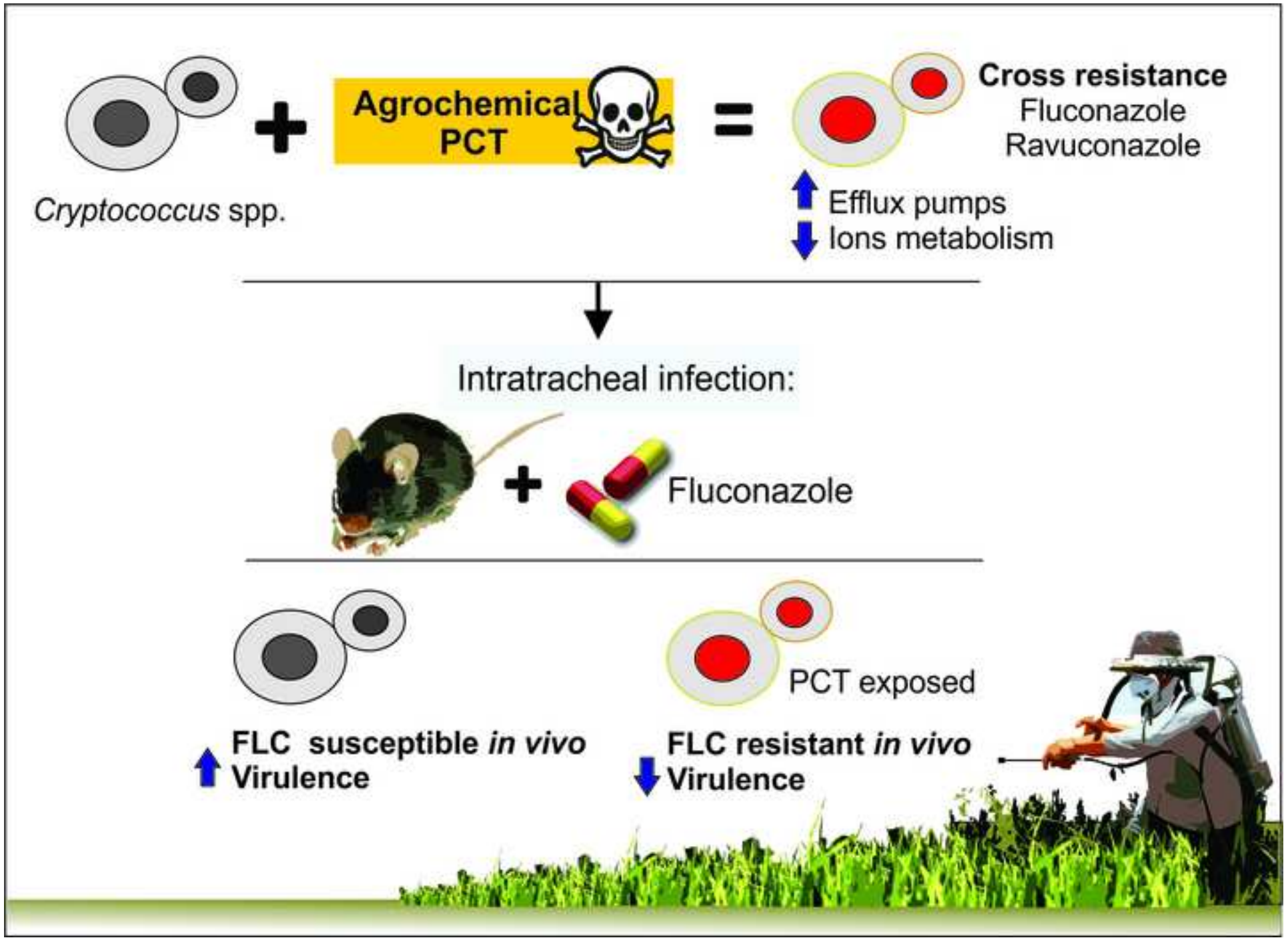
*Graphical Abstract



***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

Highlights

- Exposure to pyraclostrobin induces cross-resistance with clinical antifungals in *Cryptococcus*
- Non-azole agrochemical exposure increases expression of efflux pumps in *C. gattii*
- Pyraclostrobin-exposed yeasts are less virulent than non-exposed ones in mice
- Fluconazole is not able to treat cryptococcosis by pyraclostrobin-exposed cells.
- Agrochemicals jeopardize animal and human health by influencing fungal resistance.

From the environment to the host: how non-azole agrochemical exposure affects the antifungal susceptibility and virulence of *Cryptococcus gattii*

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Abstract

Agrochemicals such as the non-azoles, used to improve crop productivity, poses severe undesirable effects on the environment and human health. In addition, they induce cross-resistance (CR) with clinical drugs in pathogenic fungi. However, till date emphasis has been given to the role of azoles on the induction of CR. Herein, we analyzed the effect of a non-azole agrochemical, pyraclostrobin (PCT), on the antifungal susceptibility and virulence of the human and animal pathogens *Cryptococcus gattii* and *C. neoformans*. We determined the minimum inhibitory concentration (MIC) of fluconazole (FLC), itraconazole, ravuconazole, amphotericin B, and PCT on colonies: (i) that were not exposed to PCT (non adapted-NA-cultures), (ii) were exposed at the maximum concentration of PCT (adapted-A-cultures) and (iii) the adapted colonies after cultivation 10 times in PCT-free media (10 passages-10p– cultures). Our results showed that exposure to PCT induced both temporary and permanent CR to clinical azoles in a temperature-dependent manner. With the objective to understand the mechanism of induction of CR through non-azoles, the transcriptomes of NA and 10p cells from *C. gattii* R265 were analyzed. The transcriptomic analysis showed that expression of the efflux-pump genes (*AFR1* and *MDR1*) and PCT target was higher in resistant 10p cells than that in NA. Moreover, the virulence of 10p cells was reduced as compared to NA cells in mice, as observed by the differential gene expression analysis of genes related to ion-metabolism. Additionally, we observed that FLC could not increase the survival rate of mice infected with 10p cells, confirming the occurrence of permanent CR *in vivo*. The findings of the present study demonstrate that the non-azole agrochemical PCT can induce permanent CR to clinical antifungals through increased expression of efflux pump genes in resistant cells and that such phenomenon also manifests *in vivo*.

Keywords: pyraclostrobin; cross-resistance; efflux pumps; fluconazole; temperature

Introduction

The growing demand for food supply has increased the consumption of agrochemicals worldwide (Popp et al., 2013; Rigotto et al., 2014; Elahi et al., 2019). However, their intensive use leads to severe undesirable effects on the environment (Hartman et al., 2016; Cui et al., 2017) as well as human health (Rigotto et al., 2014; Elahi et al., 2019). Recently, some studies have shown that agrochemicals select microorganisms that are otherwise resistant to clinical antifungals, developing cross-resistance (CR) (Ren et al., 2017; Brilhante et al., 2019; Bastos et al., 2018; Carneiro et al., 2019). In our previous work, we showed that the triazole agrochemical tebuconazole triggers permanent and temporary CR to clinical azole drugs in *Cryptococcus* spp., both *in vitro* and *in vivo* (Bastos et al., 2018). Nevertheless, it is uncertain whether non-azole agrochemicals such as pyraclostrobin (PCT) are also able to induce CR.

Cryptococcus gattii and *Cryptococcus neoformans* are the main etiologic agents of cryptococcosis, which affect more than 200,000 people per year worldwide, with a mortality rate of 81% (Rajasingham et al., 2017; Williamson et al., 2017). These microorganisms are generally found in the plant habitats. More specifically, *C. gattii* is found in more than 50 tree species, especially in *Eucalyptus* (Chatuverdi and Chatuverdi, 2011, Prakash et al., 2018), whereas the primary niche of *C. neoformans* is associated with plants as well as with birds' feces (Cogliati et al., 2016). However, it remains unclear (i) how the interaction with plants influences the biology of these fungi (Xue et al., 2007); and (ii) how human interferences, mainly, the use of agrochemicals (Del Poeta and Casadevall, 2012), affect the biology of *Cryptococcus* spp. the way they affect *Aspergillus fumigatus* (Snelders et al., 2012; Ren et al., 2017) and *Candida* spp. (Brilhante et al., 2019).

The therapeutic arsenal used to treat cryptococcosis is composed of amphotericin B (AMB), 5-flucytosine, and the azoles fluconazole (FLC) and itraconazole (ITZ) (Perfect et al., 2010; Molloy et al., 2018). Despite the availability of reports showing increased resistance to azoles (Smith et al., 2015; Chen et al., 2016), the underlying mechanism of resistance is still unclear, especially in *C. gattii*. According to previous studies, resistance may be caused by mutations in the *ERG11* gene, which encodes the drug-target protein ERG11p, and also by the overexpression of efflux pumps, e.g. *AFR-1 (*ATP-binding cassette transporter), *AFR-2*, and *MDR11* (putative ABC multidrug resistance transporter similar to Ste6) (Basso et al., 2015; Yang et al., 2016, Cavalheiros et al., 2018).

The antifungal PCT is a strobilurin fungicide, which belongs to the quinone outside inhibitors (QoI) group. QoI alters mitochondrial respiration by binding to the Qo site of the cytochrome b, blocking the electron transfer to cytochrome c_1, which leads to the disruption of the energy cycle (Bartlett etal., 2002). Because of its high efficiency and broad-spectrum action against phytopathogenic fungi, it has been considered as an "environment-friendly fungicide". Hence, its consumption has increased worldwide including in the United States, United Kingdom, and China (Bartlett etal., 2002; Oliver and Hewitt, 2014; Gou et al., 2017).

Despite being considered low-toxic for humans, birds, mammals, and bees (Barlett, 2002), there are reports of poisoning caused by PCT (CDC, 2018). Furthermore, the widespread use of strobilurin fungicides, including PCT, can pose a potential risk to aquatic organisms, since residues of pesticides can remain in the air, soil, or water through runoff and/or leaching from soil to the surrounding waterbodies (Hartman, et al., 2016; Cui, et al., 2017).

The present study was aimed at investigating the effect of the non-azole agrochemical PCT on the susceptibility of *Cryptococcus* spp. to clinical drugs and its virulence.

Materials and Methods

Microorganisms

We used twelve strains of *C. gattii* (eight clinical and two environmental isolates, from the culture collection of the Laboratório de Micologia, at Universidade Federal de Minas Gerais, state of Minas Gerais, Brazil; and two reference strains from the culture collection of the University of Georgia, Atlanta, GA, USA) (Table 1) (Santos et al., 2012). Besides four strains of *C. neoformans* (one clinical and three reference strains) (Magalhães et al., 2013) were also used (Table 1). All isolates were maintained in Sabouraud Dextrose Broth medium with 10% glycerol, at -80°C.

Antifungal drug susceptibility testing

The minimum inhibitory concentration (MIC) of FLC (Sigma-Aldrich, St. Louis, MO), AMB (Sigma-Aldrich), and the environmental antifungal PCT (COMET®) were determined by the microdilution method (MIC^{broth}) (CLSI, 2012). The MIC of PCT was also verified by spot tests on Sabouraud Dextrose Agar (SDA) medium, supplemented with different concentrations of PCT (MIC^{solid}), as previously described (Bastos et al., 2018). The MIC^{broth} and MIC^{solid} tests were performed at 30°C and 35°C, and all the tests were performed in duplicates for each strain.

Agrochemical adaptationand cross-resistance tests (CR)

Following MIC^{solid} tests, the strains were grown on SDA medium supplemented with increasing concentrations of the pesticide. Initially, all strains were grown on a medium supplemented with PCT at a concentration of MIC/2 (sub-MIC: half the MIC value). After the colonies have developed, an inoculum containing approximately 1×10^4 to 5×10^4 fungal cells was inoculated onto SDA medium supplemented with the MIC of PCT. This process was repeated, and the strains were grown in a stepwise manner, at increasing amounts of PCT (ranging from 0.25 mg/L to 256.0 mg/L), up to the concentration at which the growth was ceased, or until the maximum limit of 256 mg/L was reached. The tests were carried out at 30°C and 35°C (Bastos et al., 2018). The highest concentration of PCT at which the fungus was capable of growing after the adaptation test was called Maximum Concentration Achieved (MCA). The ability of the microorganisms to multiply in the presence of PCT was assessed by determining the ratio between the MCA and the sub-MIC (MCA/sub-MIC).

The colonies exposed to the agrochemicals were named PCT-adapted (A), while the original ones were called non-adapted (NA). Subsequently, the MIC^{broth} of FLC, AMB, and PCT were determined for the NA and A colonies. The tests were performed at two different temperatures, 30°C and 35°C (MIC^{broth} incubation temperature), for the colonies adapted at 30°C temperature 35°C, respectively. The strain was considered cross-resistant (CR) when it showed an increased MIC for both the agrochemical and clinical drugs. The CR was further classified either as temporary or permanent depending on the restoration of susceptibility to the drugs. In temporary CR the agrochemical-adapted colonies returned to their original susceptibility and the colonies showing permanent CR stayed susceptible to the drugs even after the withdrawal of PCT for 10 passages (10p colonies) (Bastos et al., 2018).

We also tested the CR between PCT and ITZ (Sigma-Aldrich), and between PCT and ravuconazole (RVZ; Sigma-Aldrich) in PCT-adapted and 10p colonies that showed CR to FLC.

Transcriptome analysis

NA and 10p cells of *C. gattii* R265 (that demonstrated permanent CR) were grown in a YPD medium without the drugs at 30°C, for total RNA extraction (Moyrand et al., 2008), in triplicates. For sequencing, strand-specific paired-end cDNA libraries were prepared from 10 μg of total RNA, by using the Illumina mRNA-Seq-Sample Prep Kit, according to the manufacturer's instructions. cDNA fragments of ~400 bp from each library were purified and checked for their quality using a Bioanalyzer (Agilent), followed by sequencing of 100 bp fragments from both ends using an Illumina HiSeq2000 instrument. The differential gene expression was investigated using DESeq1 v1.1659, DESeq2 v1.4.160, and edgeR v3.6.161, with default settings and false discovery rate (FDR) cutoff set at 0.05. The genes with more than 10 mapped fragments in at least one library were selected, and the fold change output from DESeq2 was considered as the decisive fold change. A gene was considered significantly differentially expressed when the fold change was higher or lower than 1.5.

***In vivo* tests**

C57BL/6 male mice, aged 6-8 weeks, were used. All experimental procedures were carried out according to the standards of the Brazilian Society of Laboratory Animal Science/Brazilian College for Animal Experimentation (http://www.sbcal.org.br). The study was approved by the Ethics Committee in Animal

Experimentation of the Universidade Federal de Minas Gerais (CEUA/UFMG, protocol number 306/2015).

The animals (n=6) were anesthetized with ketamine (60mg/kg) and xylazine (10mg/kg), followed by infection with 1×10^5 non-adapted or 10p (30°C) *C. gattii* R265 cells through the intratracheal route. Some groups were treated daily with 20 mg/kg of FLC through the intraperitoneal route (the controls are represented by the untreated groups). The mice were monitored daily for survival (Ferreira et al., 2015). Further, other groups of mice were also infected with NA and 10p cells and the animals were anesthetized and euthanized after 15 days to collect the lungs. The lungs were then homogenized in phosphate buffered saline (PBS), and plated on SDA medium. After 48h of incubation at 35°C, the recovered colonies were collected and subsequently used for the MIC^{broth} test.

Statistical analysis

All statistical analysis, except for the transcriptome data, was performed with the software GraphPad Prism, version 6.00 for Windows (GraphPad Software, San Diego, CA, USA). The significance test was done by Student's t-test at $p < 0.05$. The survival curve was plotted according to Kaplan-Meier analysis, and the results were examined using the log-rank test. All analyses were repeated at least twice.

Results

Adaptation process increases resistance to PCT

Initially, we determined the MIC of FLC, AMB, and PCT for each strain of *C.gattii* and *C. neoformans* at 30ºC and 35°C. The results showed inhibition of all the strains by the drugs when the MIC was checked in the broth medium (MIC^{broth}) (data not shown) and solid medium (MIC^{solid}) (Table 1).

MIC^{solid} test revealed that all strains of *C. gattii* and *C. neoformans* were capable of growth at higher concentrations of PCT when the adaptation test was performed at 30°C (Table 1), of which, *C. gattii* L27/01 tolerated the agrochemical the most in the adaptation test, being able to grow in a medium 2,048 times richer in PCT (Table 1). However, the adaptation test at 35°C revealed that 75% of the *C. gattii* and 100% of the *C. neoformans* strains tolerated more the PCT (Table 1).

The geometric means of the ratio MCA/Sub-MIC at 30°C were observed to be higher than those at 35°C in both the species (Table 1), indicating the influence of the temperature on adaptation.

PCT exposure causes temporary and permanent cross-resistance with clinical azolesin a temperature-dependent manner

An increase (more than four folds) in the MIC of PCT was observed in 58.3% (n=7) of the *C. gattii* strains and 100% (n=4) of the *C. neoformans* (Tables 2 and 3) after the adaptation process at 30°C. However, the MIC^{broth} tests performed at 35°C identified only five strains of *C. gattii,* demonstrating less susceptibility to the pesticide. Further, all PCT-adapted colonies of *C. neoformans* adapted at 30°C were independent of the temperature variations (Table 3).

Furthermore, we tested whether the PCT exposure had affected the susceptibility of the pathogens to FLC and AMB. Tables 2 and 3 show that the geometric mean values of FLC MIC^{broth} for PCT-adapted cells at 30°C increased by more than 2.0 times for *C. gattii*. On the other hand, the geometric means of adapted cells did not differ considerably from those of NA cells (Tables 2 and 3) at 35°C for *C. gattii*, and at both temperatures for *C. neoformans*. It was observed that 41.6% (n=5) of the *C. gattii* and 25% (n=1) of the *C. neoformans* strains presented CR to FLC when the adaptation and MIC processes were performed at 30°C (Tables 2 to 4). Four (33.3%) *C. gattii* strains (R265, ATCC 24065, L24/01, and L27/01) demonstrated permanent CR, and did not return to their original susceptibility even after growing them for 10 passages in an agrochemical-free media (Tables 2 and 4). In contrast, three strains of *C. gattii* and one strain of *C. neoformans* (ATCC 62066) exhibited a temporary CR by returning to their original susceptibility after 10 passages in a medium without PCT (Tables 2to4). The permanent and temporary CR displayed by *C. gattii* R265 and 547/OTTI/94-PI-10, respectively, were verified both at 30°C and 35°C (Table 2). The PCT-adapted cells at 30°C that exhibited CR to FLC were also found to show CR to RVZ (Table 5), but not to ITZ (Table 5) and AMB (data not shown).

Additionally, the adaptation test at 35°C identified higher MIC^{broth} of PCT for two strains of *C. gattii* and one of *C. neoformans* (Table 6). However, a CR toFLC (Tables 4 and 6) and AMB (data not shown) was not observed.

Transcriptomic profile of *C. gattii* R265 10p cells is different from that of NA cells.

To investigate the permanent changes caused by the PCT exposure, we compared the RNA expression profile in NA and 10p cells of *C. gattii* R265. Both the cells were grown in a medium without PCT, at 30°C. The analysis identified 230 genes

showing differential expression, of which 110 were up-regulated and 120 were down-regulated. Though most of these genes encode hypothetical proteins (55.2%), the genes related to amino acid metabolism, oxidation-reduction process, sugar metabolism, transmembrane transport, nucleotide metabolism, ribosome biogenesis, drug transport, cell wall biosynthesis, and phosphatases were up-regulated in the 10p cells than the NA cells (Figure 1A). However, the down-regulated genes were related to the amino acid, sugar and ion metabolism, oxidation-reduction process, membrane component, transmembrane transport, and RNA metabolism (Figure 1B).

The genes like *CTR4* [solute carrier family 31 (copper transporter), member 1] and *FRE* (ferric-chelate reductase)-1 and 7, probably involved in virulence of *Cryptococcus* spp. were also down-regulated (Table 7).

Efflux pumps and genes encoding PCT target were up-regulated in 10p cells

It has been proposed that *Cryptococcus* spp. becomes less susceptible to azole drugs due to the overexpression of *ERG-11* and/or efflux-pumps genes (*AFR-1*, *AFR-2*, and *MDR11*) (Basso et al., 2015; Bastos et al., 2018). We searched for the genes mentioned above in the transcriptome data, to validate their role in the development of resistance to azoles. We found that *AFR1* and *MDR11* were up-regulated (1.5 times fold change) in the 10p cells (Table 7), suggesting their role in the development of the observed phenotype. Though, we did not observe any significant difference in the expression profile of *ERG11*, *CNBG_4400*, the gene encoding cytochrome b2, the target for the agrochemical PCT, was more expressed (2.05 times fold change) in the 10p cells (Table 7).

279 ***C. gattii* R265 10p cells are less virulent and more resistant to FLC than NA cells *in***
280 ***vivo***

281 For *in vivo* validation of the reduced virulence in the 10p cells, both NA and 10p
282 cells of *C. gattii* R265 were tested in mice. All animals infected with NA cells died
283 within 30 days of post-infection (d.p.i). On the other hand, 20% of the mice infected
284 with 10p cells were alive 60 d.p.i. revealing their lower virulence ($p<0.05$) (Figure 2).

285 It was observed that the treatment with FLC increased the survival rate of NA-
286 infected mice, as they remained alive even after 60 d.p.i. In contrast, FLC could not
287 augment the survival of the mice infected with 10p cells (Figure 1), demonstrating the
288 *in vivo* occurrence of CR.

289 In addition, we tested the MIC^{broth} for colonies recovered from the lungs of mice
290 infected with NA and 10p cells for 15 days. The colonies from animals infected with
291 10p cells were less susceptible to PCT and all the clinical azoles tested than the NA
292 colonies (Figure1), indicating that the 10p cells of *C. gattii* R265 present *in vivo* CR to
293 FLC and other azole drugs.

294

295 **Discussion**

296 The fast-growing world population calls for increasing food supply, thereby
297 increased crop productivity. In order to achieve this, the use of agrochemicals is the
298 most preferred alternative that helps to avoid losses due to pests such as insects and
299 microorganism infections (Popp et al., 2013). However, these substances may pose
300 harmful effects on human and animal health and the environment (Rigotto et al., 2014).
301 Recently, we showed that tebuconazole, an environmental triazole, causes temporary
302 and permanent CR to clinical azoles in *C. gattii* and *C. neoformans* (Bastos et al., 2018).
303 However, it is not well-understood whether non-azole agrochemicals induce the same

304 effect. In this work, we showed that the agrochemical PCT, which inhibits the activity
305 of the cytochrome b and the electron transport chain in mitochondria, is also able to
306 select cells of *Cryptococcus* that are less susceptible to azole drugs (FLC, ITZ, and
307 RVZ). PCT was chosen for this study for several reasons, including its action
308 mechanism (non-azole), extensive use (Bartlett etal., 2002; Oliver and Hewitt, 2014;
309 Gou et al., 2017), and broad-spectrum activity. It has also been reported to be used for
310 *Eucalyptus* habitats, where *Cryptococcus* spp. can be found (Chatuverdi and
311 Chatuverdi, 2011).

312 Temperature is a crucial factor that controls the growth of *Cryptococcus.* Several
313 studies have reported its involvement in several phenomena such as the generation of
314 titan cells, hyphal growth, inheritance patterns of mitochondria, capsule size, survival
315 inside avian macrophages, and general virulence (Zaragoza and Casadevall, 2004;
316 Bielska and May, 2015; Wang et al., 2015; Johnston et al. 2017; and Watkins et al.,
317 2017; Hommel et al., 2018). In the present study, we observed that the rate of CR is
318 dependent on the temperature at which the adaptation test was conducted. When the test
319 was carried out at 30°C, more strains of *C. gattii* and *C. neoformans* presented CR to
320 FLC. Additionally, the lowest temperature positively influenced the concentration of
321 PCT tolerated by the fungi. Our results show the capacity of acquiring mechanisms
322 related to CR between agrochemical and azole clinical drugs, thus supporting the earlier
323 findings. Besides, the incubation temperature during the MIC^{broth} tests was also relevant
324 to the CR exhibited by the strains adapted at 30°C. While some strains presented CR to
325 FLC, regardless of the incubation temperature, others showed increased resistance only
326 when the cells were incubated at 30°C. These results reinforce that even with higher
327 resistance in the environment at low temperatures, resistance may not manifest in
328 animal infections due to the mammalian body temperature (Bastos et al., 2018).

While PCT targets the mitochondrial metabolism, azole drugs act on the ergosterol synthesis. Therefore, the next question was why the cells that had been exposed to the non-azole agrochemical became less susceptible to clinical azoles. Association between mitochondrial metabolism deficiency and azole resistance in *Cryptococcus* has been poorly documented. Nevertheless, it has been described that *C. neoformans* becomes less susceptible to FLC when brought into contact with tetracycline, an antibiotic that interferes in the synthesis of bacterial and mitochondrial proteins (Oliver et al., 2008).

In an attempt to better understand why PCT-exposed cells become less susceptible to clinical azoles, we performed the transcriptome analysis of the NA and 10p cultures of *C. gattii* R265, which had presented permanent CR to these drugs. The upregulation of efflux-pump genes, *AFR1*, and *MDR1* in 10p cells demonstrated a hypothesis for the underlying mechanism for the development of azole resistance in 10p cells. It could be because of pumping out of the antifungal drugs out of the cell. However, it is still not clear, whether or not PCT is also pumped out by the efflux pumps of *Cryptococcus* spp.

Another well-documented mechanism of resistance to PCT in environmental fungi involves mutations in the gene encoding the drug target, *cytb* (cytochrome b) (Yin et al., 2012). In our work, 10p cells were able to express 2-fold more Cytb than NA cells, which may be the cause of the less susceptible phenotype.

Cryptococcus spp. have several virulence factors, being the following the classic ones: capsule production, ability to grow at 37°C, and production of enzymes, among them phospholipase, urease, laccase, and SOD (Bielska and May, 2015). Recently, other important virulence pathways of this genus have been studied, which include the obtention and use of metals, like iron, copper, and zinc. These ions act as cofactors of

several enzymes, and they are essential in processes like respiration. Because of their importance, microorganisms like *Cryptococcus* must be able to acquire them from the environment and from the host to ensure their growth (Silva et al., 2011). In this context, we identified that the gene *CTR4* and other possible genes involved in ion metabolism were less expressed in the 10p cells. CTR4p is a copper-transport protein, essential not only for copper homeostasis but also for the virulenceof *C. neoformans*, since a *ctr4Δ* strain is less virulent than the corresponding wild type (Waterman et al.,2012). Thus, we hypothesize that low expression of the genes related to iron and copper obtention may lead to reduced virulence in the 10p cells. The less virulent phenotype was further confirmed in the mice model.

Finally, to investigate if the drug resistance observed *in vitro* also manifests *in vivo*, we infected the mice with NA and 10p cells of *C. gattii* R265 that presented CR to all clinical azoles. We then treated some of the animals with FLC, while others were kept untreated as the control. The treatment with the drug did not change the survival of the animals infected with the 10p cells, as opposed to the mice infected with NA cells. *In vivo* CR was also confirmed in colonies recovered from the lungs of animals infected with 10p cells. They were less susceptible to PCT and azoles than those obtained from animals infected with NA cells.

Conclusion

In conclusion, fungicide PCT exposure selects cells with CR to clinical azole drugs, both *in vitro* and *in vivo*. PCT also decreased the virulence of *C. gattii* R265, after contact with the agrochemical ceased. This study demonstrates the permanent implications of non-azole agrochemicals on fungal virulence and susceptibility to drugs and indicates how anthropic action in the environment could be responsible for the evolution of resistant strains of *Cryptococcus* spp.

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We declare that we have no conflicts of interest.

Transparency declarations.

None to declare.

Author contributions

Conceived and designed the experiments: RWB, DAS, GJ. Performed the experiments: RWB, GJCF, HCSC, LVNO, LGE, APNS, FM. Analyzed the data: RWB, CM, DAS. Contributed reagents/materials/analysis tools: GJ, DAS. Contributed to the writing of the manuscript: RWB, GJ, DAS.

Legends of the figures:

Figure 1: Transcriptomic profile of *C. gattii* R265 adapted cells grown in medium without pyraclostrobin (PCT) (10p) and non-adapted (NA) cells. The function of genes (A) up- and (B) down-regulated in 10p cells compared to NA.

Figure 2: Virulence and *in vivo* cross-resistance in non-adapted (NA) and adapted cells grown in medium without agrochemical (10p). (A) 10p cells are significantly ($p<0.05$) less virulent than NA. The treatment with fluconazole (FLC) significantly ($p<0.05$) increased the survival of mice infected with NA, but not of those infected with 10p cells. (B) Cells recovered from the lungs of animals infected with 10p colonies were more resistant to pyraclostrobin (PCT), FLC, itraconazole (ITZ) and ravuconazole (RVZ), but not to amphotericin B (AMB) than those recovered from NA-infected mice. * $p<0.05$; ** $p<0.01$.

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

Strain or parameter	**MIC^{solid}(mg/L)**		**MCA (mg/L)**		**MCA/Sub-MIC^{solid}**	
C.gattii	**30°C**	**35°C**	**30°C**	**35°C**	**30°C**	**35°C**
R265 (C)	1.0	1.0	200.0	1.0	400.0	2.0
ATCC 24065 (R)	1.0	0.5	256.0	1.0	512.0	4.0
ATCC 32608 (R)	1.0	0.5	10.0	1.0	20.0	4.0
547/OTTI/94-PI-10 (E)	1.0	0.5	1.0	1.0	2.0	4.0
ICB 181 (E)	1.0	1.0	10.0	0.5	20.0	2.0
L24/01 (C)	1.0	1.0	1.0	0.5	2.0	2.0
L27/01 (C)	0.25	0.25	256.0	0.5	2,048.0	4.0
L28/02 (C)	0.25	1.0	0.5	0.5	4.0	1.0
1913/ER (C)	1.0	2.0	256.0	256.0	512.0	256.0
LMM 818 (C)	2.0	2.0	256.0	1.0	256.0	1.0
23/10893 (C)	1.0	2.0	12.0	1.0	24.0	1.0
29/10893 (C)	1.0	2.0	256.0	175.0	512.0	175.0
Range	0.25 – 2.0	0.25 – 2.0	1.0 – 256.0	1.0 – 256.0	2.0 – 2,048.0	1.0– 256.0
Geometric mean	0.84	0.94	26.71	1.93	63.63	4.60
C. neoformans	**30°C**	**35°C**	**30°C**	**35°C**	**30°C**	**35°C**
H99 (C)	1.0	1.0	2.0	2.0	4.0	4.0
ATCC 24067 (R)	0.5	2.0	2.0	2.0	8.0	2.0
ATCC 28957 (R)	1.0	2.0	10.0	2.0	20.0	2.0
ATCC 62066 (R)	1.0	2.0	2.0	2.0	4.0	2.0
Range	0.5 – 1.0	1.0 – 2.0	2.0 – 10.0	2.0	4.0 – 20.0	2.0 – 4.0
Geometric mean	0.84	1.68	2.99	2.0	7.11	2.38

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

Table 2. Minimum inhibitory concentrations (MICs.mg/L) of fluconazole and pyraclostrobin for non-adapted (NA) cells of *C. gattii* strains .PCT-adapted (A) at 30°C and PCT-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]						Pyraclostrobin[b]					
	Temperature 30°C			Temperature 35°C			Temperature 30°C			Temperature 35°C		
	NA	A	10p	NA	A	10p	NA	A	10p	NA	A	10p
R265	**8.0**	**128.0 (16X)**	**128.0 (16X)**	**8.0**	**32.0 (4X)**	**32.0 (4X)**	**1.0**	**16.0 (16X)**	**8.0 (16X)**	**0.5**	**2.0 (4X)**	**2.0 (4X)**
ATCC 24065	**4.0**	**16.0 (4X)**	**16.0 (4X)**	4.0	4.0	ND	**0.125**	**1.0 (8X)**	**1.0 (8X)**	**0.125**	**1.0 (8X)**	**1.0 (8X)**
ATCC 32608	16.0	32.0	ND	8.0	16.0	ND	2.0	4.0	ND	1.0	2.0	ND
547/OTTI/94-PI-10	**16.0**	**128.0 (8X)**	16.0	**8.0**	**32.0 (4X)**	8.0	**1.0**	**8.0 (8X)**	1.0	**1.0**	**4.0 (4X)**	1.0
ICB 181	16.0	16.0	ND	8.0	16.0	ND	**0.5**	**4.0 (8X)**	**4.0 (8X)**	**0.25**	**1.0 (4X)**	**2.0 (8X)**
L24/01	**16.0**	**64.0 (4X)**	**64.0 (4X)**	8.0	16.0	ND	**0.25**	**1.0 (4X)**	**2.0 (8X)**	**0.25**	**1.0 (4X)**	**2.0 (8X)**
L27/01	**16.0**	**64.0 (4X)**	**64.0 (4X)**	32.0	16.0	ND	**1.0**	**128.0 (128X)**	**4.0 (4X)**	2.0	1.0	ND
L28/02	32.0	64.0	ND	16.0	32.0	ND	1.0	1.0	ND	0.5	1.0	ND
1913/ER	16.0	32.0	ND	16.0	8.0	ND	8.0	8.0	ND	1.0	2.0	ND
LMM 818	16.0	16.0	ND	16.0	8.0	ND	8.0	8.0	ND	2.0	4.0	ND
23/10893	8.0	4.0	ND	8.0	4.0	ND	4.0	4.0	ND	4.0	4.0	ND
29/10933	8.0	16.0	ND	4.0	4.0	ND	**4.0**	**128.0 (32X)**	**128.0 (32X)**	4.0	4.0	ND
MIC range	4.0 – 32.0	4.0 – 128.0	ND	4.0 – 32.0	4.0 – 32.0	ND	0.125 – 8.0	1.0 – 128.0	ND	0.125 – 4.0	1.0 – 4.0	ND
Geometric mean	12.70	28.50	ND	9.51	11.98	ND	1.33	6.72	ND	0.84	1.88	ND

a: MIC endpoint considering 50% of growth inhibition; *b*: MIC endpoint considering 100% of growth inhibition. The number in parentheses shows how many times (X) the MIC value of the PCT-adapted or 10p colonies was higher (≥4X) than the MIC of the NA colonies. Values in bold indicate an increase in the MIC by at least 4X more than in theNA colonies. ND = not determined.

Table 3. Minimum inhibitory concentrations (MICs.mg/L) of fluconazole and pyraclostrobin for non-adapted (NA) cells, PCT-adapted (A) at 30°C and PCT-adapted colonies subcultured 10 times in agrochemical-free medium (10p - 10 passages) of *C. neoformans* strains. Tests were performed at 30°C and 35°C.

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

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

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

Resistance	*C. gattii*		*C. neoformans*	
	30°C	35°C	30°C	35°C
Cross- resistance (CR)	41.6	0	25	0
Temporary CR	8.3	0	25	0
Permanent CR	33.3	0	0	0

Table 5. Minimum inhibitory concentrations (MICs.mg/L) of itraconazole and ravuconazole for non-adapted (NA) cells of *C. gattii* and *C. neorofomans* strains. PCT-adapted (A) at 30°C and PCT-adapted colonies subcultured 10 times in agrochemical-free medium (10p-10 passages). Tests were performed at 30°C and 35°C.

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	0.5	1.0	1.0	0.25	0.25	0.5	**0.125**	**2.0 (16X)**	**2.0 (16X)**	**0.03**	**0.12 (4X)**	**0.12 (4X)**
ATCC 24065	0.5	1.0	0.5	0.25	0.5	0.5	**0.06**	**0.25 (4X)**	0.125	**0.03**	**0.12 (4X)**	0.03
547/OTTI/94-PI-10	0.5	1.0	ND	0.25	0.5	ND	**0.25**	**1.0 (4X)**	ND	**0.06**	**0.25 (4X)**	ND
L24/01	1.0	1.0	1.0	0.5	1.0	1.0	**0.125**	**1.0 (8X)**	**1.0 (8X)**	**0.06**	**0.5 (8X)**	**0.25 (4X)**
L27/01	0.25	0.25	0.5	0.25	0.25	0.25	**0.125**	**0.5 (4X)**	**0.5 (4X)**	**0.03**	**0.25 (4X)**	**0.25 (4X)**
C. neoformans	**NA**	**A**	**10p**	**NA**	**A**	**10p**	**NA**	**A**	**10p**	**NA**	**A**	**10p**
ATCC 62066	0.25	0.5	ND	0.25	0.5	ND	**0.03**	**0.5 (16X)**	ND	**0.031**	**0.12 (4X)**	ND

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

Table 6. Minimum inhibitory concentrations (MICs.mg/L) of fluconazole and pyraclostrobin for non-adapted (NA) cells of *C. gattii* and *C. neoformans* strains.PCT-adapted (A) at 35°C and PCT-adapted colonies subcultured 10 times in agrochemical-free medium (10p - 10 passages). Tests were performed at 35°C.

Strain or parameter	**Fluconazole**[a]			**Pyraclostrobin**[b]		
C. gattii	**NA**	**A**	**10p**	**NA**	**A**	**10p**
R265	8.0	8.0	ND	0.5	1.0	ND
ATCC 24065	4.0	4.0	ND	0.125	0.25	ND
ATCC 32608	8.0	8.0	ND	1.0	1.0	ND
547/OTTI/94-PI-10	8.0	16.0	ND	1.0	2.0	ND
ICB 181	8.0	8.0	ND	**0.25**	**1.0 (4X)**	0.5
L24/01	8.0	16.0	ND	0.25	0.25	ND
L27/01	32.0	16.0	ND	**2.0**	**8.0 (4X)**	**8.0 (4X)**
L28/02	16.0	16.0	ND	0.5	1.0	ND
1913/ER	16.0	16.0	ND	1.0	2.0	ND
LMM 818	16.0	8.0	ND	2.0	4.0	ND
23/10893	8.0	4.0	ND	4.0	8.0	ND
29/10933	4.0	4.0	ND	4.0	2.0	ND
MIC range	4.0 – 32.0	4.0 – 16.0	ND	0.125 – 4.0	0.25 – 8.0	ND
Geometric mean	9.51	8.97	ND	0.84	1.49	ND
C. neoformans	**NA**	**A**	**10p**	**NA**	**A**	**10p**
H99	8.0	16.0	ND	0.5	0.5	ND
ATCC 24067	4.0	8.0	ND	**1.0**	**16.0 (16X)**	**4.0 (4X)**
ATCC 28957	2.0	4.0	ND	0.25	0.5	ND
ATCC 62066	4.0	4.0	ND	1.0	1.0	ND
MIC range	2.0 – 8.0	4.0 – 16.0	ND	0.25 – 1.0	0.5 – 16.0	ND
Geometric mean	4.0	6.72	ND	0.59	1.41	ND

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

Table 7. List of down-regulated genes involved in ion metabolism and up-regulated genes that could be involved in drug resistance of *C. gattii* R265 10p cells.

ORF	Genes identification	Function	10p/NA Fold change
CNBG_0560	Solute carrier family 31 (copper transporter), member 1*(CTR4)*	Copper ion transmembrane transport	-2.45
CNBG_6082	Ferric-chelate reductase 7 (*FRE7*)	Oxidoreductase activity	-1.92
CNBG_9038	Ferric-chelate reductase	Oxidoreductase activity	-1.62
CNBG_2627	Ferric-chelate reductase 1 (*FRE1*)	Oxidoreductase activity	-1.62
CNBG_4400	Cytochrome b2, mitochondrial (*cytb*)	Respiration (target of pyraclostrobin)	2.058
CNBG_1200	ATP-binding cassette transporter (*AFR-1*)	Drug transport	1.566
CNBG_1138	Putative abc multidrug resistance transporter with similarity to Ste6 (*MDR11*)	Drug transport	1.526

Figures 1 and 2

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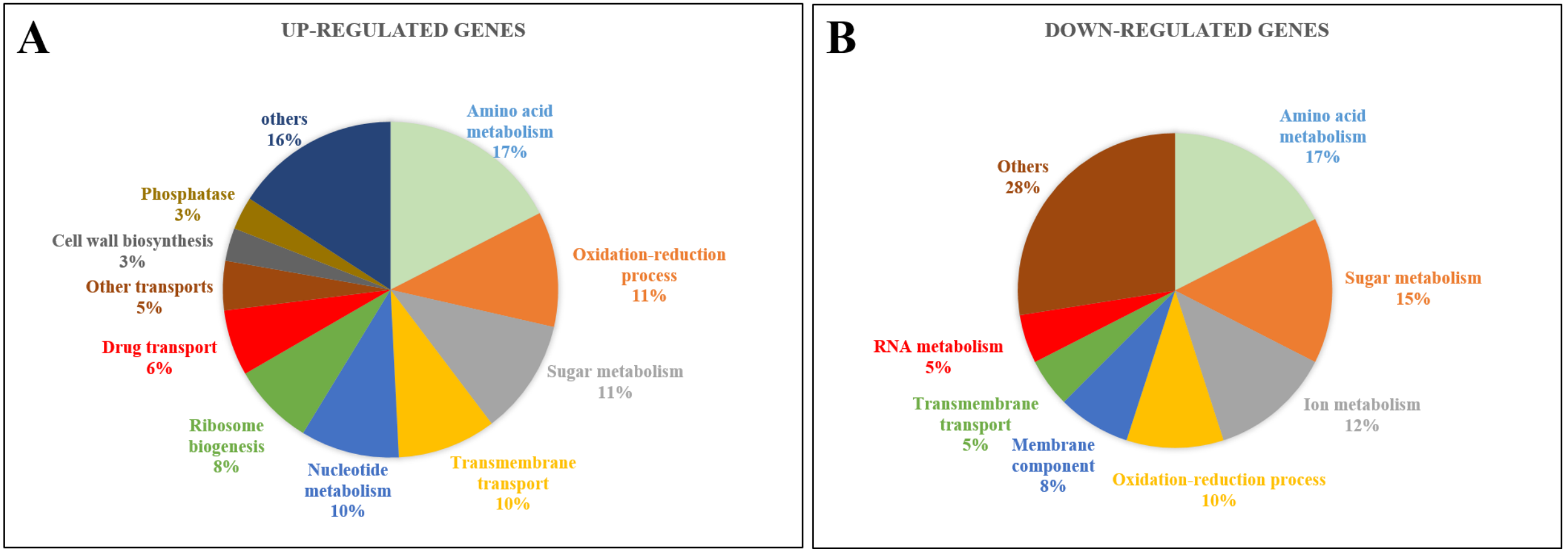

Figure 1: Transcriptomic profile of *C. gattii* R265 10p cells is different from that of NA cells.. Function of genes up- (A) and down-regulated in 10p cells compared to NA.

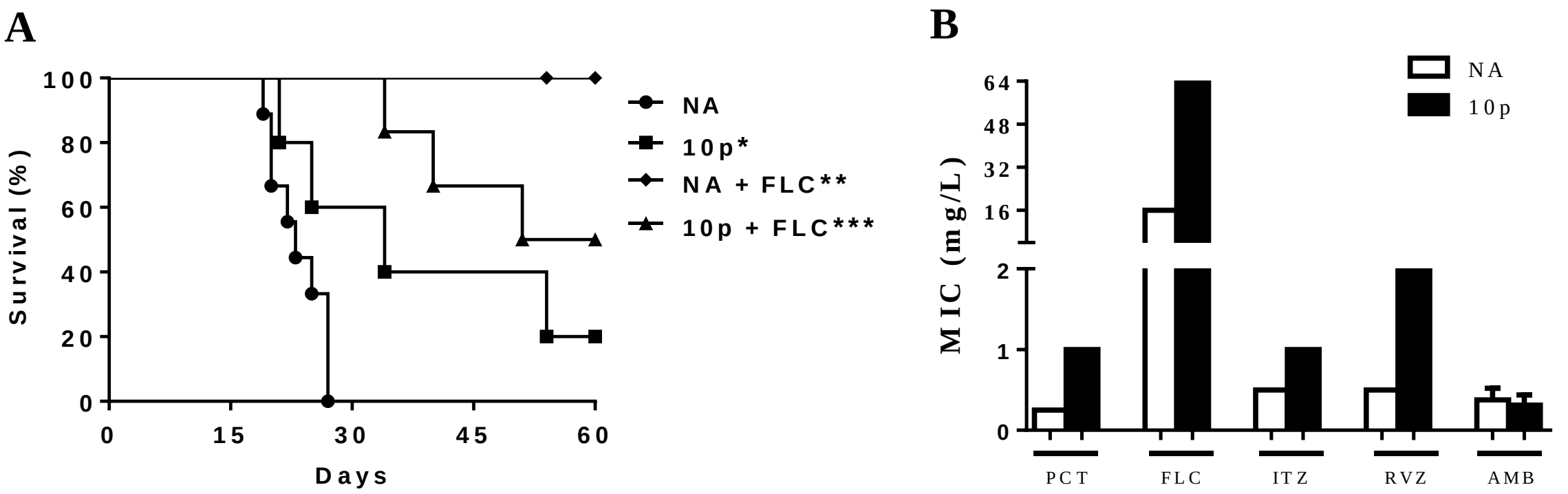

Figure 2: Virulence and *in vivo* Cross-resistance in non-adapted (NA) and adapted cells subcultivated in medium without agrochemical (10p). A) 10p cells are significatvely ($p<0.05$) less virulent than NA. The treatment with fluconazole (FLC) significatvely ($p<0.05$) increased the survival of mice infected with NA, but not of those infected with 10p cells. B) Cells recovered from the lungs of animals infected with 10p colonies were more resistant to pyraclostrobin (PCT), fluconazole (FLC), itraconazole (ITZ) and ravuconazole (RVZ), but not to amphotericin B (AMB), than those recovered from NA-infected mice. ($p>0.05$). * $p<0.05$; ** $p<0.01$ compared to NA group.