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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

Received 7 June 2017 **Returned for modification** 2 August 2017 **Accepted** 26 October 2017

Accepted manuscript posted online 6 November 2017

Citation 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 cross-resistance to clinical drugs and affects morphophysiology and virulence of *Cryptococcus gattii* and *C. neoformans*. Antimicrob Agents Chemother 62:e01179-17. https://doi.org/10.1128/AAC.01179-17.

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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]

Strain	MIC (μg/ml) at indicated temp (geometric mean)[b]											
	Fluconazole						Tebuconazole					
	30°C			35°C			30°C			35°C		
	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]

	MIC at indicated temp[b]											
	Itraconazole						Ravuconazole					
	30°C			35°C			30°C			35°C		
Strain	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]

	MIC (μg/ml) (geometric mean)[b]					
	Fluconazole			Tebuconazole		
Strain	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.

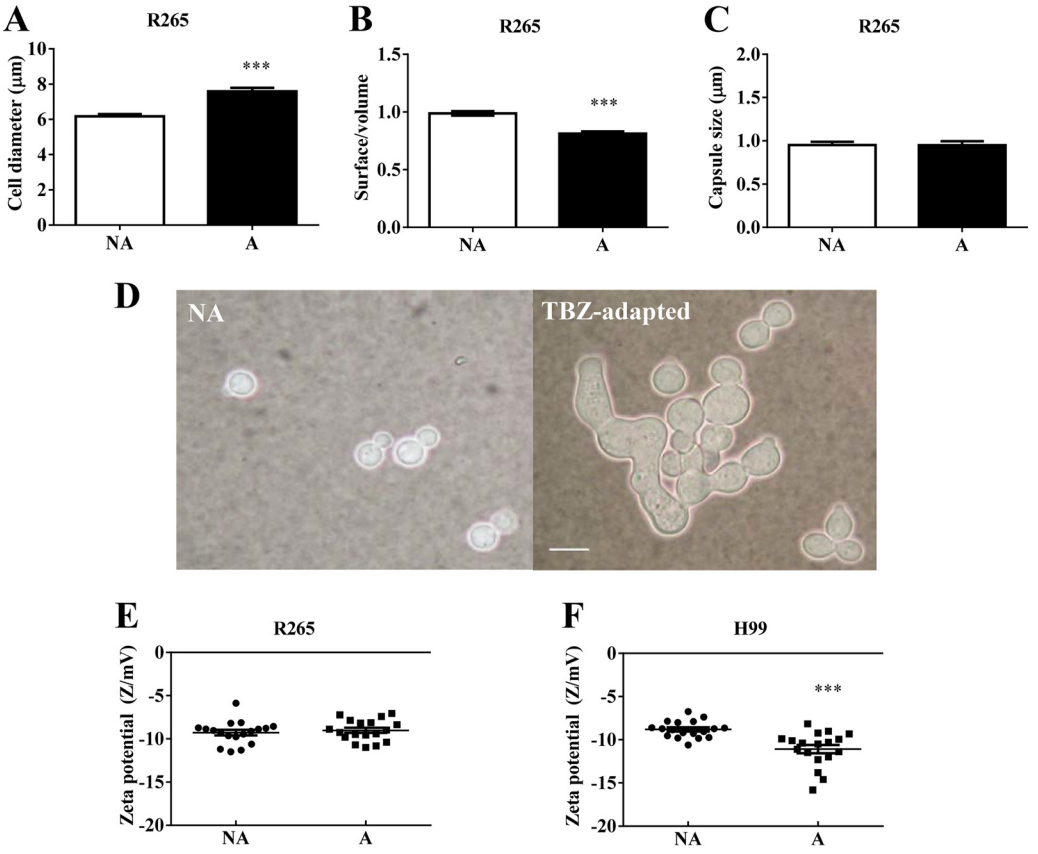

FIG 1 TBZ exposure causes morphophysiological changes in *Cryptococcus gattii* R265 and *C. neoformans* H99. (A to C) TBZ-adapted cells of *C. gattii* R265 exhibited increased cell diameter (A) and decreased surface/volume ratio (B) but unaltered capsule thickness (C) compared to nonadapted (NA) cells. (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).

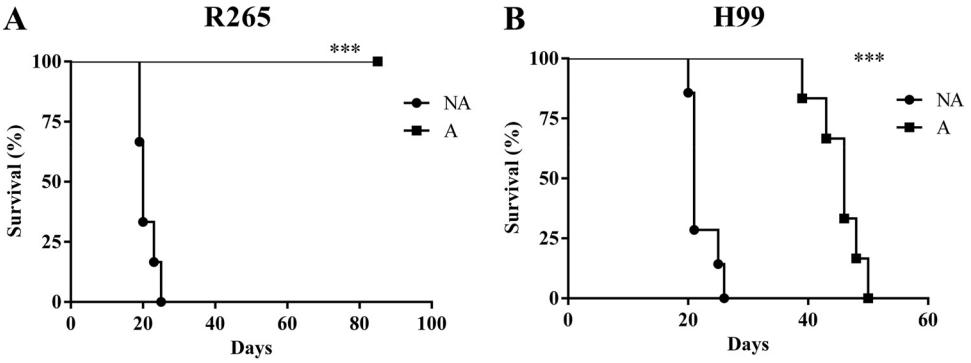

FIG 2 TBZ exposure decreases virulence in *Cryptococcus gattii* R265 and *C. neoformans* H99. C57BL/6 mice were infected by the intratracheal route with 1×10^5 CFU of nonadapted (NA) and TBZ-adapted (A) cells. The survival curve showed that animals infected with TBZ-adapted cells of *C. gattii* R265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

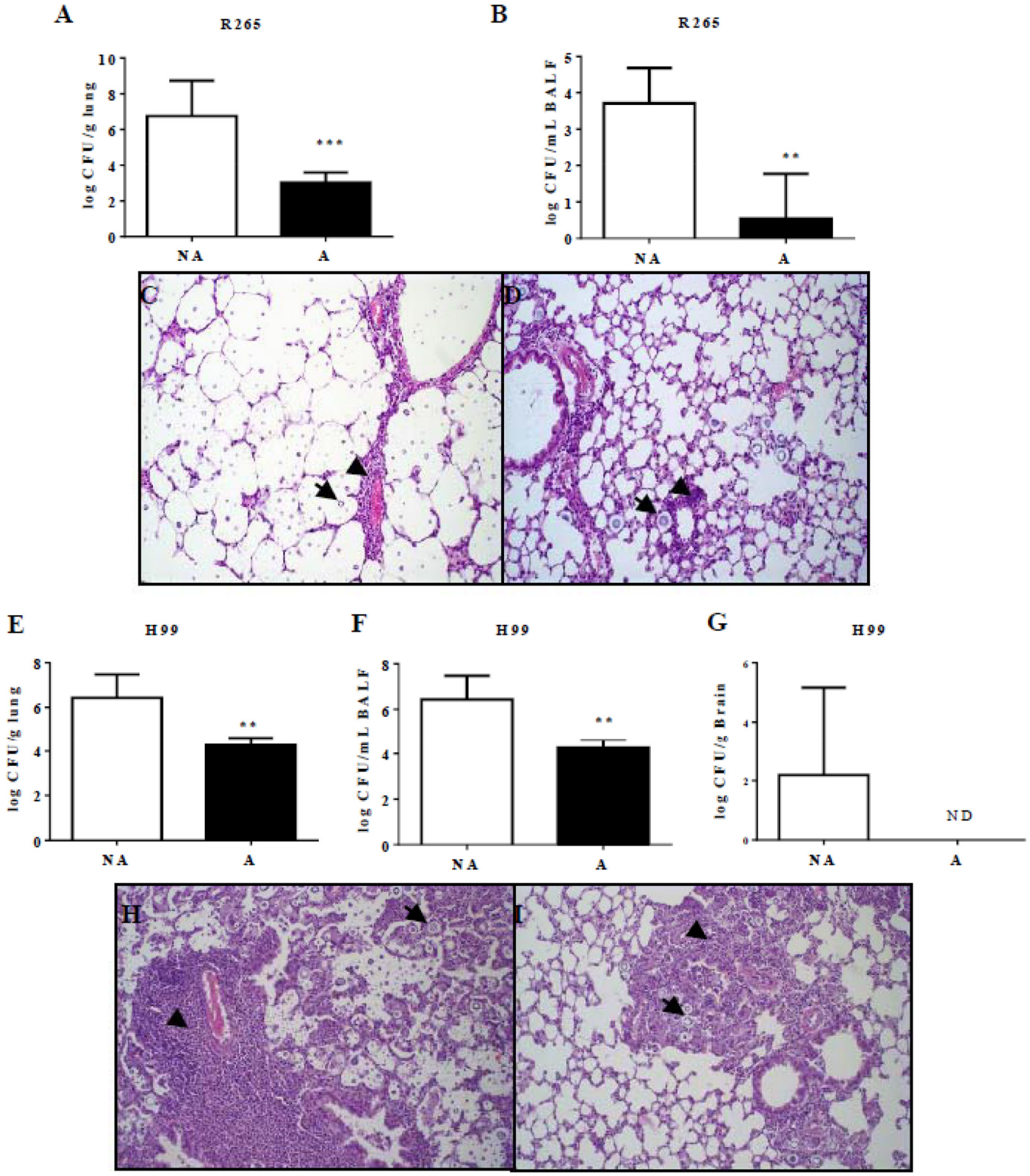

FIG 3 Animals infected with TBZ-adapted cells exhibit lower fungal load in the lungs, BALF, and brain and decreased lung inflammation. C57BL/6 mice were infected by the intratracheal route with 1×10^5 CFU of nonadapted (NA) and TBZ-adapted (A) cells for the determination of the CFU level per gram and 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.

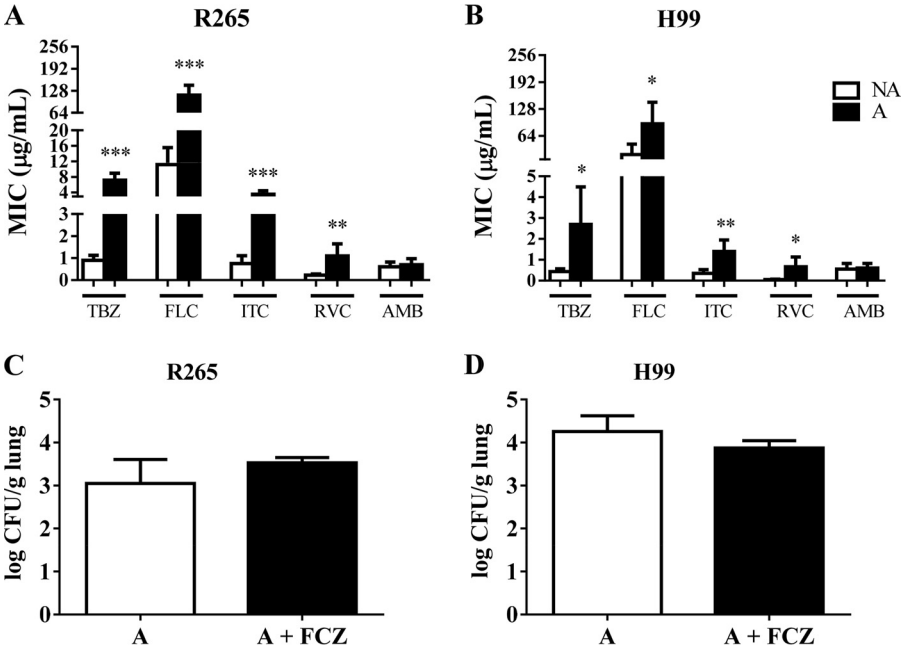

FIG 4 TBZ exposure causes antifungal resistance *in vivo*. The MICs of tebuconazole (TBZ), fluconazole (FLC), itraconazole (ITC), ravuconazole (RVC), and amphotericin B (AMB) were determined for colonies recovered from lungs of animals that had been infected with nonadapted (NA) and TBZ-adapted (A) cells 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-

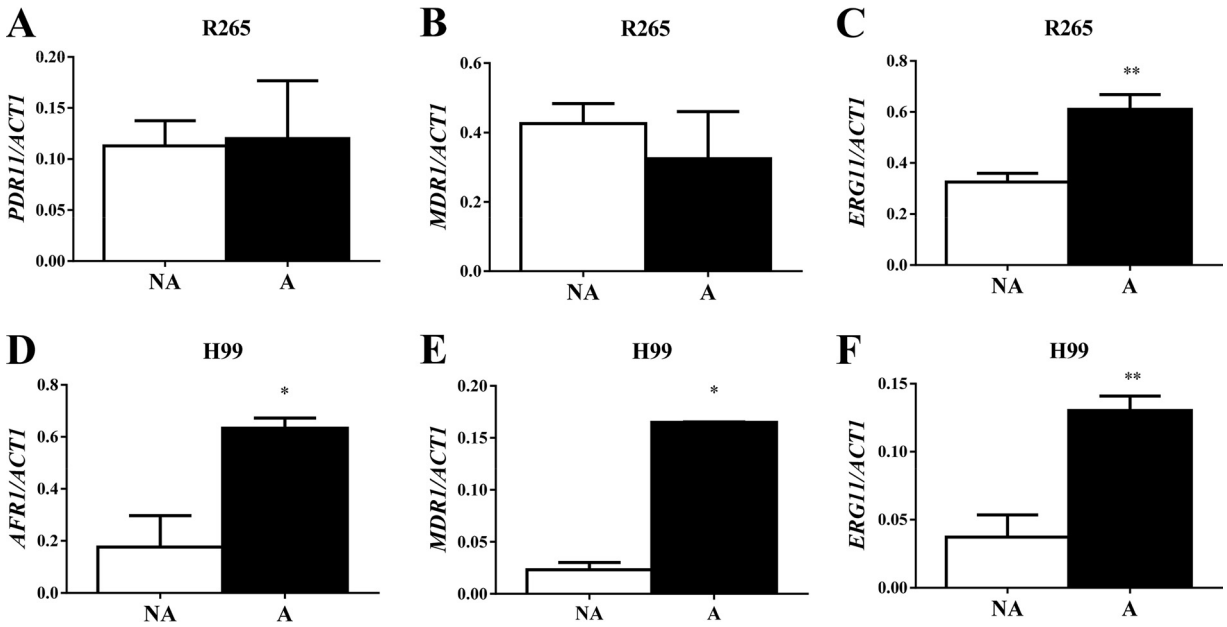

FIG 5 TBZ exposure changes in the expression of efflux pumps and *ERG11* genes. Expression of *PDR1* (A) and *MDR1* (B), but not that of *ERG11* (C), was altered by tebuconazole exposure in *C. gattii* R265. In *C. neoformans* H99, the levels of expression of *AFR1* (D), *MDR1* (E), and *ERG11* (F) were 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.

ACKNOWLEDGMENTS

This study was supported by Fundação de Amparo a Pesquisa do Estado de Minas Gerais—FAPEMIG (grant APQ-00727-16) and Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq (grant 403006/2016-3). R.W.B. received fellowships from CNPq and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (PDSE-CAPES-88881.131683/2016-01). D.A.S. is a research fellow of the CNPq (grant 305154/2014-1).

We declare that we have no conflicts of interest.

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