Environmental triazole induces cross-resistance to clinical drugs and affects morphophysiology and virulence of *Cryptococcus gattii* and *C. neoformans*

Running tittle: Effects of environmental triazole in *Cryptococcus*

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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 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, ravuconazole), but not with amphotericin B. In some strains, CR was observed even after the exposure ceased. Further, tebuconazole exposure changed the morphology, including pseudohyphae formation in *C. neoformans* H99, and 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 non-exposed 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 the increased of expression of the *ERG11* in both species, and efflux pumps (*AFR1* and *MDR1*) in *C. neoformans*. Our study supports the idea that agrochemical usage can significantly affect human pathogens present in the environment by affecting their resistance to clinical drugs.

Key words. Agrochemical, antifungal cross-resistance, fluconazole, tebuconazole, pseudohyphae.

INTRODUCTION

Losses of crops due to pests are a major problem that must be faced by agriculture to achieve increased food production (1). One of the most-used 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 inhibits the 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 are the effects of agrochemicals on human pathogens present in the environment and (ii) what are the indirect effects on human and animal health. Some studies have shown that the 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 and if 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, are also used in some

cases (11). Although antibiotic therapy is frequently effective, there are important drawbacks. 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* spp. strains with increased tolerance to these drugs is increasing (13, 14).

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

RESULTS

Antifungal drug susceptibility testing and screening of subpopulations more tolerant to 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 MCA/$Sub\text{-}MIC^{solid}$ ratios at 30 and 35°C (the higher the ratio, more passages through tebuconazole-containing media occurred). When the adaptation was performed at 30°C,

38% (5) of *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 being almost 3-fold higher than when the test was performed at 35°C (Table 1).

Tebuconazole-adapted **colonies present cross-resistance with fluconazole and other azole drugs**

Tables 2 and 3 show the MIC in liquid medium (MIC^{broth}) for fluconazole and tebuconazole for non-adapted (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 two dilutions of the MIC for tebuconazole compared to the MIC of NA colonies (Tables 2 and 3). Overall, 61.5% (8) of *C. gattii* (Table 2) and 100% (4) of *C. neoformans* (Table 3) tebuconazole-adapted cells became more resistant to the antifungal environment 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% (5) of *C. gattii* (Table 2) and 50% (2) of *C. neoformans* strains (Table 3) tebuconazole-adapted colonies became more resistant to tebuconazole.

We then tested whether tebuconazole adaptation can also decrease susceptibility to fluconazole. Geometric means of tebuconazole-adapted colonies 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 selected populations with

cross-resistance (CR). Thirty eight percent (5) of *C. gattii* and 100% (4) of *C. neoformans* tebuconazole-adapted colonies presented higher MICs for fluconazole, as well as for tebuconazole (Tables 2–4). Four and three strains of *C. gattii* (R265, ATCC 32608, L27/01, and 196L/03) and *C. neoformans* (H99, ATCC 24067, and ATCC 62066), respectively, returned to the original phenotype when grown in medium without the agrochemical (10p) (Tables 2 and 3) and this was named as "temporary CR". This phenomenon occurred dependently (ATCC 32608, L27/01 and ATCC 62066) or independently (R265, 196L/03, H99 and ATCC 24067) on the temperature for different strains (Tables 2–4). The other strain of *C. gattii* (ATCC 24065) and *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 a "permanent CR" (Tables 3–5). This profile also appears to be temperature-dependent (Table 2–4).

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 only exhibited altered susceptibility to ravuconazole when the test was performed at 30°C (Table 5), confirming the importance of temperature in the resistance process.

We also performed tebuconazole-adaptation at 35°C using the same procedure (Tables 6 and 7). Overall, 42% (6) of *C. gattii* strains exhibited an increased MIC to tebuconazole after tebuconazole adaptation (Table 6) and 23% (3) presented CR with fluconazole. For two strains (R265 and 23/10893), the phenotype reverted after

subcloning several times on non-selective 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 MIC to tebuconazole, but lacked CR with fluconazole (Tables 4 and 7).

The *Cryptococcus* spp. 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, exhibited an increased MIC only for fluconazole (one isolated from *C. gattii* L24/01 adapted at 30°C and one isolated from *C. neoformans* ATCC 62066 adapted at 35°C) (Tables 2 and 7). This phenomenon also has been observed when an environmental non-azole antifungal agent is 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, 70–90% tebuconazole-adapted 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 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 tebuconazole-adapted cells of the same strain (Fig. 2A and B). 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 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 disseminated into the brain in 40% of infected animals, unlike the tebuconazole-adapted cells (Fig. 3G).

Histopathology analysis confirmed the decreased virulence of tebuconazole-adapted colonies. Mice infected with non-adapted cells of *C. gattii* R265 presented a moderate to intense and diffuse amount of extracellular yeasts in the alveolar and bronchial lumen (Fig. 3C). This was associated with discrete mixed perivascular inflammatory infiltrate with predominance of neutrophils, and multifocal and discrete

alveolar thickening due to mononuclear inflammatory infiltrate (Fig. 3C). Mice infected with non-adapted *C. neoformans* H99 demonstrated an intense and diffuse amount of yeasts in the pulmonary parenchyma (Fig. 3H). We also observed an intense mixed perivascular inflammatory infiltrate and intense and multifocal to diffuse mixed inflammatory infiltrate with predominance of macrophages and multinucleated giant cells in the alveolar space (Fig. 3H). However, the animals infected with adapted cells exhibited a reduced amount of yeasts observed 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 (non-infected mice).

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 for all azole drugs in colonies recovered from the lungs of animals infected with tebuconazole-adapted cells was significantly ($p < 0.05$) higher than that for drugs recovered from animals infected with NA cells (Fig. 4A and B). Furthermore, fluconazole did not reduce the fungal burden in the lungs ($p>0.05$) from mice (Fig. 4C and D), which 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 for azole drugs for the adapted versus non-adapted *C. gattii* R265 and *C. neoformans* H99. Figure 5

shows that the gene expression levels of *ERG-11*, but not those of the efflux pumps *PDR-11* and *MDR-1* (Fig. 5A-C), were significantly ($p < 0.05$) higher in adapted cells than non-adapted of *C. gattii* R265. However, in *C. neoformans* H99, all genes (*ERG11*, *AFR-1*, and *MDR-1*) were expressed at a higher level ($p < 0.05$) in tebuconazole-exposed cells (Fig. 5D-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*, to clinical drugs (fluconazole and itraconazole) and less virulence in a murine model when compared with tolerance and virulence of cells not exposed to the agrochemical. We also tested an azole drug currently in clinical trials (ravuconazole) (21) to study whether there would be CR with drugs not yet commercially available, which was confirmed.

First, we showed that previous exposure to tebuconazole selects cells with permanent 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 pumps genes (*AFR1*, *AFR2*, *PDR11*, and *MDR1*) (24, 27) and, in some cases, overexpression of the target of these drugs, ERG11p (25). Here, we showed that the mechanism of azole tolerance in *C. gattii* and *C. neoformans* can be different in strains that presented temporary CR, such H99 and R265. We observed increased expression of efflux pumps and *ERG11* in *C. neoformans* H99, but only of *ERG11* in *C. gattii* R265. These results demonstrated that although the adapted cells of both species exhibited increased MICs for all the same azole drugs, the mechanism for each species, and possibly for each strain, was different. 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 original cells (25). In *Candida parapsilosis*, Rocha and colleagues reported that the exposure to the agrochemical tetraconazole selected cells more resistant to azoles 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 (28, 29). 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) (28, 30) and the mechanism of resistance can be different than that for fluconazole (31). More studies should determine the mechanisms involved in the permanent CR caused by agrochemical exposure.

Temperature is a critical factor that the human pathogenic fungi must overcome to cause illness (32). 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 to the pesticide compared when the experiment was performed 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 (33). 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 less 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 (31). Usually, cells with a higher diameter and a smaller capsule are less virulent (20, 23, 34). Here, we observed that tebuconazole-adapted cells, which exhibited higher diameters than those of NA cells, were less virulent in both species. Morphological analysis also showed that tebuconazole-adapted cells of *C. neoformans* H99 exhibited pseudohyphae formation. Pseudofilamentation happens when budding cells do not fully separate, so a chain of bound cells is formed (35). This process is common in *Candida* (35), but rare and little-studied in *Cryptococcus.* Pseudofilamentation seems to be a response to overcome environmental stresses (36), but during this process, the cells became less virulent (37), as our results demonstrated. This decreased virulence may occur because cell surface molecules become differentially presented, and they cannot be phagocytosed by macrophages (38) and do not reach the central nervous system (35, 37). The zeta potential data confirmed that pseudohyphae formation 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 that pseudohyphae do not reach the central nervous system (35, 37), which explains why these cells are less virulent than NA cells.

Other studies have reported that strains with secondary resistance to fluconazole are less virulent than susceptible strains (23, 39), whereas strains that are heteroresistant to itraconazole and fluconazole are more virulent (20, 40). In most of these cases, morphological changes are crucial for altered virulence (20, 23, 39). In itraconazole-heteroresistant cells, increased virulence has been attributed to decreased cell size (20), the inverse of what was observed in the cross-resistant cells in this study (cells were bigger and less virulent). Our study reinforced 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 on the virulence and *in vivo* resistance of *Cryptococcus* spp.

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) (17). We also used four strains of *C. neoformans* (one clinical and three reference strains) (Table 1) (18). All isolates were maintained on Sabouraud Dextrose Agar (SDA) at 4°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 (morphophysiological, virulence changes, *in vivo* antifungal resistance, and RT-PCR).

Antifungal drug susceptibility testing

The minimum inhibitory concentrations (MICs) for 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}) (19). The MIC for tebuconazole also was determined by spot tests on SDA supplemented with different concentrations of the pesticide (MIC^{solid}) (20). For the spot tests, cell suspensions containing 1–5 × 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 to tebuconazole)

After performing susceptibility testing on solid medium (MIC^{solid}), the strains were grown on SDA with increasing concentrations of the pesticide. Initially, all strains were grown on medium supplemented with tebuconazole at a concentration of the MIC/2 (sub-MIC). After one week, an inoculum using at least five colonies was prepared in sterile saline, and the transmittance (530 nm) of the suspensions was adjusted to 75–77% ($1–5 \times 10^6$ fungal cells). Subsequently, 10 μL of this suspension was inoculated on a medium containing tebuconazole at the MIC. After one 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 or until cultivation at 256 μg/mL. These tests were performed both at 30 and 35°C. The colonies that were exposed to tebuconazole were named tebuconazole-adapted (A) colonies and the original colonies (no exposure) were named non-adapted (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} for fluconazole, amphotericin B, and tebuconazole was determined for non-adapted and tebuconazole-adapted colonies. The test was performed at 30 and 35°C for the colonies adapted at 30°C and at 35°C for the colonies adapted at 35°C. A strain was considered cross-resistant when it presented decreased susceptibility both to 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 increased MIC^{broth} (at least four times) were mixed, and then were subcultured every 48 h on SDA plates without tebuconazole for 10 passages (10p colonies) (20). 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) (21) for the tebuconazole-adapted and 10p colonies that showed CR with fluconazole.

Morphometric and zeta potential analysis.

Non-adapted and tebuconazole-adapted colonies were grown on SDA and SDA supplemented with tebuconazole at 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 (20). 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 (22).

Ethics statement, virulence, and cross-resistance *in vivo*

C57BL/6 male mice, 6–8 weeks-old, 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.cobea.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) and xylazine (10 mg/kg) in sterile saline. Next, each animal received 30 µL of 1×10^5 cells of *C. gattii* R265 or *C. neoformans* H99 by the intratracheal route. The mice were monitored daily for survival (20).

Other groups of animals were infected and euthanized under anesthesia 15 days post-inoculation 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 colony-forming unit per gram (CFU/g) or mL (23). We also determined the MIC^{broth} for 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.

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. Fifteen days post-inoculation, the animals were euthanized and the lungs collected for CFU/g determination.

RNA extraction and RT-PCR analysis

Non-adapted and adapted cells of *C. gattii* R265 and *C. neoformans* H99 were grown on SDA and SDA + 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 DNaseI-treated RNA was used for reverse-transcription (RT) using the QuantiTect Reverse Transcription (Qiagen) kit. Subsequently, the cDNAs were PCR-amplified in the presence of dCTP (α33P) (Perkin Elmer) with the primers for the following genes: *ACT1*, *AFR1*, and *MDR1* for *C. neoformans* and *ACT1*, *MDR1* (24), and *PDR11* (25) 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) (26).

Statistical analyses

All statistical analyses were performed using GraphPad Prism, version 6.00, for Windows (GraphPad Software, San Diego, CA, USA) with $p < 0.05$ considered significant. The results of antifungal experiments (*in vitro* and *in vivo*), morphometric analysis, zeta potential, quantification of CFU/g organs, or BALF and RT-PCR were analyzed by Student's t test. Survival curves were plotted by 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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Transparency declarations.

None to declare.

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FIG 1. TBZ-exposure causes morphophysiological changes in *Cryptococcus gattii* R265 and *C. neoformans* H99. TBZ adapted-cells of *C. gattii* R265 exhibited increased cell diameter (A) and decreased surface/volume ratio (B), but capsule thickness was maintained (C), compared to those of non-adapted (NA) cells. *C. neoformans* H99 presented the pseudohyphae form after TBZ-adaptation (D). India ink suspension was used. Bar = 10 μm. 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 = non-adapted; A = TBZ-adapted; ** $p < 0.01$; *** $p < 0.001$.

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 non-adapted (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 non-adapted (NA) and TBZ-adapted (A) cells for the CFU/g determination and histopathology analysis. 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. Lung histopathology analysis showed that NA cells caused more inflammation (C) than A cells (D). 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 intense inflammation in the lungs (H) when compared to those of animals infected with A cells (I). **p < 0.01; *** p < 0.001. ND = not detected. NA = non-adapted; 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 for tebuconazole (TBZ), fluconazole (FLC), itraconazole (ITC), ravuconazole (RVC), and amphotericin B (AMB) was determined for colonies recovered from lungs of animals infected with non-adapted (NA) and TBZ-adapted (A) cells of *C. gattii* R265 (A) or *C. neoformans* H99 (B) and treated (A + FLC) or not (A) with FLC (10 mg/kg). After 15 days, the animals were euthanized and the lungs were collected for CFU/g determination. There was no statistical difference in the fungal load in the lungs of animals infected with TBZ-adapted cells of *C. gattii* R265 (C) or *C. neoformans* H99 (D) treated or not with FLC. * p < 0.05; ** p < 0.01; ***p < 0.001.

FIG. 5. TBZ-exposure changes in the expression of efflux pumps and *ERG-11* 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 expression of *AFR1* (D), *MDR1* (E) and *ERG11* (F) was increased in adapted cells versus that of non-adapted cells. NA = non-adapted; A = TBZ-adapted; * p < 0.05; ** p < 0.01.

627 **Table 1.** Screening of *C. gattii* and *C. neoformans* subpopulations with increased
628 tolerance to tebuconazole (TBZ-adaptation).
629

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)	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	0.5 – 8.0	0.75 – 10.0	0.5 – 16.0	1.5 – 10.0	0.5 – 8.0
Geometric mean	1.37	1.30	3.80	1.81	5.53	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	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

630 MIC^{solid}: Minimum Inhibitory Concentration in solid medium for tebuconazole before the
631 adaptation process, MCA: Maximum Concentration Achieved of tebuconazole in the TBZ-
632 adaptation test. C, clinical strain; Reference strain; E, environmental.

633 **Table 2.** Minimum inhibitory concentrations (MICs, µg/mL) for fluconazole and tebuconazole of *C. gattii* strains of non-adapted (NA), TBZ-adapted (A) at

634 30°C and TBZ-adapted colonies subcultured 10 times in agrochemical-free medium (10p - 10 passages). Tests were performed at 30°C and 35°C.

Strain or Parameter	Fluconazole[a]						Tebuconazole[a]					
	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**	**64.0 (8X)**	16.0	**8.0**	**64.0 (8X)**	16,0	**0.5**	**4.0 (8X)**	1.0	**1.0**	**4.0 (4X)**	2.0
ATCC 24065	**4.0**	**32.0 (8X)**	**32.0 (8X)**	4.0	8.0	ND	**0.5**	**4.0 (8X)**	**4.0 (8X)**	0.5	1.0	ND
ATCC 32608	**16.0**	**64.0 (4X)**	32.0	8.0	16.0	ND	**0.5**	**2.0 (4X)**	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 (4X)**	1.0	**0.25**	**2.0 (8X)**	0.25
L24/01	**16.0**	**64.0 (4X)**	16.0	8.0	16.0	ND	4.0	4.0	ND	1.0	2.0	ND
L27/01	**16.0**	**64.0 (4X)**	16.0	32.0	32.0	ND	**0.5**	**4.0 (8X)**	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 (4X)**	0.125	**0.125**	**0.5 (4X)**	0.125
196L/03	**16.0**	**128.0 (8X)**	16.0	**16.0**	**128.0 (8X)**	16.0	**1.0**	**8.0 (8X)**	1.0	**1.0**	**8.0 (8X)**	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 (8X)**	0.25	**0.125**	**1.0 (8X)**	0.25
29/10933	8.0	16.0	ND	4.0	4.0	ND	0.25	0.25	ND	0.25	0.25	ND
MIC range	4.0 – 32.0	8.0 – 128.0	ND	4.0 – 32.0	4.0 – 128.0	ND	0.125– 4.0	0.25 – 8.0	ND	0.125– 2.0	0.25 – 8.0	ND
Geometric mean	12.92	35.60	ND	9.90	17.80	ND	0.56	1.79	ND	0.56	1.37	ND

635

636 a: MIC endpoint considering 50% of growth inhibition. The number in parentheses shows how many times (X) the MIC value of the TBZ-adapted or 10p
637 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
638 determined.

639 **Table 3**. Minimum inhibitory concentrations (MICs, µg/mL) for fluconazole and tebuconazole of *C. neoformans* strains of non-adapted (NA),TBZ-adapted
640 (A) at 30°Cand TBZ-adapted colonies subcultured 10 times in agrochemical-free medium (10p-10 passages). Tests were performed at 30°C and 35°C.

Strain or parameter	Fluconazole[a]						Tebuconazole[a]					
	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**	**128.0 (8X)**	32.0	**8.0**	**64.0 (8X)**	8.0	**1.0**	**4.0 (4X)**	1.0	**0.25**	**2.0 (8X)**	0.25
ATCC 24067	**16.0**	**64.0 (4X)**	16.0	**4.0**	**32.0 (8X)**	4.0	**0.5**	**2.0 (4X)**	0.5	**0.25**	**1.0 (4X)**	0.25
ATCC 28957	**4.0**	**16.0 (4X)**	**32.0 (8X)**	2.0	4.0	ND	**0.5**	**4.0 (8X)**	**2.0 (4X)**	0.5	0.5	ND
ATCC 62066	**4.0**	**16.0 (4X)**	4.0	4.0	4.0	ND	**0.25**	**1.0 (4X)**	0.5	0.5	0.5	ND
MIC range	4.0 – 16.0	16.0 – 128.0	ND	2.0 – 8.0	4.0 – 64.0	ND	0.25 – 1.0	1.0 – 4.0	ND	0.25 – 0.5	0.5 – 2.0	ND
Geometric mean	8.0	38.05	ND	4.0	13.45	ND	0.5	2.38	ND	0.35	0.84	ND

641 a: MIC endpoint considering 50% of growth inhibition. The number in parentheses shows how many times (X) the MIC value of the TBZ-adapted or 10p
642 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
643 determined.

644 **Table 4.** Percentage (%) of Cross-resistance (CR) between TBZ and FCZ presented for *C. gattii*

645 and *C. neoformans* strains after TBZ-adaptation at 30 and 35°C.

Resistance	*C. gattii*		*C. neoformans*	
	30°C	35°C	30°C	35°C
Cross- Resistance (CR)	38%	21%	100%	7%
Temporary CR	31%	15%	75%	7%
Permanent CR	7%	7%	25%	0

646

647 **Table 5.** Minimum inhibitory concentrations (MICs,µg/mL) for itraconazole and ravuconazole of *C. gattii* and *C. neoformans* strains of non-adapted (NA),

648 TBZ-adapted (A) at 30°C and TBZ-adapted colonies subcultured 10 times in agrochemical-free medium (10p-10 passages). Tests were performed at 30°C and

649 35°C.

650

Strain	Itraconazole[a]						Ravuconazole[a]					
	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	**0.25**	**1.0 (4X)**	0.25	**0.25**	**1.0 (4X)**	0.5	**0.125**	**2.0 (16X)**	0.06	**0.03**	**0.5 (16X)**	0.03
ATCC 24065	0.5	1.0	1.0	0.25	0.5	1.0	**0.06**	**2.0 (32X)**	**2.0 (32X)**	**0.03**	**0.125 (4X)**	**0.125 (4X)**
ATCC 32608	0.5	1.0	ND	0.5	0.5	ND	**0.125**	**0.5 (4X)**	ND	0.125	0.125	ND
L27/02	0.5	1.0	ND	0.25	0.5	ND	**0.125**	**1.0 (8X)**	ND	**0.03**	**0.125 (4X)**	ND
196L/03	0.5	1.0	ND	0.5	1.0	ND	**0.25**	**2.0 (8X)**	ND	**0.125**	**2.0 (16X)**	ND
C.neoformans	**NA**	**A**	**10p**	**NA**	**A**	**10p**	**NA**	**A**	**10p**	**NA**	**A**	**10p**
H99	**0.125**	**1.0 (8X)**	ND	**0.125**	**1.0 (8X)**	ND	**0.06**	**1.0 (16X)**	ND	**0.06**	**0.5 (8X)**	ND
ATCC 24067	0.5	1.0	ND	0.25	0.5	ND	**0.125**	**1.0 (8X)**	ND	**0.015**	**0.25 (16X)**	ND
ATCC 28957	**0.25**	**1.0 (4X)**	ND	**0.125**	**0.5 (4X)**	ND	**0.06**	**0.5 (8X)**	ND	0.015	0.03	ND
ATCC 62066	0.5	1.0	ND	0.25	0.25	ND	**0.03**	**0.5 (16X)**	ND	0.03	0.03	ND

a: MICendpoint considering 50% of growth inhibition. The number in parentheses shows how many times (X) the MIC value of the TBZ-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 6. Minimum inhibitory concentrations (MICs, µg/mL) for fluconazole and tebuconazole of *C. gattii* strains of non-adapted (NA),TBZ-adapted (A) at 35°Cand TBZ-adapted colonies subcultured 10 times in agrochemical-free medium (10p-10 passages). Tests were performed at 35°C.

Strain or parameter	Fluconazole[a]			Tebuconazole[a]		
	NA	**A**	**10p**	**NA**	**A**	**10p**
R265	**8.0**	**32.0 (4X)**	16.0	**1.0**	**4.0 (4X)**	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 (4X)**	**1.0 (4X)**
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 (8X)**	0.25
196L/03	**16.0**	**256.0 (16X)**	**256.0 (16X)**	**1.0**	**8.0 (8X)**	**16.0 (16X)**
LMM 818	16.0	16.0	ND	0.25	0.25	ND
23/10893	**8.0**	**32.0 (4X)**	4.0	**0.125**	**2.0 (16X)**	0.125
29/10933	4.0	8.0	ND	**0.25**	**1.0 (4X)**	0.5
MIC range	4.0 – 32.0	4.0 – 256.0	ND	0.125 – 2.0	0.25 – 8.0	ND
Geometric mean	9.90	17.80	ND	0.56	1.53	ND

a: MIC endpoint considering 50% of growth inhibition. The number in parentheses shows how many times (X) the MIC value of the TBZ-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.

664 **Table 7.** Minimum inhibitory concentrations (MICs, µg/mL) for fluconazole and tebuconazole of *C.*
665 *neoformans* strains of non-adapted (NA),TBZ-adapted (A) at 35°Cand TBZ-adapted colonies subcultured
666 10 times in agrochemical-free medium (10p-10 passages). Tests were performed at 35°C.

Strain or parameter	Fluconazole[a]			Tebuconazole[a]		
	NA	A	10p	NA	A	10p
H99	**8.0**	**32.0 (4X)**	16.0	**0.25**	**1.0 (4X)**	**2.0 (8X)**
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 (8X)**	4.0	1.0	2.0	ND
MIC range	4.0 – 8.0	8.0 – 32.0	ND	0.25 – 1.0	1.0 – 2.0	ND
Geometric mean	5.65	16.00	ND	0.50	1.19	ND

667 a: MIC endpoint considering 50% of growth inhibition. The number in parentheses shows how many times
668 (X) the MIC value of the TBZ-adapted or 10p colonies was higher (≥4X) than the MIC of the NA colonies.
669 Values highlighted indicate increased MIC by at least 4X more than NA colonies. ND = not determined.
670
671 **Table 8.** Minimum inhibitory concentrations (MICs, µg/mL) for itraconazole and ravuconazole
672 of *C. gattii* and *C. neoformans* strains of non-adapted (NA), TBZ-adapted (A) at 35°C and TBZ-
673 adapted colonies subcultured 10 times in agrochemical-free medium (10p-10 passages). Tests
674 were performed at 35°C.

Strain	Itraconazole[a]			Ravuconazole[a]		
C. gattii	**NA**	**A**	**10p**	**NA**	**A**	**10p**
R265	0.25	0.25	0.25	**0.03**	**0.5 (16X)**	ND
196L/03	0.5	1.0	1.0	**0.125**	**1.0 (8X)**	**1.0 (8X)**
23/10893	0.5	0.5	ND	0.03	0.06	ND
C. neoformans	**NA**	**A**	**10p**	**NA**	**A**	**10p**
H99	**0.25**	**2.0 (8X)**	ND	**0.03**	**0.5 (16X)**	ND

675 a: MIC endpoint considering 50% of growth inhibition. The number in parentheses shows how many times
676 (X) the MIC value of the TBZ-adapted or 10p colonies was higher (≥4X) than the MIC of the NA colonies.
677 Values highlighted indicate increased MIC by at least 4X more than NA colonies. ND = not determined.
678

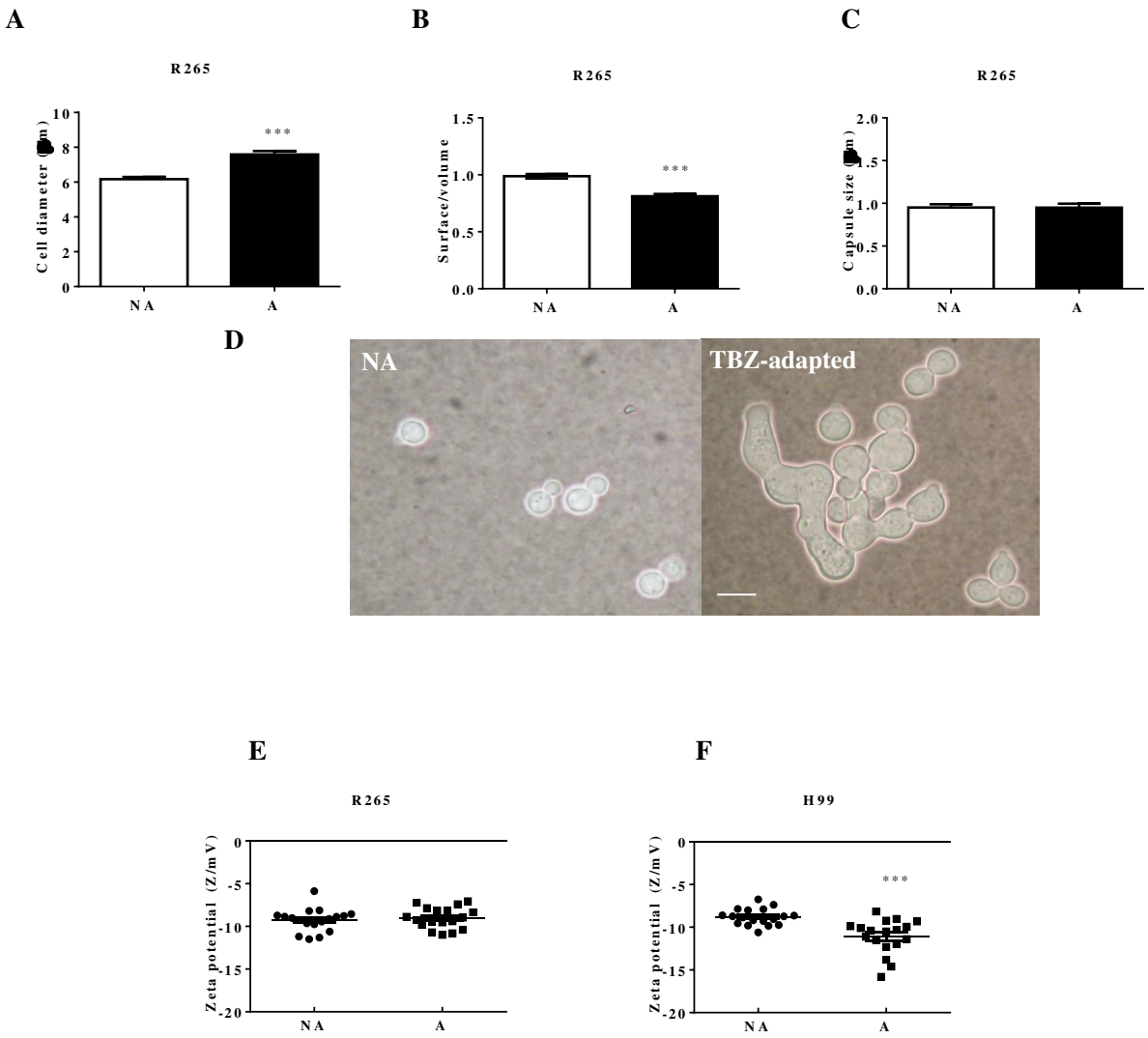


FIG 1. TBZ-exposure caused morpho-physiological changes in *Cryptococcus gattii* R265 and *C. neoformans* H99. TBZ adapted- cells of *C. gattii* had the cell diameter incresead (A) and the surfasse/volume ratio (B) decreased, but maintained the thickness of capsule (C), compared to nonadapted (NA) cells. *C. neoformans* H99 presented pseudohyphae form after TBZ-adaptation (D). India ink suspension was used. Bar=10µm. The electronegativity of the cellular surface was not altered in *C. gattii* R265 (E), however, it increased for TBZ-adapted cells of *C. neoformans* H99 (F). NA=nonadapted; A=TBZ-adapted; ** p<0.01; *** p<0.001.

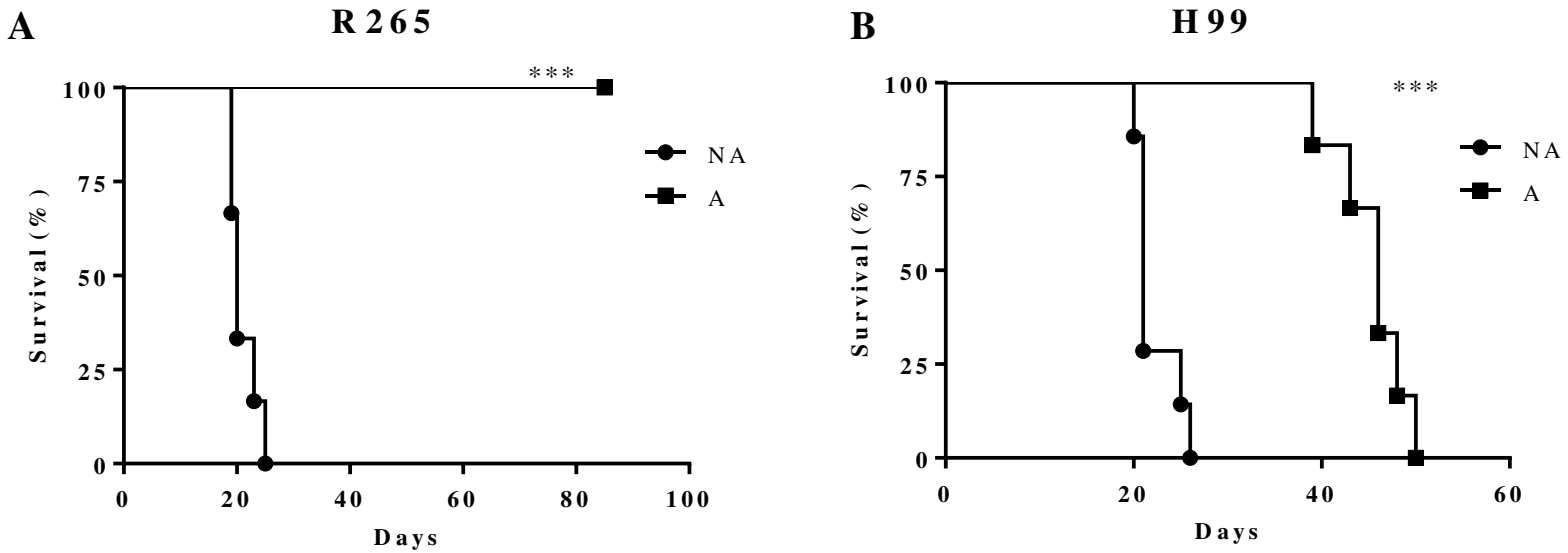


FIG 2. TBZ-exposure caused decreased of virulence in *Cryptococcus gattii* R265 and *C. neoformans* H99. C57BL/6 mice were infected by intratracheal route with $1x10^5$ CFU of non-adapted -NA- and TBZ-adapted -A- cells. The survival curve showed that animals infected with TBZ-adapted cells of *C. gattii* R265 (A) and *C. neoformans* H99 (B) survived more than those infected with NA cells.

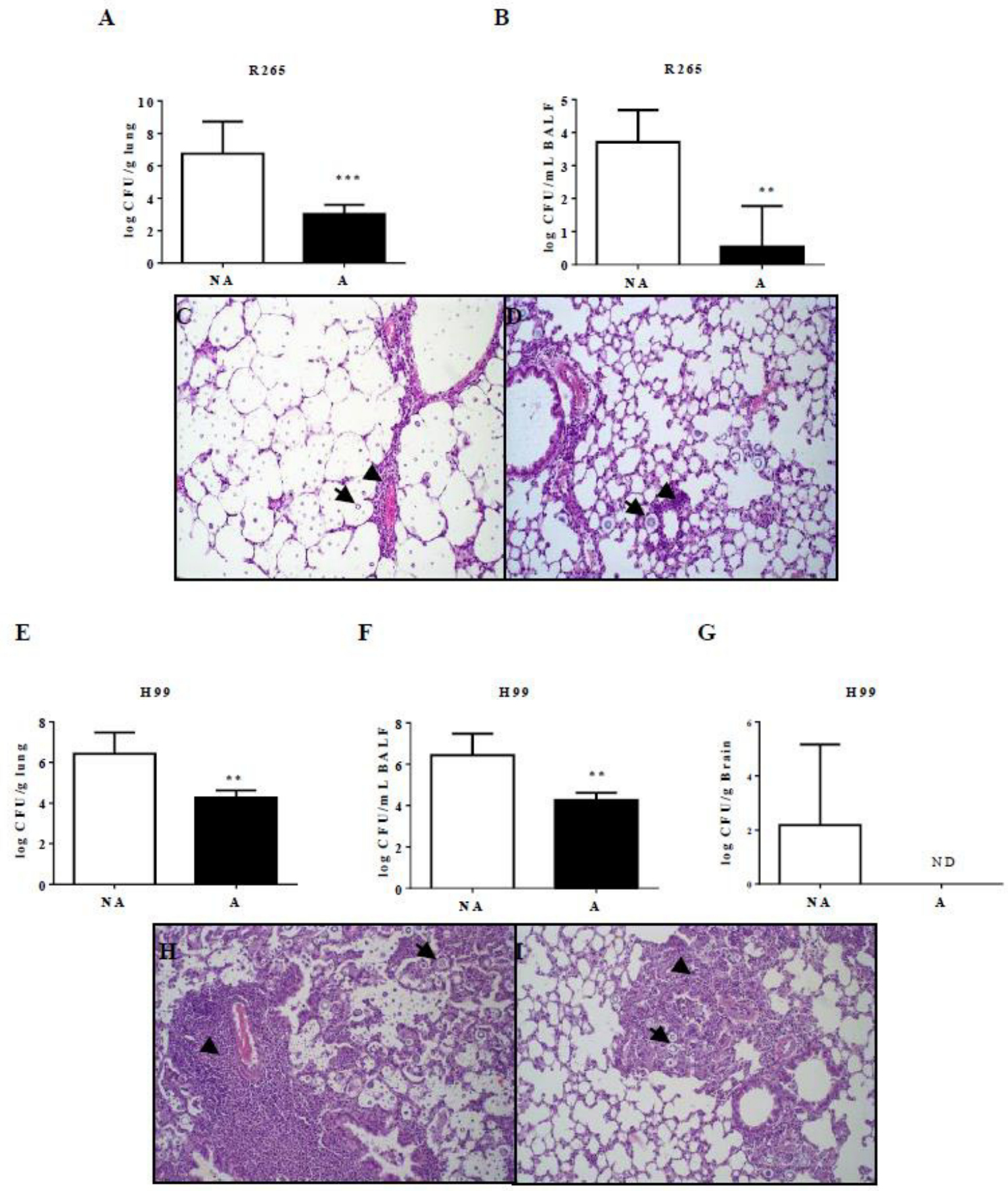


FIG 3. Animals infected with TBZ-adapted cells had lower fungal load in the lungs, BALF and brain and decreased of inflamation in the lungs. C57BL/6 mice were infected by intratracheal route with $1x10^5$ CFU of non-adapted -NA- and TBZ-adapted -A- cells for the CFU/g determination and histophatoly analysis. After 15 days of infection, it was observed higher fungal burden in the lungs (A) and Bronchoalveolar lavage fluid (BALF) (B) of animals infected with NA cells of *C. gattii* R265. The histopathology analysis of the lungs showed that NA cells caused more inflamation (C) than A cells (D). Animals infected with NA cells of *C. neoformans* also had higher load fungal in the lungs (E), BALF (F) and brain (G) and intense inflamation in the lungs (H) when compared to 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 head arrows indicate the inflammatory infiltrate.

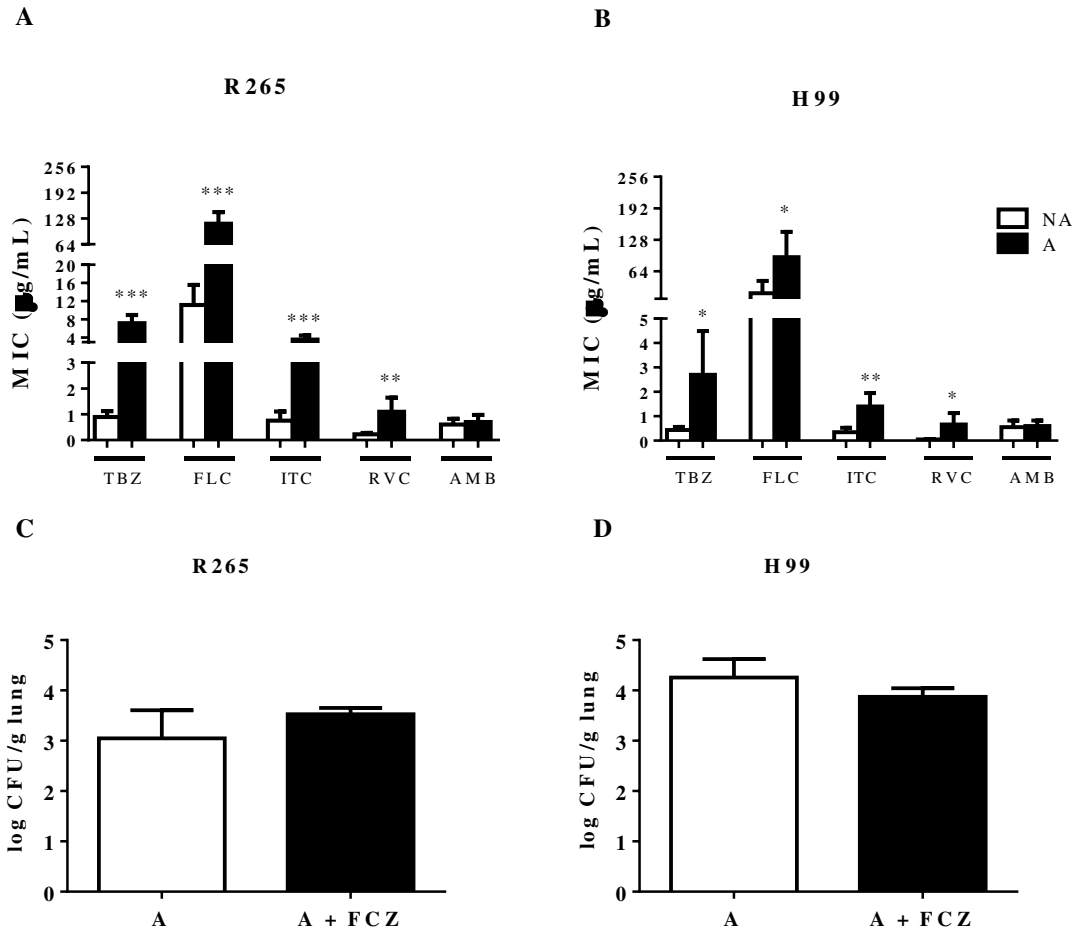


FIG. 4. TBZ-exposure caused antifungal resistance *in vivo*. The MIC for tebuconazole (TBZ), fluconazole (FLC), itraconazole (ITC), ravuconazole (RVC) and amphotericin B (AMB) was determined for colonies recovered from lungs of animals infected with non-adapted –NA- and TBZ-adapted -A- cells of *C. gattii* R265 (A) or *C. neoformans* H99 (B) and treated (A + FLC) or not (A) with FLC (10mg/Kg). After 15 days, the animals were euthanized and the lungs were collected to CFU/g determination. There was not statistical difference in the fungal load in the lungs of animals infected with TBZ-adapted cells of *C. gattii* R265 (C) or *C. neoformans* H99 (D) treated or not with FLC. * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

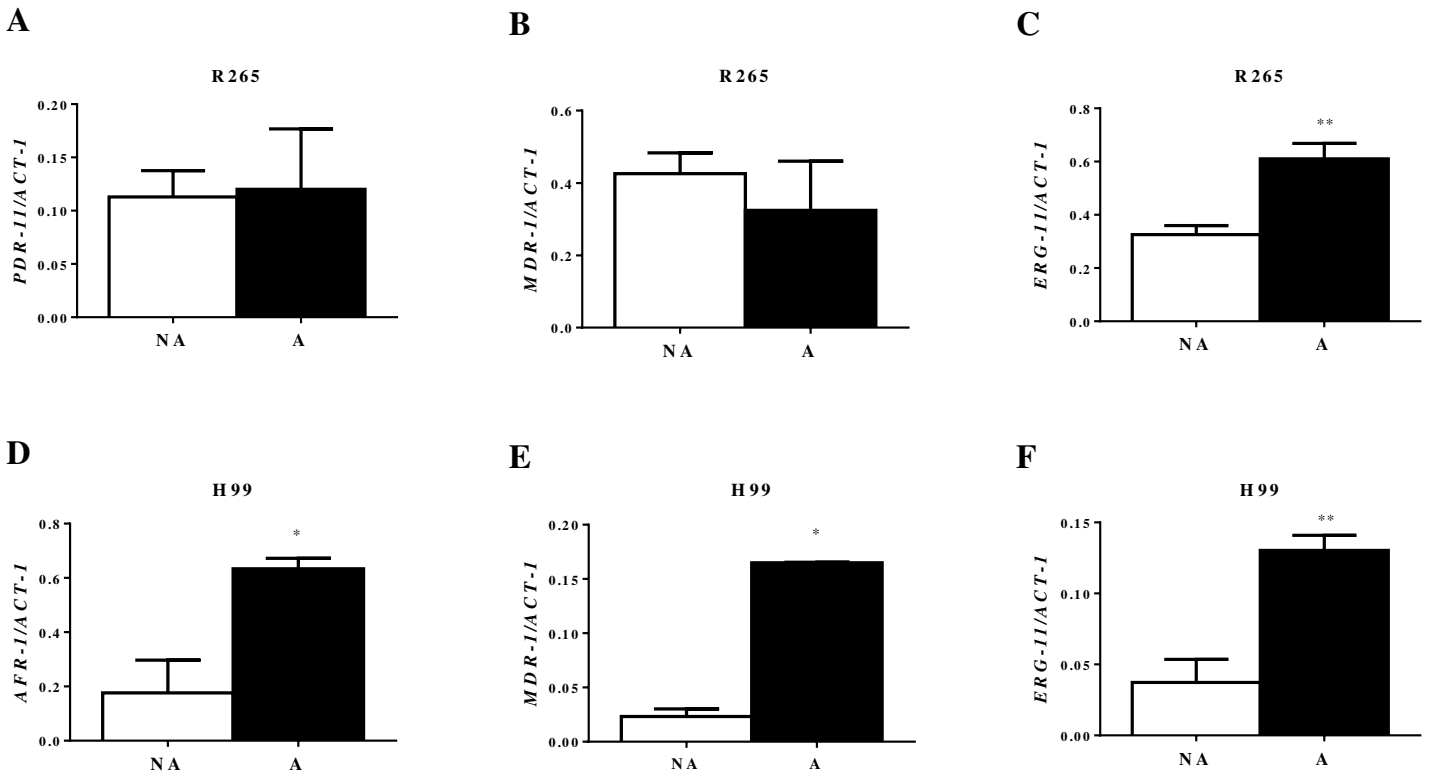


FIG. 5. Expression of *PDR1* (A) and *MDR1* (B), but not that of *ERG11* (C), was altered by tebuconazole exposure in *C. gattii* R265. In *C. neoformans* H99, the expression of *AFR1* (D), *MDR1* (E) and *ERG11* (F) was increased in adapted cells versus that of non-adapted cells. NA = non-adapted; A = TBZ-adapted; * $p < 0.05$; ** $p < 0.01$.