



HAL
open science

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

Rafael Wesley Bastos, Hellem Cristina Silva Carneiro, Lorena Vívien Neves Oliveira, Karen Maia Rocha, Gustavo José Cota Freitas, Marliete Carvalho Costa, Thaís Furtado Ferreira Magalhães, Vanessa Silva Dutra Carvalho, Cláudia Emanuela Rocha, Gabriella Freitas Ferreira, et al.

► **To cite this version:**

Rafael Wesley Bastos, Hellem Cristina Silva Carneiro, Lorena Vívien Neves Oliveira, Karen Maia Rocha, Gustavo José Cota Freitas, et al.. Environmental Triazole Induces Cross-Resistance to Clinical Drugs and Affects Morphophysiology and Virulence of *Cryptococcus gattii* and *C. neoformans*. *Antimicrobial Agents and Chemotherapy*, 2018, 62 (1), pp.e01179-17. 10.1128/AAC.01179-17 . pasteur-02652442

HAL Id: pasteur-02652442

<https://pasteur.hal.science/pasteur-02652442>

Submitted on 15 Jun 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

3

4 Running title: Effects of environmental triazole in *Cryptococcus*

5

6 Rafael Wesley Bastos¹, Hellem Cristina Silva Carneiro¹, Lorena Vívien Neves de
7 Oliveira¹, Karen Maia Rocha¹, Gustavo José Cota Freitas¹, Marliete Carvalho Costa¹,
8 Thaís Furtado Ferreira Magalhães¹, Vanessa Silva Dutra de Carvalho¹, Cláudia
9 Emanuela Rocha², Gabriella Freitas Ferreira³, Tatiane Alves de Paixão², Frédérique
10 Moyrand⁴, Guilhem Janbon⁴, Daniel Assis Santos^{1*}

11

12

13 ¹ Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal
14 de Minas Gerais, Belo Horizonte, Minas Gerais, Brasil.

15 ² Departamento de Patologia Geral, Instituto de Ciências Biológicas, Universidade Federal
16 de Minas Gerais, Belo Horizonte, Minas Gerais, Brasil.

17 ³ Departamento de Farmácia, Universidade Federal de Juiz de Fora - Campus
18 Governador Valadares, Governador Valadares, Brasil.

19 ⁴ Département de Mycologie, Institut Pasteur, Paris, France.

20

21 *Corresponding author: Daniel Assis Santos, Departamento de Microbiologia, Instituto
22 de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos,
23 6627, Pampulha, Belo Horizonte, Minas Gerais, Brazil, 31270-901. E-mail:
24 das@icb.ufmg.br or dasufmg@gmail.com; Fax: +55 31 3409 2733.

25

26 **Abstract**

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

48

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

51 **INTRODUCTION**

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

57 Tebuconazole (TBZ), an agrochemical triazole, has a broad spectrum of action
58 and is used to treat or prevent diseases in fruits, cereals, and vegetables. Tebuconazole
59 inhibits the fungal sterol-(lanosterol)-14- α demethylase, an enzyme that participates in
60 ergosterol synthesis (3).

61 Evidence clearly shows that pesticides can cause problems for human health (2,
62 4, 5). It is unclear, however, (i) what are the effects of agrochemicals on human
63 pathogens present in the environment and (ii) what are the indirect effects on human and
64 animal health. Some studies have shown that the exposure to environmental antifungals
65 selects subpopulations of medically important pathogens that are less susceptible to
66 clinical drugs (6–8). However, these studies did not describe the effects of pesticides on
67 the virulence of these pathogens after exposure and if resistance is maintained *in vivo*.

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

74 Cryptococcosis is treated with amphotericin B combined with fluconazole (FLC)
75 and/or 5-flucytosine. Other azole drugs, such as itraconazole, are also used in some

76 cases (11). Although antibiotic therapy is frequently effective, there are important
77 drawbacks. Amphotericin B and 5-flucytosine are nephrotoxic and hepatotoxic,
78 respectively, and they are not available in all countries (12). Regarding azole drugs,
79 especially fluconazole, the isolation of *Cryptococcus* spp. strains with increased
80 tolerance to these drugs is increasing (13, 14).

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

86

87 **RESULTS**

88

89 **Antifungal drug susceptibility testing and screening of subpopulations more** 90 **tolerant to tebuconazole (tebuconazole adaptation)**

91 As expected, all *C. gattii* and *C. neoformans* strains were inhibited by the drugs
92 tested: fluconazole, amphotericin B, and tebuconazole, at temperatures of 30 and 35°C
93 (Data not shown). All strains were also sensitive to tebuconazole when the MIC was
94 determined in solid medium (MIC^{solid}) (Table 1).

95 Further, we determined whether the strains were capable of growing in higher
96 concentrations of tebuconazole in a stepwise manner, and we studied whether the
97 temperature would affect this adaptation. Tables 1 and 2 show the Maximum
98 Concentration Achieved (MCA) of tebuconazole in the tebuconazole adaptation test and
99 MCA/Sub-MIC^{solid} ratios at 30 and 35°C (the higher the ratio, more passages through
100 tebuconazole-containing media occurred). When the adaptation was performed at 30°C,

101 38% (5) of *C. gattii* strains were able to grow in a concentration 10 times higher than
102 before the adaptation ($MCA/ Sub-MIC^{solid} = 10.0$) and the geometric mean of the ratio
103 was 5.53 (Table 1). However, when the tests were carried out at 35°C, the strains grew
104 in a lower concentration of tebuconazole (geometric mean = 2.78), demonstrating that
105 temperature affected the adaptation process (Table 1). The same phenomenon was
106 observed for *C. neoformans* strains, with a geometric mean of $MCA/Sub-MIC^{solid}$ ratio
107 at 30°C being almost 3-fold higher than when the test was performed at 35°C (Table 1).

108

109 Tebuconazole-adapted **colonies present cross-resistance with fluconazole and other** 110 **azole drugs**

111 Tables 2 and 3 show the MIC in liquid medium (MIC^{broth}) for fluconazole and
112 tebuconazole for non-adapted (NA) and tebuconazole-adapted (A) colonies of *C. gattii*
113 and *C. neoformans*, respectively, when the adaptation was performed at 30°C. Despite
114 adaptation, not all *C. gattii* strains exhibited alterations of at least two dilutions of the
115 MIC for tebuconazole compared to the MIC of NA colonies (Tables 2 and 3). Overall,
116 61.5% (8) of *C. gattii* (Table 2) and 100% (4) of *C. neoformans* (Table 3) tebuconazole-
117 adapted cells became more resistant to the antifungal environment than the NA colonies
118 when the tests were performed at the same adaptation temperature. In contrast, when
119 MIC assays were carried out at 35°C using cells adapted at 30°C, 38% (5) of *C. gattii*
120 (Table 2) and 50% (2) of *C. neoformans* strains (Table 3) tebuconazole-adapted
121 colonies became more resistant to tebuconazole.

122 We then tested whether tebuconazole adaptation can also decrease susceptibility
123 to fluconazole. Geometric means of tebuconazole-adapted colonies at 30°C and 35°C
124 increased almost 3-fold and 2-fold for *C. gattii* and 5-fold and 3-fold for *C. neoformans*,
125 respectively (Tables 2 and 3). Additionally, the adaptation selected populations with

126 cross-resistance (CR). Thirty eight percent (5) of *C. gattii* and 100% (4) of *C.*
127 *neoformans* tebuconazole-adapted colonies presented higher MICs for fluconazole, as
128 well as for tebuconazole (Tables 2–4). Four and three strains of *C. gattii* (R265, ATCC
129 32608, L27/01, and 196L/03) and *C. neoformans* (H99, ATCC 24067, and ATCC
130 62066), respectively, returned to the original phenotype when grown in medium without
131 the agrochemical (10p) (Tables 2 and 3) and this was named as “temporary CR”. This
132 phenomenon occurred dependently (ATCC 32608, L27/01 and ATCC 62066) or
133 independently (R265, 196L/03, H99 and ATCC 24067) on the temperature for different
134 strains (Tables 2–4). The other strain of *C. gattii* (ATCC 24065) and *C. neoformans*
135 (ATCC 28957), which showed CR with fluconazole, did not return to the original
136 susceptibility phenotype even after growth in tebuconazole-free medium, demonstrating
137 a “permanent CR” (Tables 3–5). This profile also appears to be temperature-dependent
138 (Table 2–4).

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

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

151 subcloning several times on non-selective medium. For one strain (196L/03) the
152 phenotype was stable even after several subcultures on agrochemical-free medium
153 (Tables 4 and 6). However, only adapted cells from *C. neoformans* H99 exhibited an
154 increased MIC to tebuconazole, but lacked CR with fluconazole (Tables 4 and 7).

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

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

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

169

170 **Tebuconazole-adaptation induced morphophysiological changes**

171 Tebuconazole adaptation caused morphological changes in *Cryptococcus* spp.
172 The adapted cells of *C. gattii* R265 exhibited a significantly increased diameter (p
173 <0.05) (Fig. 1A) and a decreased surface/volume ratio (Fig. 1B) compared to NA cells
174 ($p<0.05$), but the capsule size was maintained (Fig. 1C). Strikingly, 70–90%
175 tebuconazole-adapted cells of *C. neoformans* H99 presented elongated, irregular shapes,

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

180 In contrast, adaptation and the morphological changes did not affect the growth
181 rate in SDA (Data not shown).

182

183 **Tebuconazole adaptation decreased the virulence of *C. gattii* R265 and *C.*** 184 ***neoformans* H99 in C57BL/6 mice**

185 Mice infected with NA cells of *C. gattii* R265 and *C. neoformans* H99
186 succumbed significantly ($p < 0.05$) earlier than those infected with tebuconazole-adapted
187 cells of the same strain (Fig. 2A and B). Interestingly, R265-adapted cells were not able
188 to kill the animals, even after 80 days (Fig. 2A).

189 Additionally, animals were infected for a better characterization of the disease
190 caused by the different cells. The fungal burden in the lungs (Fig. 3A and E) and
191 bronchoalveolar lavage fluid (BALF) (Fig. 3B and F) was significantly higher ($p < 0.05$)
192 in mice infected with NA cells in both strains. *C. gattii* R265 cells were not detected in
193 the brain of any infected animal; however, NA cells of *C. neoformans* H99 disseminated
194 into the brain in 40% of infected animals, unlike the tebuconazole-adapted cells (Fig.
195 3G).

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

201 alveolar thickening due to mononuclear inflammatory infiltrate (Fig. 3C). Mice infected
202 with non-adapted *C. neoformans* H99 demonstrated an intense and diffuse amount of
203 yeasts in the pulmonary parenchyma (Fig. 3H). We also observed an intense mixed
204 perivascular inflammatory infiltrate and intense and multifocal to diffuse mixed
205 inflammatory infiltrate with predominance of macrophages and multinucleated giant
206 cells in the alveolar space (Fig. 3H). However, the animals infected with adapted cells
207 exhibited a reduced amount of yeasts observed in lung parenchyma, and consequently a
208 significant reduction in associated inflammation (Fig. 3D and I). No change was
209 observed in the lungs of control group mice (non-infected mice).

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

214

215 **Tebuconazole adaptation caused antifungal cross-resistance *in vivo***

216 The MIC for all azole drugs in colonies recovered from the lungs of animals
217 infected with tebuconazole-adapted cells was significantly ($p < 0.05$) higher than that
218 for drugs recovered from animals infected with NA cells (Fig. 4A and B). Furthermore,
219 fluconazole did not reduce the fungal burden in the lungs ($p > 0.05$) from mice (Fig. 4C
220 and D), which was also observed in histology analyses (Data not shown).

221

222 **Tebuconazole adaptation induced different mechanisms of resistance in *C. gattii* 223 and *C. neoformans***

224 We investigated the mechanism involved in the increased MIC for azole drugs
225 for the adapted versus non-adapted *C. gattii* R265 and *C. neoformans* H99. Figure 5

226 shows that the gene expression levels of *ERG-11*, but not those of the efflux pumps
227 *PDR-11* and *MDR-1* (Fig. 5A-C), were significantly ($p < 0.05$) higher in adapted cells
228 than non-adapted of *C. gattii* R265. However, in *C. neoformans* H99, all genes (*ERG11*,
229 *AFR-1*, and *MDR-1*) were expressed at a higher level ($p < 0.05$) in tebuconazole-
230 exposed cells (Fig. 5D-F).

231

232

233 **DISCUSSION**

234 In this study, we showed that exposing *C. gattii* and *C. neoformans* to the
235 triazole agrochemical tebuconazole resulted in greater tolerance, *in vitro* and *in vivo*, to
236 clinical drugs (fluconazole and itraconazole) and less virulence in a murine model when
237 compared with tolerance and virulence of cells not exposed to the agrochemical. We
238 also tested an azole drug currently in clinical trials (ravuconazole) (21) to study whether
239 there would be CR with drugs not yet commercially available, which was confirmed.

240 First, we showed that previous exposure to tebuconazole selects cells with
241 permanent or temporary CR with fluconazole. The major mechanism of resistance to
242 azole drugs that has been demonstrated for *Cryptococcus* spp. is the overexpression of
243 efflux pumps genes (*AFR1*, *AFR2*, *PDR11*, and *MDR1*) (24, 27) and, in some cases,
244 overexpression of the target of these drugs, ERG11p (25). Here, we showed that the
245 mechanism of azole tolerance in *C. gattii* and *C. neoformans* can be different in strains
246 that presented temporary CR, such H99 and R265. We observed increased expression of
247 efflux pumps and *ERG11* in *C. neoformans* H99, but only of *ERG11* in *C. gattii* R265.
248 These results demonstrated that although the adapted cells of both species exhibited
249 increased MICs for all the same azole drugs, the mechanism for each species, and
250 possibly for each strain, was different. Another study confirmed that resistance

251 mechanisms can be strain-dependent, demonstrating that one azole-heteroresistant strain
252 of *C. gattii* expressed more *PDR11* and *ERG11* than original cells (25). In *Candida*
253 *parapsilosis*, Rocha and colleagues reported that the exposure to the agrochemical
254 tetraconazole selected cells more resistant to azoles drugs because of overexpression of
255 efflux pumps, but they did not observe altered *ERG11* expression (7).

256 Tebuconazole exposure also caused changes in azole susceptibility that were
257 observed even after 10 passages on agrochemical-free medium. We called this
258 phenomenon permanent CR, and it can indicate that a mutation could have occurred to
259 confer resistance to the cells. Moreover, a study with *Aspergillus fumigatus* exposed to
260 environmental antifungals, including tebuconazole, showed that *ERG11* mutation
261 causes resistance to fluconazole and other azoles, except for itraconazole (8). Other
262 studies also showed that CR between fluconazole and itraconazole is not common (28,
263 29). These phenomena may occur because itraconazole, in addition to inhibiting
264 ERG11p, also inhibits NADH-dependent 3-ketosteroid reductase (an enzyme that
265 catalyzes one of the last reactions of ergosterol synthesis) (28, 30) and the mechanism
266 of resistance can be different than that for fluconazole (31). More studies should
267 determine the mechanisms involved in the permanent CR caused by agrochemical
268 exposure.

269 Temperature is a critical factor that the human pathogenic fungi must overcome
270 to cause illness (32). To test whether temperature can also affect the process of
271 acquiring resistance, the strains were exposed to tebuconazole and incubated at 30 or
272 35°C. When adaptation was carried out at the lower temperature, the strains supported
273 higher concentrations of the drug and more strains became tolerant to the pesticide
274 compared when the experiment was performed at 35°C. This indicates that the
275 temperature of 30°C is more favorable for the fungus to develop resistance, probably

276 because the optimal temperature of growth of *Cryptococcus* is around 25°C (33). In
277 addition to the importance of temperature during the adaptation process, we showed that
278 it is relevant for determining MIC (incubation temperature). The tebuconazole-adapted
279 colonies grown at 30°C were less tolerant to the drugs than colonies grown at 35°C,
280 indicating that resistance in the environment may not occur *in vivo* because of the body
281 temperature of endothermic animals. These data may also explain, at least in part, why
282 *Cryptococcus* spp. are not considered to be a major problem in antimicrobial resistance
283 in clinical practice (12).

284 The morphological characteristics of *Cryptococcus* cells influence their
285 virulence (31). Usually, cells with a higher diameter and a smaller capsule are less
286 virulent (20, 23, 34). Here, we observed that tebuconazole-adapted cells, which
287 exhibited higher diameters than those of NA cells, were less virulent in both species.
288 Morphological analysis also showed that tebuconazole-adapted cells of *C. neoformans*
289 H99 exhibited pseudohyphae formation. Pseudofilamentation happens when budding
290 cells do not fully separate, so a chain of bound cells is formed (35). This process is
291 common in *Candida* (35), but rare and little-studied in *Cryptococcus*.
292 Pseudofilamentation seems to be a response to overcome environmental stresses (36),
293 but during this process, the cells became less virulent (37), as our results demonstrated.
294 This decreased virulence may occur because cell surface molecules become
295 differentially presented, and they cannot be phagocytosed by macrophages (38) and do
296 not reach the central nervous system (35, 37). The zeta potential data confirmed that
297 pseudohyphae formation can cause changes in cell surface molecules that influence the
298 electronegativity of the cell surface.

299 To better characterize the influence of tebuconazole exposure on virulence, we
300 analyzed the microscopic changes and the fungal quantity in the lungs. The higher

301 fungal burden in the lungs and BALF of animals infected with NA cells of both species
302 agreed with the survival curve data. Further, we detected fungus in the brain only of the
303 animals infected with NA cells of *C. neoformans* H99. These results support those of
304 previous studies that pseudohyphae do not reach the central nervous system (35, 37),
305 which explains why these cells are less virulent than NA cells.

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

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

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

324

325

326 MATERIALS AND METHODS

327

328 Microorganisms and study design

329 We used 13 strains of *C. gattii* (nine clinical and two environmental isolates, all
330 from the culture collection of the Laboratório de Micologia da Universidade Federal de
331 Minas Gerais, Minas Gerais, Brazil, and two reference strains from the culture
332 collection of the University of Georgia, Atlanta, GA) (Table 1) (17). We also used four
333 strains of *C. neoformans* (one clinical and three reference strains) (Table 1) (18). All
334 isolates were maintained on Sabouraud Dextrose Agar (SDA) at 4°C.

335 Antifungal susceptibility, tebuconazole adaptation, and cross-resistance tests
336 were performed for all strains. The *C. gattii* R265 and *C. neoformans* H99 strains were
337 chosen for further tests (morphophysiological, virulence changes, *in vivo* antifungal
338 resistance, and RT-PCR).

339

340 Antifungal drug susceptibility testing

341 The minimum inhibitory concentrations (MICs) for fluconazole (FLC) (Sigma-
342 Aldrich, St. Louis, MO), amphotericin B (AMB) (Sigma-Aldrich), and the agricultural
343 fungicide tebuconazole (TBZ) (ALTERNE[®]) were determined using the microdilution
344 method proposed by the Clinical and Laboratory Standards Institute (CLSI) M27-A3
345 method (MIC^{broth}) (19). The MIC for tebuconazole also was determined by spot tests on
346 SDA supplemented with different concentrations of the pesticide (MIC^{solid}) (20). For the
347 spot tests, cell suspensions containing $1-5 \times 10^4$ cells were plated onto SDA plates
348 containing different concentrations of tebuconazole (from 0.125 to 256.0 µg/mL). The
349 growth pattern was determined after 72 h of incubation. The MIC^{broth} and MIC^{solid} tests
350 were performed at two different incubation temperatures: 30 and 35°C. All tests were

351 performed in duplicate for each strain and the tests were repeated at least twice to
352 confirm the results.

353

354 **Tebuconazole adaptation (Screening for subpopulations more tolerant to**
355 **tebuconazole)**

356 After performing susceptibility testing on solid medium (MIC^{solid}), the strains
357 were grown on SDA with increasing concentrations of the pesticide. Initially, all strains
358 were grown on medium supplemented with tebuconazole at a concentration of the
359 $MIC/2$ (sub-MIC). After one week, an inoculum using at least five colonies was
360 prepared in sterile saline, and the transmittance (530 nm) of the suspensions was
361 adjusted to 75–77% ($1-5 \times 10^6$ fungal cells). Subsequently, 10 μ L of this suspension
362 was inoculated on a medium containing tebuconazole at the MIC. After one week, the
363 process was repeated and the strains were grown, in a stepwise manner, at increasing
364 concentrations of tebuconazole until the concentration where the growth ceased or until
365 cultivation at 256 μ g/mL. These tests were performed both at 30 and 35°C. The colonies
366 that were exposed to tebuconazole were named tebuconazole-adapted (A) colonies and
367 the original colonies (no exposure) were named non-adapted (NA) colonies.

368 The highest concentration of tebuconazole that the fungus was capable of
369 growing in after the tebuconazole adaptation tests was called the Maximum
370 Concentration Achieved (MCA). We also quantified the ability of the microorganisms
371 to grow in the presence of the agrochemical by determining the MCA to sub-MIC ratio
372 (MCA/sub-MIC).

373

374

375

376 **Cross-resistance tests (CR)**

377 The MIC^{broth} for fluconazole, amphotericin B, and tebuconazole was determined
378 for non-adapted and tebuconazole-adapted colonies. The test was performed at 30 and
379 35°C for the colonies adapted at 30°C and at 35°C for the colonies adapted at 35°C. A
380 strain was considered cross-resistant when it presented decreased susceptibility both to
381 tebuconazole and clinical drugs.

382 To test the stability of the cross-resistance to fluconazole and tebuconazole, at
383 least five colonies of each adapted strain that showed increased MIC^{broth} (at least four
384 times) were mixed, and then were subcultured every 48 h on SDA plates without
385 tebuconazole for 10 passages (10p colonies) (20). Next, we determined the MIC^{broth} for
386 10p colonies.

387 We also tested the CR between tebuconazole and itraconazole (ITC) (Sigma-
388 Aldrich) and ravuconazole (RVC) (Sigma-Aldrich) (an azole in Phase II trials) (21) for
389 the tebuconazole-adapted and 10p colonies that showed CR with fluconazole.

390

391 **Morphometric and zeta potential analysis.**

392 Non-adapted and tebuconazole-adapted colonies were grown on SDA and SDA
393 supplemented with tebuconazole at MCA, respectively, for 72 h at 30°C. Subsequently,
394 the cells were visualized in a suspension in India ink with an optical microscope
395 (Axioplan; Carl Zeiss) and the slides were photographed using a Coolpix 4500 (Nikon)
396 digital camera. The capsule and diameter of at least 50 cells with regular form were
397 measured using ImageJ 1.40 g software (<http://rsb.info.nih.gov/ij/>; National Institutes of
398 Health, NIH, Bethesda, MD). In addition, the surface-to-volume ratio (S/V) was
399 calculated using the formula $3/r$, where r is the radius (20). For cells with irregular form,
400 qualitative analyses were performed. The zeta potentials of the NA and A yeast cells

401 were calculated using a zeta potential analyzer (Zetasizer NanoZS90; Malvern, United
402 Kingdom) as described previously (22).

403

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

405 C57BL/6 male mice, 6–8 weeks-old, were used for animal experiments. All
406 experimental procedures were carried out according to the standards of the Brazilian
407 Society of Laboratory Animal Science/Brazilian College for Animal Experimentation
408 (available at <http://www.cobea.org.br/>). The study was approved by the Ethics
409 Committee in Animal Experimentation of the Universidade Federal de Minas Gerais
410 (CEUA/UFMG, protocol # 306 / 2015).

411 The animals (six per group) were anesthetized by intraperitoneal (i.p.) injection
412 with ketamine hydrochloride (60 mg/kg) and xylazine (10 mg/kg) in sterile saline. Next,
413 each animal received 30 μ L of 1×10^5 cells of *C. gattii* R265 or *C. neoformans* H99 by
414 the intratracheal route. The mice were monitored daily for survival (20).

415 Other groups of animals were infected and euthanized under anesthesia 15 days
416 post-inoculation to obtain lungs, bronchoalveolar lavage fluid (BALF), and brain tissue.
417 The organ homogenates and BALF were plated onto SDA to determine the fungal
418 burden, expressed as colony-forming unit per gram (CFU/g) or mL (23). We also
419 determined the MIC^{broth} for tebuconazole, fluconazole, itraconazole, ravuconazole, and
420 amphotericin B for the colonies recovered from the lungs. Moreover, lungs were
421 collected, fixed in formalin, embedded in paraffin, sectioned, and stained with
422 hematoxylin-eosin (HE) for histopathological analysis.

423 To test the antifungal cross-resistance *in vivo*, mice infected with tebuconazole-
424 adapted *C. gattii* R265 and *C. neoformans* H99 received 10 mg/kg of fluconazole daily

425 by the intraperitoneal route. Fifteen days post-inoculation, the animals were euthanized
426 and the lungs collected for CFU/g determination.

427

428 **RNA extraction and RT-PCR analysis**

429 Non-adapted and adapted cells of *C. gattii* R265 and *C. neoformans* H99 were
430 grown on SDA and SDA + tebuconazole plates, respectively, at 30°C. After 72 h, the
431 colonies were collected and the RNA was extracted using Trizol reagent (Invitrogen)
432 following the manufacturer's instructions. Total RNA (5 µg) was subjected to DNase I
433 treatment (Roche), and then 1 µg of the DNaseI-treated RNA was used for reverse-
434 transcription (RT) using the QuantiTect Reverse Transcription (Qiagen) kit.
435 Subsequently, the cDNAs were PCR-amplified in the presence of dCTP (α 33P) (Perkin
436 Elmer) with the primers for the following genes: *ACT1*, *AFR1*, and *MDR1* for *C.*
437 *neoformans* and *ACT1*, *MDR1* (24), and *PDR11* (25) for *C. gattii*. PCR products were
438 resolved on a 7.5% polyacrylamide gel and quantified using a Typhoon 9200 imager
439 and ImageQuant 5.2 software (Molecular dynamics) (26).

440

441 **Statistical analyses**

442 All statistical analyses were performed using GraphPad Prism, version 6.00, for
443 Windows (GraphPad Software, San Diego, CA, USA) with $p < 0.05$ considered
444 significant. The results of antifungal experiments (*in vitro* and *in vivo*), morphometric
445 analysis, zeta potential, quantification of CFU/g organs, or BALF and RT-PCR were
446 analyzed by Student's t test. Survival curves were plotted by Kaplan-Meier analysis and
447 results were analyzed using the log rank test. All tests, including animal experiments,
448 were repeated at least twice.

449

450 **Acknowledgements**

451 This study was supported by Fundação de Amparo a Pesquisa do Estado de
452 Minas Gerais - FAPEMIG (Grant APQ-00727-16) and Conselho Nacional de
453 Desenvolvimento Científico e Tecnológico - CNPq (Grant 403006/2016-3). RWB
454 received fellowships from CNPq and Coordenação de Aperfeiçoamento de Pessoal de
455 Nível Superior (PDSE-CAPES -88881.131683/2016-01). DAS is a research fellow of
456 the CNPq (Grant 305154/2014-1).

457

458 **Transparency declarations.**

459 None to declare.

460

461 **REFERENCES**

462

- 463 1. Wu W, Ma B. 2015. Integrated nutrient management (INM) for sustaining crop productivity
464 and reducing environmental impact: a review. *Sci Total Environ* 512-513: 415-427.
- 465 2. Rigotto RM, Vasconcelos DP, Rocha MM. 2014. Pesticide use in Brazil and problems for
466 public health. *Cad Saude Publica* 30: 1360-1362.
- 467 3. Kwork IMR, Loeffler L. 1993. The biochemical mode of action of some newer azole
468 fungicides. *Pest Management Science* 39: 1-11.
- 469 4. Health COE. 2012. Pesticide exposure in children. *Pediatrics* 130: e1757-1763.
- 470 5. Crinnion WJ. 2009. Chlorinated pesticides: threats to health and importance of detection.
471 *Altern. Med. Rev* 14: 347-359.
- 472 6. Serfling A, Wohlrab J, Deising HB. 2007. Treatment of a clinically relevant plant-pathogenic
473 fungus with an agricultural azole causes cross-resistance to medical azoles and potentiates
474 caspofungin efficacy. *Antimicrob Agents Chemother* 51: 3672-3676.

475 7. Rocha MF, Alencar LP, Paiva MA, Melo LM, Bandeira SP, Ponte YB, Sales JA, Guedes GM,
476 Castelo-Branco DS, Bandeira TJ, Cordeiro RA, Pereira-Neto WA, Brandine GS, Moreira JL,
477 Sidrim JJ, Brilhante RS. 2016. Cross-resistance to fluconazole induced by exposure to the
478 agricultural azole tetraconazole: an environmental resistance school? *Mycoses* 59: 281-290.

479 8. Ren J, Jin X, Zhang Q, Zheng Y, Lin D, Yu Y. 2017. Fungicides induced triazole-resistance in
480 *Aspergillus fumigatus* associated with mutations of TR46/Y121F/T289A and its appearance in
481 agricultural fields. *J Hazard Mater* 326: 54-60.

482 9. Kronstad JW, Attarian R, Cadieux B, Choi J, D'Souza CA, Griffiths EJ, Geddes JM, Hu G,
483 Jung WH, Kretschmer M, Saikia S, Wang J. 2011. Expanding fungal pathogenesis: *Cryptococcus*
484 breaks out of the opportunistic box. *Nat Rev Microbiol* 9: 193-203.

485 10. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. 2009.
486 Estimation of the current global burden of cryptococcal meningitis among persons living with
487 HIV/AIDS. *AIDS* 23: 525-530.

488 11. Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ, Harrison TS,
489 Larsen RA, Lortholary O, Nguyen MH, Pappas PG, Powderly WG, Singh N, Sobel JD, Sorrell
490 TC. 2010. Clinical practice guidelines for the management of cryptococcal disease: 2010 update
491 by the infectious diseases society of america. *Clin Infect Dis* 50: 291-222.

492 12. Perfect JR, Bicanic T. 2015. Cryptococcosis diagnosis and treatment: What do we know now?
493 *Fungal Genet Biol* 78: 49-54.

494 13. Smith KD, Achan B, Hullsiek KH, McDonald TR, Okagaki LH, Alhadab AA, Akampurira A,
495 Rhein JR, Meya DB, Boulware DR, Nielsen K, ASTRO-CM/COAT Team. 2015. Increased
496 Antifungal Drug Resistance in Clinical Isolates of *Cryptococcus neoformans* in Uganda.
497 *Antimicrob Agents Chemother* 59: 7197-7204.

498 14. Chen YC, Chang TY, Liu JW, Chen FJ, Chien CC, Lee CH, Lu CH. 2015. Increasing trend of
499 fluconazole-non-susceptible *Cryptococcus neoformans* in patients with invasive cryptococcosis: a
500 12-year longitudinal study. *BMC Infect Dis* 15: 277.

501 15. Springer DJ, Ren P, Raina R, Dong Y, Behr MJ, McEwen BF, Bowser SS,
502 Samsonoff WA, Chaturvedi S, Chaturvedi V. 2010. Extracellular fibrils of pathogenic yeast
503 *Cryptococcus gattii* are important for ecological niche, murine virulence and human neutrophil
504 interactions. PLoS One 5: e10978.

505 16. Del Poeta M, Casadevall A. 2012. Ten challenges on *Cryptococcus* and cryptococcosis.
506 Mycopathologia 173: 303-310.

507 17. Santos JR, Gouveia LF, Taylor EL, Resende-Stoianoff MA, Pianetti GA, César IC, Santos
508 DA. 2012. Dynamic interaction between fluconazole and amphotericin B against *Cryptococcus*
509 *gattii*. Antimicrob Agents Chemother 56: 2553-2558.

510 18. Magalhães TF, da Silva CM, de Fátima A, da Silva DL, Modolo LV, Martins CV, Alves RB,
511 Ruiz AL, Longato GB, de Carvalho JE, de Resende-Stoianoff MA. 2013. Hydroxyaldimines as
512 potent in vitro anticryptococcal agents. Lett Appl Microbiol 57: 137-143.

513 19. CLINICAL AND LABORATORY STANDARDS INSTITUTE. 2012. Reference method for
514 broth dilution antifungal susceptibility testing of yeasts: fourth informational supplement, CLSI
515 document M27- S4. Clinical and Laboratory Standards Institute, Wayne, PA.

516 20. Ferreira GF, Santos JR, Costa MC, Holanda RA, Denadai AM, Freitas GJ, Santos AR,
517 Tavares PB, Paixão TA, Santos DA. 2015. Heteroresistance to itraconazole alters the morphology
518 and increases the virulence of *Cryptococcus gattii*. Antimicrob Agents Chemother 59: 4600-4609.

519 21. Allen D, Wilson D, Drew R, Perfect J. 2015. Azole antifungals: 35 years of invasive
520 fungal infection management. Expert Rev Anti Infect Ther 13: 787-798.

521 22. Nosanchuk JD, Cleare W, Franzot SP, Casadevall A. 1999. Amphotericin B and fluconazole
522 affect cellular charge, macrophage phagocytosis, and cellular morphology of *Cryptococcus*
523 *neoformans* at subinhibitory concentrations. Antimicrob Agents Chemother 43: 233-239.

524 23. Santos JR, Holanda RA, Frases S, Bravim M, Araujo GeS, Santos PC, Costa MC, Ribeiro MJ,
525 Ferreira GF, Baltazar LM, Miranda AS, Oliveira DB, Santos CM, Fontes AC, Gouveia LF,
526 Resende-Stoianoff MA, Abrahão JS, Teixeira AL, Paixão TA, Souza DG, Santos DA. 2014.

527 Fluconazole alters the polysaccharide capsule of *Cryptococcus gattii* and leads to distinct
528 behaviors in murine Cryptococcosis. PLoS One 9: e112669.

529 24. Basso LR, Gast CE, Bruzual I, Wong B. 2015. Identification and properties of plasma
530 membrane azole efflux pumps from the pathogenic fungi *Cryptococcus gattii* and *Cryptococcus*
531 *neoformans*. J Antimicrob Chemother 70: 1396-1407.

532 25. Sykes JE, Hodge G, Singapuri A, Yang ML, Gelli A, Thompson GR 3rd. 2017. *In vivo*
533 development of fluconazole resistance in serial *Cryptococcus gattii* isolates from a cat. Med
534 Mycol 55: 396-401.

535 26. Gonzalez-hilarion S, Paulet d, Lee KT, Hon CC, Lechat P, Mogensen E, Moyrand F, Proux C,
536 Barboux R, Bussotti G, Hwang J, Coppée JY, Bahn YS, Janbon G. 2016. Intron retention-
537 dependent gene regulation in *Cryptococcus neoformans*. Scientific Reports 6: 32252.

538 27. Yang ML, Uhrig J, Vu K, Singapuri A, Dennis M, Gelli A, Thompson GR. 2016. Fluconazole
539 Susceptibility in *Cryptococcus gattii* Is Dependent on the ABC Transporter Pdr11. Antimicrob
540 Agents Chemother 60: 1202-1207.

541 28. Espinel-Ingroff A, Aller AL, Canton E, Castañón-Olivares LR, Chowdhary A, Cordoba S,
542 Cuenca-Estrella M, Fothergill A, Fuller J, Govender N, Hagen F, Illnait-Zaragozi MT, Johnson E,
543 Kidd S, Lass-Flörl C, Lockhart SR, Martins MA, Meis JF, Melhem MS, Ostrosky-Zeichner L,
544 Pelaez L, Pfaller MA, Schell WA, St-Germain G, Trilles L, Turnidge J. 2012. *Cryptococcus*
545 *neoformans*-*Cryptococcus gattii* species complex: an international study of wild-type
546 susceptibility endpoint distributions and epidemiological cutoff values for fluconazole,
547 itraconazole, posaconazole, and voriconazole. Antimicrob Agents Chemother 56: 5898-5906.

548 29. Trpković A, Pekmezović M, Barać A, Crnčević Radović L, Arsić Arsenijević V. 2012. *In*
549 *vitro* antifungal activities of amphotericin B, 5-fluorocytosine, fluconazole and itraconazole
550 against *Cryptococcus neoformans* isolated from cerebrospinal fluid and blood from patients in
551 Serbia. J Mycol Med 22: 243-248.

552 30. Vanden Bossche H, Marichal P, Le Jeune L, Coene MC, Gorrens J, Cools W. 1993. Effects of
553 itraconazole on cytochrome P-450-dependent sterol 14 alpha-demethylation and reduction of 3-
554 ketosteroids in *Cryptococcus neoformans*. Antimicrob Agents Chemother 37: 2101-2105.

555 31. Sionov E, Chang YC, Garraffo HM, Dolan MA, Ghannoum MA, Kwon-Chung KJ. 2012.
556 Identification of a *Cryptococcus neoformans* cytochrome P450 lanosterol 14 α -demethylase
557 (Erg11) residue critical for differential susceptibility between fluconazole/voriconazole and
558 itraconazole/posaconazole. Antimicrob Agents Chemother 56: 1162-1169.

559 32. Bielska E, May RC. 2016. What makes *Cryptococcus gattii* a pathogen? FEMS Yeast Res 16:
560 fov106.

561 33. Howard DH. 1961. Some factors which affect the initiation of growth of *Cryptococcus*
562 *neoformans*. J Bacteriol 82: 430-435.

563 34. Okagaki LH, Strain AK, Nielsen JN, Charlier C, Baltes NC, Chrétien F, Heitman J, Dromer
564 F, Nielsen K. 2010. Cryptococcal cell morphology affects host cell interactions and
565 pathogenicity. PLoS Pathog 6: e1000953.

566 35. Trevijano-Contador N, Rueda C, Zaragoza O. 2016. Fungal morphogenetic changes inside the
567 mammalian host. Semin Cell Dev Biol 57: 100-109.

568 36. Lee SC, Phadke S, Sun S, Heitman J. 2012. Pseudohyphal growth of *Cryptococcus*
569 *neoformans* is a reversible dimorphic transition in response to ammonium that requires Amt1 and
570 Amt2 ammonium permeases. Eukaryot Cell 11: 1391-1398.

571 37. Zhai B, Zhu P, Foyle D, Upadhyay S, Idnurm A, Lin X. 2013. Congenic strains of the
572 filamentous form of *Cryptococcus neoformans* for studies of fungal morphogenesis and virulence.
573 Infect Immun 81: 2626-2637.

574 38. Lin J, Idnurm A, Lin X. Morphology and its underlying genetic regulation impact the
575 interaction between *Cryptococcus neoformans* and its hosts. 2015. Med Mycol 53: 493-504.

576 39. Rossi SA, Trevijano-Contador N, Scorzoni L, Mesa-Arango AC, de Oliveira HC,
577 Werther K, de Freitas Raso T, Mendes-Giannini MJ, Zaragoza O, Fusco-Almeida AM.

578 2016. Impact of Resistance to Fluconazole on Virulence and Morphological Aspects of
579 *Cryptococcus neoformans* and *Cryptococcus gattii* Isolates. Front Microbiol 7: 153.

580 40. Sionov E, Chang YC, Garraffo HM, Kwon-Chung KJ. 2009. Heteroresistance to
581 fluconazole in *Cryptococcus neoformans* is intrinsic and associated with virulence.
582 Antimicrob Agents Chemother 53: 2804-2815.

583

584 **FIG 1. TBZ-exposure causes morphophysiological changes in *Cryptococcus gattii***
585 **R265 and *C. neoformans* H99.** TBZ adapted-cells of *C. gattii* R265 exhibited increased
586 cell diameter (A) and decreased surface/volume ratio (B), but capsule thickness was
587 maintained (C), compared to those of non-adapted (NA) cells. *C. neoformans* H99
588 presented the pseudohyphae form after TBZ-adaptation (D). India ink suspension was
589 used. Bar = 10 μ m. The electronegativity of the cellular surface was not altered in *C.*
590 *gattii* R265 (E), however, it was increased in TBZ-adapted cells of *C. neoformans* H99
591 (F). NA = non-adapted; A = TBZ-adapted; ** p < 0.01; *** p < 0.001.

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

597 **FIG 3. Animals infected with TBZ-adapted cells exhibit lower fungal load in the**
598 **lungs, BALF, and brain and decreased**
599 **lung inflammation.** C57BL/6 mice were infected by the intratracheal route with $1 \times$
600 10^5 CFU of non-adapted (NA) and TBZ-adapted (A) cells for the CFU/g determination
601 and histopathology analysis. After 15 days of infection, higher fungal burden was

602 observed in the lungs (A) and bronchoalveolar lavage fluid (BALF) (B) of animals
603 infected with NA cells of *C. gattii* R265. Lung histopathology analysis showed that NA
604 cells caused more inflammation (C) than A cells (D). Animals infected with NA cells of
605 *C. neoformans* H99 also exhibited a higher fungal load in the lungs (E), BALF (F), and
606 brain (G) and intense inflammation in the lungs (H) when compared to those of animals
607 infected with A cells (I). **p < 0.01; *** p < 0.001. ND = not detected. NA = non-
608 adapted; A = TBZ-adapted. Arrows indicate yeast in the lungs and arrowheads indicate
609 the inflammatory infiltrate.

610

611 **FIG 4. TBZ-exposure causes antifungal resistance *in vivo*.** The MICs for
612 tebuconazole (TBZ), fluconazole (FLC), itraconazole (ITC), ravuconazole (RVC), and
613 amphotericin B (AMB) was determined for colonies recovered from lungs of animals
614 infected with non-adapted (NA) and TBZ-adapted (A) cells of *C. gattii* R265 (A) or *C.*
615 *neoformans* H99 (B) and treated (A + FLC) or not (A) with FLC (10 mg/kg). After 15
616 days, the animals were euthanized and the lungs were collected for CFU/g
617 determination. There was no statistical difference in the fungal load in the lungs of
618 animals infected with TBZ-adapted cells of *C. gattii* R265 (C) or *C. neoformans* H99
619 (D) treated or not with FLC. * p < 0.05; ** p < 0.01; ***p < 0.001.

620

621 **FIG. 5. TBZ-exposure changes in the expression of efflux pumps and *ERG-11***
622 **genes.** Expression of *PDR1* (A) and *MDR1* (B), but not that of *ERG11* (C), was altered
623 by tebuconazole exposure in *C. gattii* R265. In *C. neoformans* H99, the expression of
624 *AFR1* (D), *MDR1* (E) and *ERG11* (F) was increased in adapted cells versus that of non-
625 adapted cells. NA = non-adapted; A = TBZ-adapted; * p < 0.05; ** p < 0.01.

626

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}	
	30°C	35°C	30°C	35°C	30°C	35°C
<i>C. gattii</i>						
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
<i>C. neoformans</i>						
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 ($\geq 4X$) 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, $\mu\text{g/mL}$) for fluconazole and tebuconazole of *C. neoformans* strains of non-adapted (NA), TBZ-adapted
 640 (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 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 ($\geq 4X$) 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	<i>C. gattii</i>		<i>C. neoformans</i>	
	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, $\mu\text{g/mL}$) 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		
	NA	A	10p	NA	A	10p	NA	A	10p	NA	A	10p
<i>C. gattii</i>												
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
<i>C. neoformans</i>												
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

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

655

656 **Table 6.** Minimum inhibitory concentrations (MICs, $\mu\text{g/mL}$) for fluconazole and tebuconazole
 657 of *C. gattii* strains of non-adapted (NA), TBZ-adapted (A) at 35°C and TBZ-adapted colonies
 658 subcultured 10 times in agrochemical-free medium (10p-10 passages). Tests were performed at
 659 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

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

663

664 **Table 7.** Minimum inhibitory concentrations (MICs, $\mu\text{g/mL}$) for fluconazole and tebuconazole of *C.*
 665 *neoformans* strains of non-adapted (NA), TBZ-adapted (A) at 35°C and 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 ($\geq 4X$) 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, $\mu\text{g/mL}$) 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		
	NA	A	10p	NA	A	10p
<i>C. gattii</i>						
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
<i>C. neoformans</i>						
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 ($\geq 4X$) 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

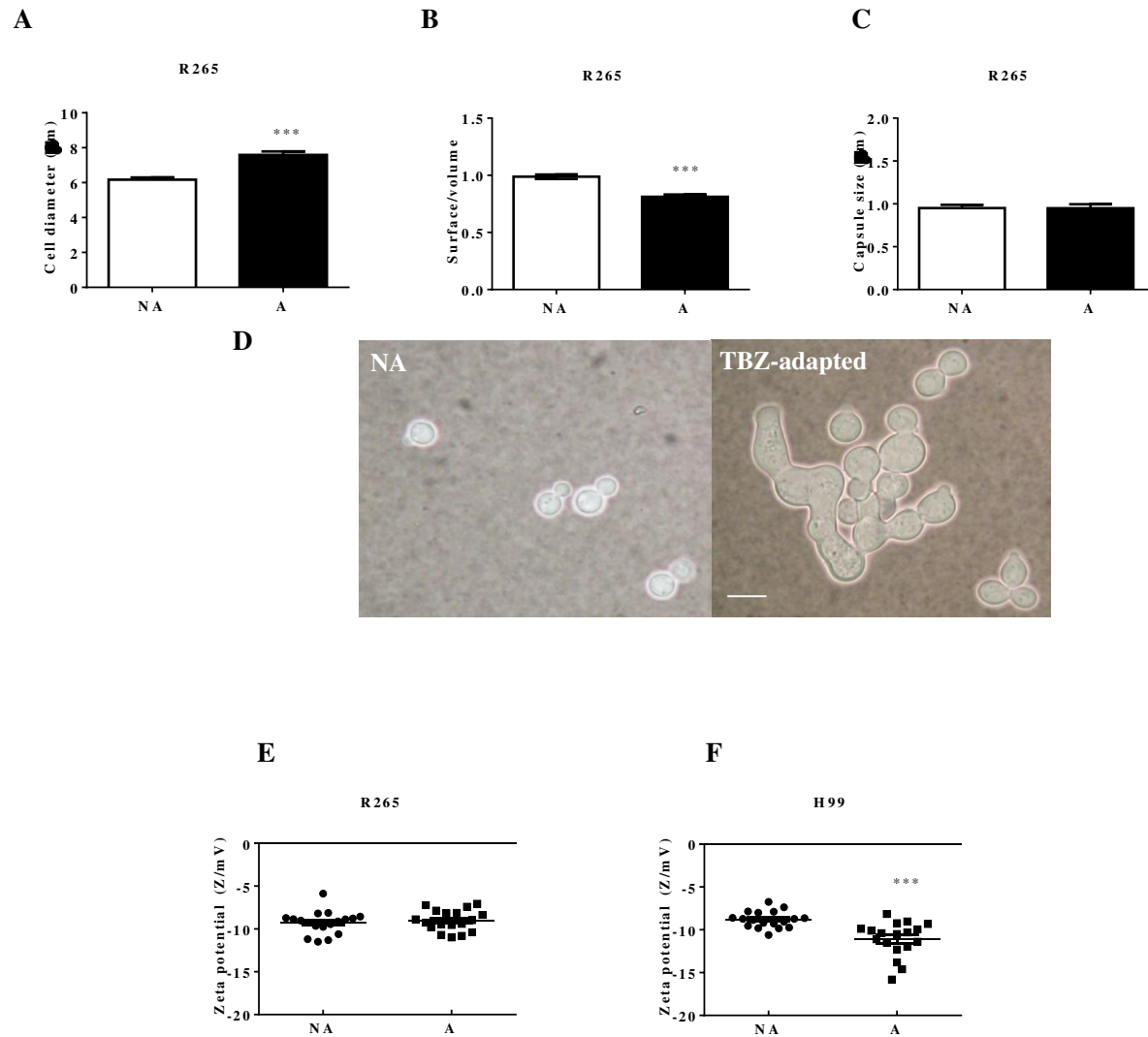


FIG 1. TBZ-exposure caused morpho-physiological changes in *Cryptococcus gattii* R265 and *C. neoformans* H99. TBZ adapted- cells of *C. gattii* had the cell diameter increased (A) and the surface/volume ratio (B) decreased, but maintained the thickness of capsule (C), compared to nonadapted (NA) cells. *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.

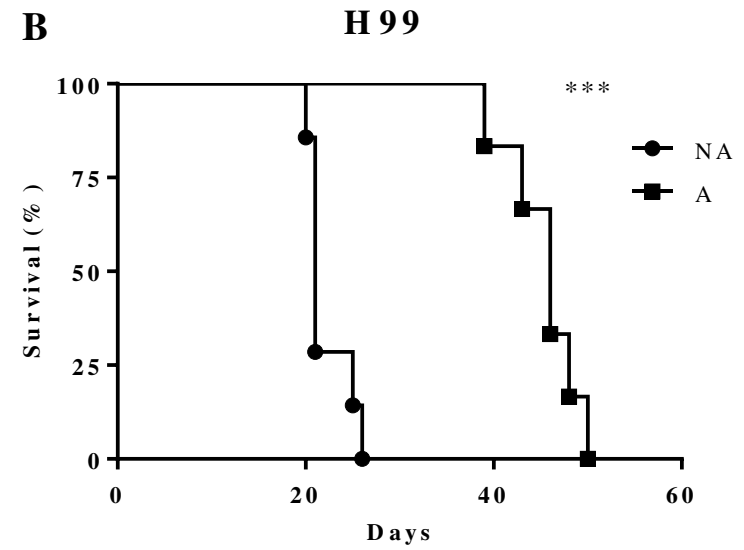
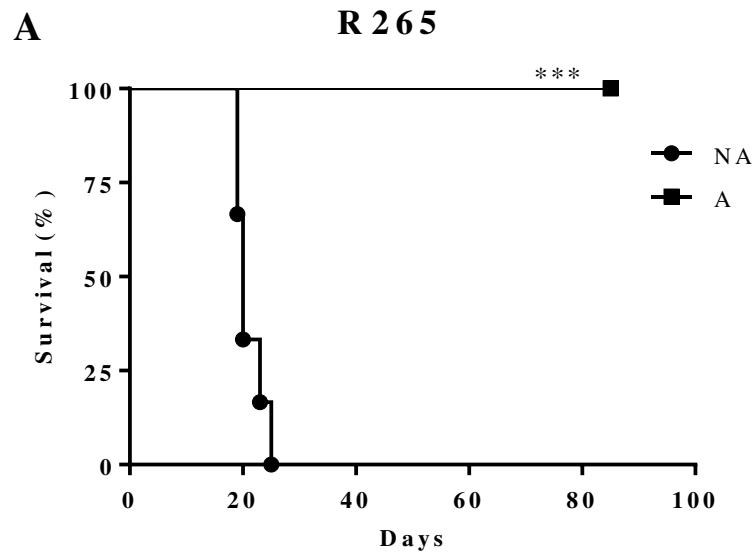
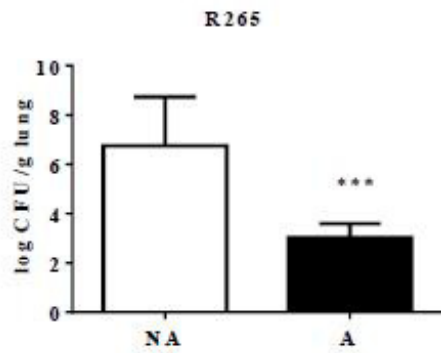
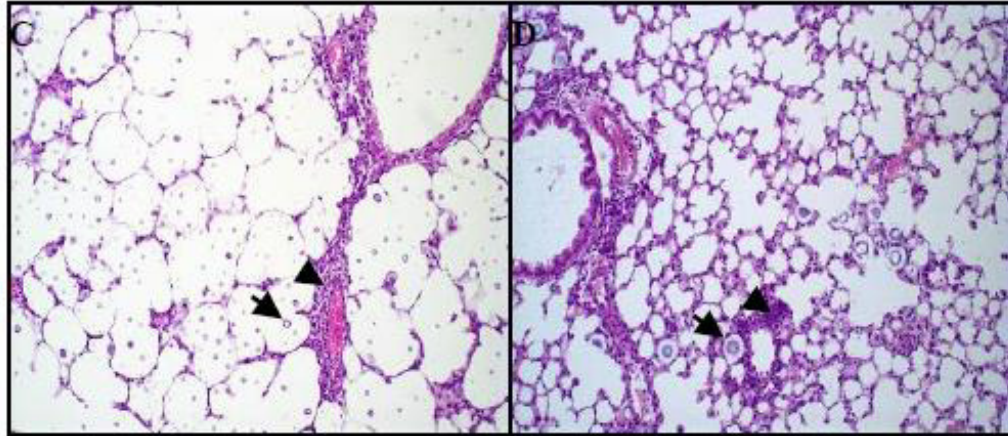
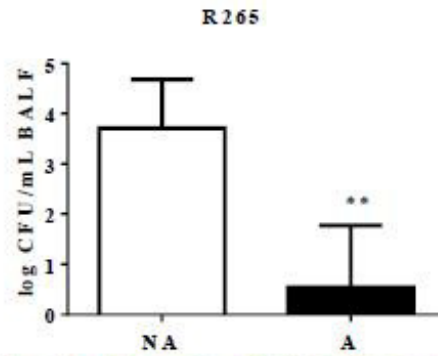


FIG 2. TBZ-exposure caused decreased of virulence in *Cryptococcus gattii* R265 and *C. neoformans* H99. C57BL/6 mice were infected by intratracheal route with 1×10^5 CFU of non-adapted -NA- and TBZ-adapted -A- cells. The survival curve showed that animals infected with TBZ-adapted cells of *C. gattii*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

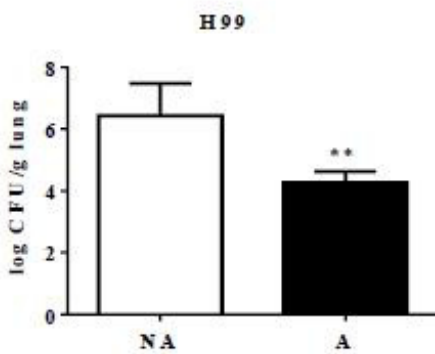
A



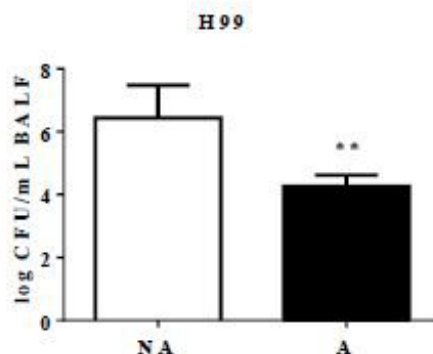
B



E



F



G

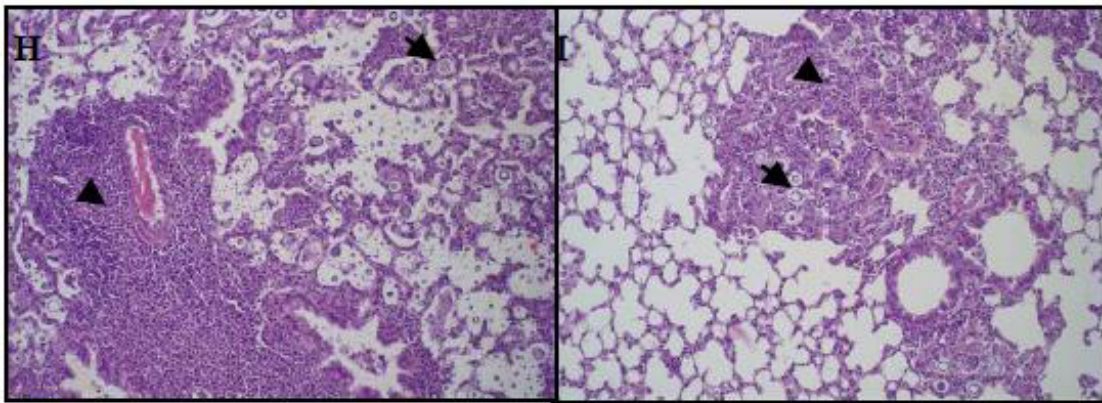
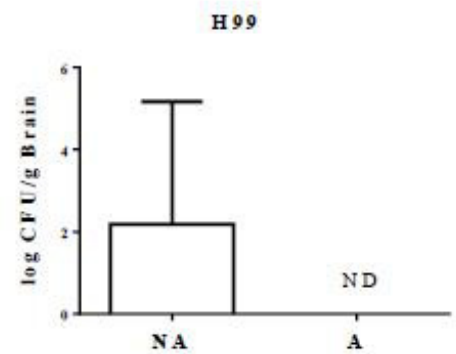


FIG 3. Animals infected with TBZ-adapted cells had lower fungal load in the lungs, BALF and brain and decreased of inflammation in the lungs. C57BL/6 mice were infected by intratracheal route with 1×10^5 CFU of non-adapted -NA- and TBZ-adapted -A- cells for the CFU/g determination and histopathology analysis. After 15 days of infection, 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 inflammation (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 inflammation 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.

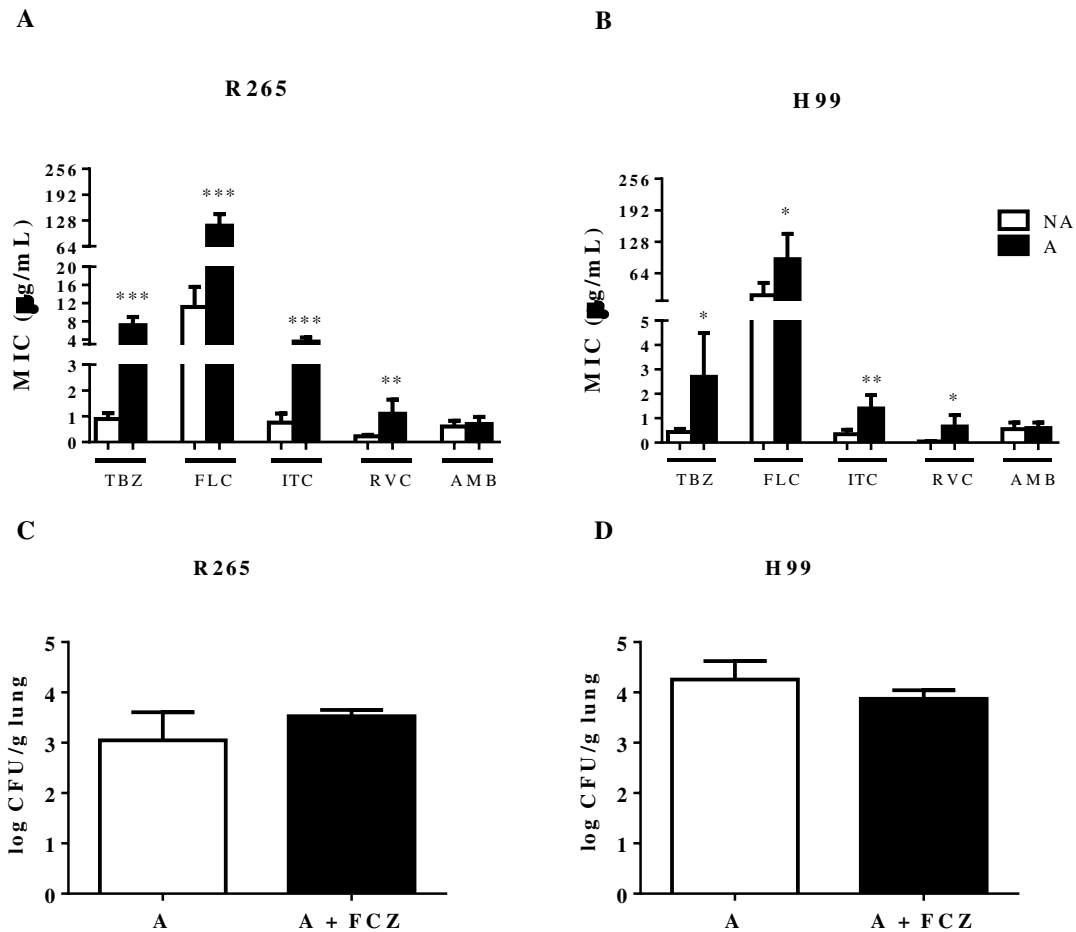


FIG. 4. TBZ-exposure caused antifungal resistance *in vivo*. The MIC for tebuconazole (TBZ), fluconazole (FLC), itraconazole (ITC), ravuconazole (RVC) and amphotericin B (AMB) was determined for colonies recovered from lungs of animals infected with non-adapted -NA- and TBZ-adapted -A- cells of *C. gattii* 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.

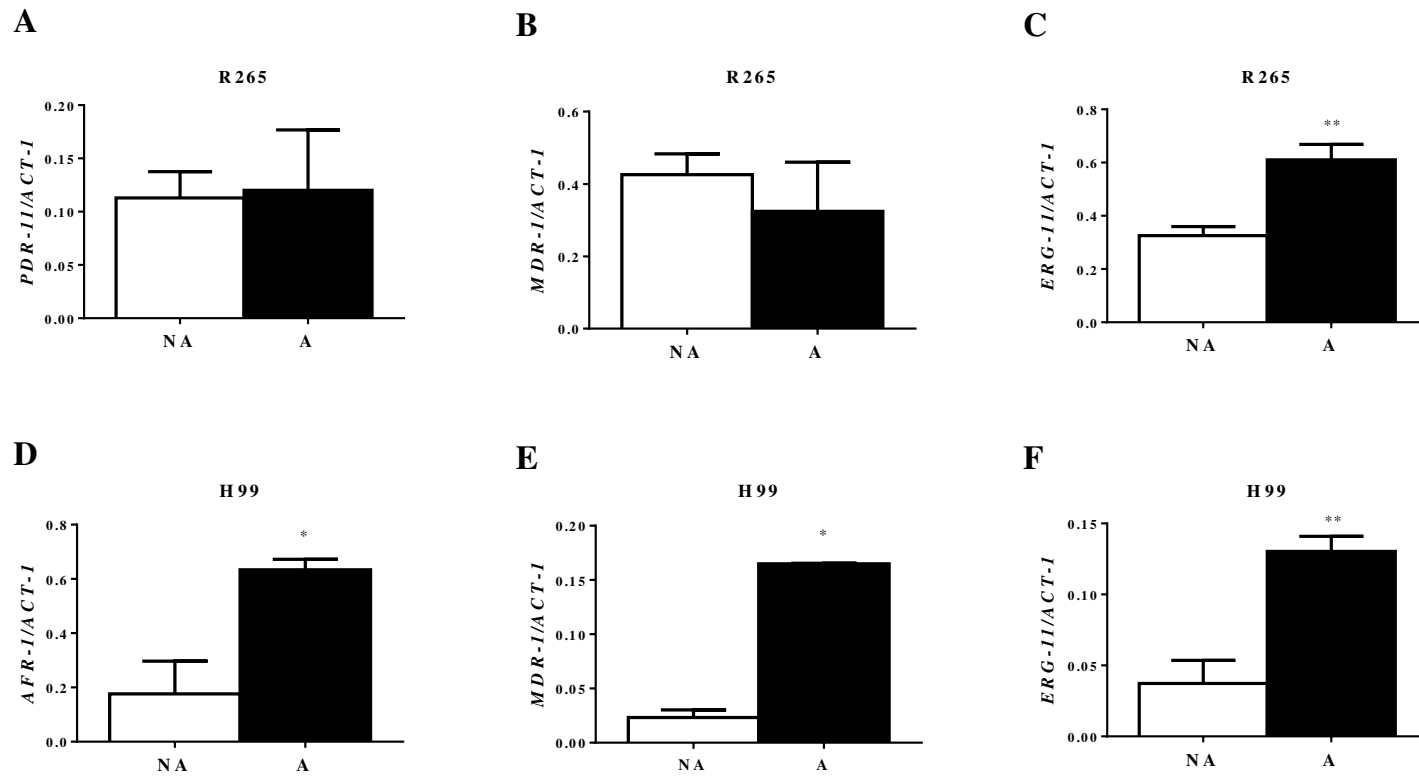


FIG. 5. Expression of *PDR1* (A) and *MDR1* (B), but not that of *ERG11* (C), was altered by tebuconazole exposure in *C. gattii* R265. In *C. neoformans* H99, the expression of *AFR1* (D), *MDR1* (E) and *ERG11* (F) was increased in adapted cells versus that of non-adapted cells. NA = non-adapted; A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