ativam as vias relacionadas a expressão dessas bombas (genes *Pdrs- pleiotropic drug resistance*) sob disfunção mitocondrial ainda é desconhecido, mas uma hipótese tem sido criada em estudos com *S. cerevisiae*. A hipótese que tenta explicar a conexão entre aumento de bombas de efluxo e metabolismo mitocondrial é complexa e envolve o metabolismo de lipídeos e a perda de DNA mitocondrial (Shingu-Vazquez e Traven, 2011).

Em *S. cerevisiae*, a expressão dos genes que condificam PDRs, envolvidos na multirresistência a drogas, é regulada pelos mesmos fatores que regulam a transcrição de genes envolvidos com o metabolismo de esfingolipídeos e fosfolipídeos de membrana. Além disso, as bombas PDRs na membrana plasmática são importantes no transporte de lipídeos da parte interna para a parte externa da membrana plasmática, contribuindo, assim, para a assimetria da membrana, fenômeno conhecido como flop de fosfolipídeos. Dessa forma, fica claro a interconexão entre metabolismo lipídico da membrana e síntese de bombas de efluxo (Shahi e Moye-Rowley, 2009; Paul e Moye-Rowley, 2014).

S. cerevisiae e *Candida glabrata*, ao contrário de *Candida albicans* e *C. neoformans*, podem sobreviver e replicar mesmo com a perda do DNA mitocondrial (mtDNA). Além disso, mutantes resistentes a drogas comumente perdem o mtDNA. A perda desse componente celular, contudo, causa disfunção mitocondrial nessas células. Várias subunidade do complexo respirarório presente na membrana interna da mitocôndria são codificados por genes presentes no mtDNA. Depois da perda do mtDNA, a correta montagem dessas subunidades na membrana interna da mitocôndria é comprometida. Além disso, mutantes que possuem problemas relacionados a montagem correta das proteínas da cadeia transportadora de elétrons possuem, também, alterações dos níveis e presença de lipídeos nas membranas mitocondriais. Postula-se que a disfunção mitocondrial causada pela perda de mtDNA causa alteração da posição de enzimas importantes para o metabolismo de lipídeos da membrana interna da mitocôndria, como a Pds1p, uma fosfatidilserina descarboxilase que converte fosfatidilserina em fosfatidiletanoamina. O acúmulo de Pds1p, por exemplo, no espaço intermembranoso seria capaz de ativar algum indutor desconhecido que, por sua vez, ativaria os fatores de transcrição Pdr1p e Pdr3p, responsáveis pela regulação dos genes *Pdrs* e de genes que condificam enzimas envolvidas no metabolismo de lipídeos. Dessa forma, a disfunção mitocondrial causada pela perda do mtDNA seria responsável pelo

aumento da resistência a drogas em *S. cerevisiae* e *Candida glabrata* (Shingu-Vazquez e Traven, 2011).

Em *Cryptococcus* spp a relação entre metabolismo mitocondrial e resistência a multidrogas é menos compreendida que em *S. cerevisiae* e *Candida* (Panepinto *et al.*, 2010). O que se tem relatado é que *C. neoformans* quando exposto à tetraciclina, um antibiótico que interfere na síntese de proteínas bacterianas e mitocondriais, se torna mais tolerante ao FLC (Oliver *et al.*, 2008). Além disso, mutantes de *C. neoformans* (*Δvad1* - *DEAD-box RNA helicase*) com defificência respisratória devido a diminuição da expressão de *TUF-4* (*mitochondrial translation elongation fator*) são mais tolerantes ao FLC. A superexpressão de *TUF-4* em *Δvad*, por sua vez, não só restaura o "crescimento respiratório" (as células passam a ser capazes de crescerem na presença de fontes de carbono como as linhagem selvagem), como a tolerância ao FLC (Panepinto *et al.*, 2010). A maior tolerância de *Δvad1* ao FLC pode ser associada a maior expressão de *sre1* nessas células comparadas às células da linhagem selvagem, que são menos tolerantes (Panepinto *et al.*, 2010). *sre1* é um gene homólogo a *SREBP* (*Sterol-Response Element Binding Protein)* em *Schyzossacharomyces pombae* que é ativado na presença de FLC quando há acúmulo dentro da célula de metabólitos tóxicos devido a inbição da 14-α demetilase, ou devido a hipóxia. Deleção de *sre1* diminui profundamente a tolerância de *C. neoformans* ao FLC (Chang *et al.*, 2007; Hughes *et al.*, 2007). Dessa forma, esses dados indicam que a relação entre a deficiência do metabolismo mitocondrial e a tolerância a drogas em *Cryptococcus* pode também estar relacionado com o metabolismo de lipídeos, já que mutantes com deficiência respiratória superexpressam o regulador *sre1* (Panepinto *et al.*, 2010).

Para tentar entender o porquê células adaptadas a PCT se tornam mais tolerantes a FLC, ITC e RVC, nós realizamos o transcriptoma das colônicas NA e 10p da linhagem R265 de *C. gattii*. A colônia 10p advinda de *C. gattii* R265 apresentou resistência cruzada permanente com FLC, ITC e RVC, além da PCT, e por isso foi escolhida. Nós decidimos, também, realizar o transcriptoma das células cultivas nas mesmas condições, que foram: em meio líquido YPD a 30°C sob agitação a 150 rpm (rotação por minuto). Dessa forma, a intenção foi avaliar as alterações entre as duas culturas que se mantinham mesmo após o contato com o agroquímico ter cessado.

Os resultados do transcriptoma mostraram que 354 genes foram expressos diferencialmente, sendo 203 genes da cultura 10p menos expressos que a cultura NA e 151 mais expressos.

As células da cultura 10p expressaram mais os genes de bomba de efluxo, *AFR1* e *MDR1*, o que nos leva a acreditar que quando essas células entram em contato com drogas antifúngicas elas poderiam bombear essas drogas para fora da célula e, o que justificaria o aumento da tolerância ao FLC nessa cultura comparada a cultura NA. Além disso, as células 10p expressaram mais o gene *CNBG_4693*, que é homólogo a *PDR16* em *C. neoformans* H99, uma bomba de efluxo que é importante para a heterorresistência ao FLC (Ngamskulrungroj *et al*., 2012). Contudo, três genes (*CNBG_4708, CNBG_6088* e *CNBG_9590* (*pdr-5*)) que codificam transportadores do tipo ABC, com provável função de bombeamento de drogas, foram menos expressos em 10p do que em NA. Nem todos os genes que codificam bombas de efluxo parecem ser importantes para a tolerância ao FLC em *Cryptococcus neoformans*. Ngamskulrungroj e colaboradores (2012) e Ko e colaboradores (2009) mostraram que a deleção dos genes *CNAG_07799* e *CNAG_05150* (Ngamskulrungroj *et al*., 2009) e *pdr5-2* e *pdr5-3* (Ko *et al*., 2009), que codificam transportadores do tipo ABC, não altera a tolerância de *C. neoformans* H99 ao FLC, ao passo que a deleção de *pdr-5* causa uma leve diminuição da tolerância (Ko *et al*., 2009). Dessa forma, nós especulamos que os genes que codificam transportadores do tipo ABC menos expressos em 10p não seriam importantes no processo de tolerância ao FLC, uma vez que *CNBG_4708, CNBG_6088* e *CNBG_9590* de *C. gattii* R265 são genes homólogos aos de *CNAG_07799, pdr-5* e *pdr5-2* em *C. neoformans* H99, respectivamente. Vale ressaltar que esse cenário pode se modificar na presença do FLC ou de PCT, uma vez que o cultivo das células para o sequenciamento do RNA foi feito sem a adição de drogas.

Ainda não se sabe se PCT pode ser bombeado por bombas de efluxo de *Cryptococcus*. O mecanismo de resistência mais comum em fungos ambientais é mutação no gene que codifica o alvo da droga, *cytb* (citocromo b) (Yin *et al*., 2012). No nosso trabalho, as culturas 10p, que são mais tolerantes a PCT, foram capazes de expressar duas vezes mais *cytb* que a cultura NA. Esse resultado indica que a cultura 10p de *C. gattii* R265 é mais tolerante a PCT provavelmente porque superxpressa o gene que codifica o alvo do agroquímico.

É importante salientar que tanto a expressão dos genes das bombas de efluxo como de *cytb* nas células da cultura 10p foi maior que as de NA na ausência da indução direta por PCT. Isso sugere, mais uma vez, que mutações poderiam ter ocorrido ou em fatores de transcrição que controlam tais genes ou mesmo na região promotora dos genes. Outra hipótese seria alterações epignéticas que poderiam estar induzindo a maior

expressão desses genes, mesmo na ausência do agroquímico. Para tentar responder essas questões em estudos futuros, nós sequenciamos o DNA genômico da cultura NA e 10p.

As drogas ambientais além de causarem alterações na tolerância a antimicrobianos clínicos causaram alterações da morfologia (diâmetro, S/V e cápsula) e da carga da superfície celular em *Cryptococcus*. As maiores alterações de morfologia aconteceram na linhagem *C. neoformans* H99 e *C. gatiii* 196L/03 que formaram pseudohifas ao serem expostas a TBZ e PCT, respectivamente. Os mecanismos envolvidos no processo de pseudofilamentação, em *Cryptococcus*, ainda não estão bem elucidados, porém, sabe-se que todos os cinco mutantes para os cinco genes (Cbk1, Kik1, Mob2, Tao3 e Sog2) da via RAM, que é importante para a citocinese celular, produzem pseudofilamentos constitutivamente, indicando a importância dessa via nesse fenômeno (Walton *et al.*, 2006; Lin *et al.*, 2015). No ambiente e no hospedeiro, a formação de pseudohifas parece ser uma estratégia de sobrevivência para escapar de predadores naturais ou de defesas do hospedeiro, e para se adaptar a condições de estresse nutricional (Lee *et al.*, 2012). Estudos com amebas demonstraram que, na presença desse protozoário, as leveduras podem formar pseudofilamentos, o que evitam a sua fagocitose (Bunting *et al.*, 1979). Além disso, já foram encontradas hifas e pseudohifas de *C. neoformans* em tecidos de pacientes com criptococose (Williamson *et al.*, 1996; Gazzoni *et al.*, 2010). Entretanto, mutantes com maior capacidade de formação de pseudohifas são menos virulentos e não disseminaram para o cérebro (Zhai *et al.*, 2013). Logo, a formação de pseudohifas contribui para a persistência do patógeno no hospedeiro, mas não participa de sua disseminação (Trevijano-Contador *et al.*, 2016). Nesse trabalho, nós observamos que a linhagem adaptada de *C. neoformans* H99, que forma pseudohifa na presença de TBZ, foi menos virulenta que a forma leveduriforme (não adaptada) em modelo murino, confirmando os dados da literatura.
Apesar de não formar pseudohifa, a cultura adaptada em TBZ de *C. gattii* R265 também foi menos virulentar que a cultura original. Nesse caso, a adaptação causou aumento do diâmentro celular e diminuição da razão S/V. Têm sido descrito que células menores são mais virulentas que as maiores devido a sua maior capacidade de translocação no animal e seu maior potencial de realizar trocas nutricionais rápidas com meio externo (Ferreira *et al.*, 2015). Contudo, mais estudos precisam ser realizados no intuito de se entender o porquê da diminuição da virulência diante à exposição ao TBZ.

Nós também avaliamos a virulência das culturas NA e 10p, advinda da cultura adaptada em PCT, de *C. gattii* R265. A cultura 10p, que apresentou resistência cruzada permanente com todos os azólicos clínicos usados nesse estudo, demonstrou-se menos

virulenta que a original. Em relação a morfologia, as células 10p apresentaram um diâmetro celular menor e uma razão S/V maior que as células NA. Seguindo o racicíonio descrito anteriormente, esperava-se que as células 10p fossem mais virulentas que as NA, o que não ocorreu. Esse resultado indica que outros mecanismos de virulência além da variação morfológica poderiam estar sendo responsáveis pela alteração da virulência na cultura 10p.

Cryptococcus spp possui vários fatores de virulência, sendo os clássicos: produção de cápsula, capacidade de crescer a 37°C, produção de enzimas como fosfolipase, urease, lacase, SOD, etc (Kronstad *et al.*, 2011). Recentemente outras vias importantes para a virulência tem sido estudadas nesse gênero, como as vias de obtenção e utilização de metais, como o ferro, cobre e zinco. O ferro, na forma dos aglomerados de heme e ferro-enxofre, é essencial como cofactor de várias enzimas, carreadoras de oxigênio e sistemas de transferência de elétrons envolvidos em funções celulares vitais que vão desde a respiração até a replicação do DNA (Schaible e Kaufmann, 2004). O cobre é um íon metálico redoxativo essencial para a maioria dos organismos aeróbicos, que também serve como cofator catalítico e estrutural para enzimas envolvidas na geração de energia, aquisição de ferro, transporte de oxigênio e metabolismo celular, entre outros processos (Kim *et al.*, 2008). O zinco também é um metal crucial, pois está no centro catalítico de numerosas enzimas e desempenha papéis importantes na funcionalidade de uma grande variedade de proteínas (Van Ho *et al.*, 2002). Devido a importância desses íons, os micro-organismos, incluindo *Cryptococcus*, precisam adquirí-los no ambiente e no hospedeiro a fim de garantir o crescimento (Silva etl al., 2011).

No nosso trabalho, os dados de transcriptoma demonstraram que cinco genes possivelmente envolvidos na absorção de ferro e cobre da cultura 10p foram menos expressos do que a cultura NA, são eles: *FRE1*, *FRE7* e *CNBG_9038* (homólogo a *FRE4* em *C. neoformans*), *CTR4* e *CNBG_1601* (homólogo a *CTR2* em *C. neoformans*).

Saikia e colaboradores (2014) estudaram a relação entre as redutases de ferro e virulência em *C. neoformans* e observaram que os mutantes *Δfre2* e *Δfre2Δfre4* eram menos virulentos do que a linhagem selvagem. De maneira semelhante, *Δctr4* é menos virulento que a linhagem selvagem (Waterman *et al.*, 2012). Dessa forma, nossa hipótese é que menor expressão dos genes relacionados a obtenção de ferro e cobre poderiam explicar, em parte, a diminuição da virulência na cultura 10p comparada a NA. Mais estudos precisam ser realizados a fim de se entender melhor essa relação e no

intuito de avaliar outros fatores de virulência como melanização e produção de enzimas como fosfolipase, urease e SOD.

Por fim, acredita-se que esse trabalho conseguiu demosntrar (i) que outros agroquímicos, especialmente os que são vastamente utilizados, ocasionam resistência cruzada com antifúngicos clínicos não somente em *C. gattii* como também em (ii) *C. neoformans*; (iii) que a temperatura pode influenciar esse fenômeno; (iv) que a resistência cruzada está relacionada com o aumento da expressão de bombas de efluxo e dos genes alvos das drogas e (v) que a exposição a agroquímicos causa diminuição da virulência dos fungos estudados.

7- CONCLUSÃO

A exposição aos agroquímicos tebuconazol e piraclostrobina aumenta a tolerabilidade a antifúngicos clínicos *in vitro* e *in vivo*, altera a morfo-fisiologia de forma linhagem-dependente e diminui a virulência de *C. gattii* e *C. neoformans* em modelo experimental murino.

8 – PERSPECTIVAS

- Realizar a análise do genoma do DNA sequenciado das culturas *C. gattii* R265 e *C. neoformans* H99 não-adaptadas e adaptadas em TBZ.

-Analisar o genoma das culturas *C. gattii* R265 não-apdatada, adaptada em PCT e 10p e relacioná-lo com o transcriptoma.

-Realizar microscopia eletrônica de transmissão para estudar o efeito da exposição à PCT sobre as mitocôndrias na linhagem *C. gattii* R265.

-Caracteriazar melhor a virulência das culturas NA e 10p (advinda da cultura adaptada em PCT) de *C. gattii* R265 por meio de determinação da carga fúngica no pulmão, LBA, cérebro e análises histopatológicas do pulmão.

-Estudar outros fatores de virulência a fim de se compreender a diminuição da virulência da cultura 10p de *C. gattii* R265.

-Investigar se a menor expressão de genes envolvidos na aquisição de íons na cultura 10p está relacionada com a diminuição da capacidade desse organismo de obter tais metais.

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