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Original article

Cross-resistance to fluconazole induced by exposure to the agricultural azole tetraconazole: an environmental resistance school?

Marcos Fábio Gadelha Rocha,[1,2] L. P. Alencar,[2] M. A. N. Paiva,[2] Luciana Magalhães Melo,[2] Silviane Praciano Bandeira,[1] Y. B. Ponte,[2] Jamille Alencar Sales,[2] G. M. M. Guedes,[1] D. S. C. M. Castelo-Branco,[1] T. J .P. G. Bandeira,[3] R. A. Cordeiro,[1] W. A. Pereira-Neto,[1] G. S. Brandine,[1] José Luciano Bezerra Moreira,[1] José Júlio Costa Sidrim[1] and Raimunda Sâmia Nogueira Brilhante[1]

[1]*Department of Pathology and Legal Medicine, Postgraduate Program in Medical Microbiology, Specialized Medical Mycology Center, Federal University of Ceará, Fortaleza, Ceará, Brazil,* [2]*School of Veterinary Medicine, Postgraduate Program in Veterinary Sciences, State University of Ceará, Fortaleza, Ceará, Brazil and* [3]*School of Medicine, Christus College – Unichristus, Fortaleza, Ceará, Brazil*

Summary

This study aimed to investigate the influence of tetraconazole and malathion, both used in agricultural activities, on resistance to fluconazole, itraconazole and voriconazole in *Candida parapsilosis* ATCC 22019. The susceptibility to tetraconazole, malathion, fluconazole, itraconazole and voriconazole, through broth microdilution. Then, 12 independent replicates, were separated and exposed to four treatment groups, each one containing three replicates: G1: tetraconazole; G2: malathion; G3: fluconazole (positive control); G4: negative control. Replicates from G1, G2 and G3, were exposed to weekly increasing concentrations of tetraconazole, malathion and fluconazole, respectively, ranging from MIC/2 to 32 × MIC, throughout 7 weeks. The exposure to tetraconazole, but not malathion, decreased susceptibility to clinical azoles, especially fluconazole. The tetraconazole-induced fluconazole resistance is partially mediated by the increased activity of ATP-dependent efflux pumps, considering the increase in antifungal susceptibility after the addition of the efflux pump inhibitor, promethazine, and the increase in rhodamine 6G efflux and *CDR* gene expression in the G1 replicates. Moreover, *MDR* expression was only detected in G1 and G3 replicates, suggesting that *MDR* pumps are also involved in tetraconazole-induced fluconazole resistance. It is noteworthy that tetraconazole and fluconazole-treated replicates behaved similarly, therefore, resistance to azoles of clinical use may be a consequence of using azoles in farming activities.

Key words: *Candida* sp., azole resistance, environmental resistance, tetraconazole, efflux pumps, *CDR* genes.

Correspondence: R. S. N. Brilhante, Department of Pathology and Legal Medicine, Postgraduate Program in Medical Microbiology, Specialized Medical Mycology Center, Federal University of Ceará, Fortaleza, Ceará, Brazil.
Tel.: 55 85 3366-8319. Fax: 55 85 3366-8303.
E-mail: brilhante@ufc.br

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Introduction

The overuse and misuse of antibacterial drugs are most likely responsible for the emergence of antimicrobial resistance in bacteria, especially in areas where resistant species are commonly isolated, such as hospital waste,[1] untreated urban wastewater[2] and livestock production and aquaculture effluent.[3,4] Similarly, the detection of antifungal resistance is often associated with patients receiving prolonged antifungal

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treatment. However, antifungal resistance also affects microbial populations from aquatic and terrestrial environments, commonly involving hosts with no previous history of antifungal exposure.[4–7]

Although fluconazole resistance is more frequently reported, resistance to other azoles, such as ketoconazole, itraconazole and voriconazole, also occurs.[4–7] This phenomenon is phenotypically stable, and in human strains, is the result of the action of several mechanisms, alone or combined.[8–10] The increased expression of transmembrane transporters of the ATP-binding cassette family (ABC) is described as the main mechanism for azole resistance in *Candida* spp. ABC efflux pumps generally have low substrate specificity, and produce energy through ATP hydrolysis to remove a wide variety of hydrophobic compounds from the cell, including azoles.[11–13]

In addition to the ABC pumps, the increased expression of the multidrug resistant (*MDR*) gene of the major facilitator superfamily, which encodes proton-dependent efflux pumps, is associated with specific fluconazole resistance.[14] This gene is controlled by the transcription factor Mrr1p (multidrug resistant regulator), and over-expressed in resistant clinical strains.[11,14–16]

In addition, resistance may also be mediated by the increased expression of *ERG11* gene, which leads to higher concentrations of the target enzyme Erg11p,[17–19] hence, requiring greater amount of drug to inhibit fungal growth.[11,20] Moreover, azole resistance may be mediated by mutations in *ERG11* gene, decreasing the affinity between azoles and the enzyme Erg11p.[17,18,20] Finally, azole resistance may also be associated with decreased activity of the enzyme Δ5,6-desaturase (Erg3p), encoded by *ERG3* gene, leading to the decreased production of 14α-metilergosta-8,24(28)-dien-3β,6α-diol, a toxic metabolite, hence promoting yeast survivability.[11,21]

The reports of azole resistance among *Candida* strains from animal and environmental sources with little or no contact with human hosts and no previous history of intentional antifungal exposure[4,5,7,22,23] drew our attention, especially because this resistance occurs at higher rates, when compared to human strains. Based on the idea that resistance is established through the evolution of microorganisms in response to environmental stresses,[24] it was hypothesised that these strains have developed azole resistance in response to the direct or indirect exposure to drugs used in agriculture. Thus, the antifungal susceptibility of one azole-susceptible standard *Candida* strain was assessed, after continuous exposure to compounds used in agriculture (one azole derivative and one organophosphate). Hence, the aim of this study was to investigate the contribution of tetraconazole and malathion for the *in vitro* development of azole resistance, using *C. parapsilosis* ATCC 22019 as a model strain.

Materials and methods

Tested strain

The strain *C. parapsilosis* ATCC 22019, which is susceptible to azoles of clinical use, was grown on potato dextrose agar for 48 h, from one single colony. The susceptibility of this strain to the antifungal drugs and the tested compounds was determined,[25] prior to beginning the experiments.

Antifungal susceptibility testing

The minimum inhibitory concentrations (MIC) of the antifungal drugs (fluconazole, itraconazole and voriconazole) and the tested agricultural compounds (tetraconazole and malathion) against one isolated colony of *C. parapsilosis* ATCC 22019, after growth on potato dextrose agar, were determined according to the document M27-A3, as recommended by the Clinical and Laboratory Standards Institute – CLSI.[25] The susceptibility was investigated through the broth microdilution assay, in 96-well microplates, with a final volume of 200 μL of RPMI buffered at pH 7 with 0.165 mol l^{-1} MOPS. The tested concentration ranges were 0.125–512 μg ml^{-1} for fluconazole (Zoltec, Pfizer, Brazil), 0.125–2048 μg ml^{-1} for tetraconazole (Domark 100 EC; Isagro SpA, Segrate, Milano, Italy) and malathion (Malathion 500 EC; De Sangosse Agrochemical, Curitiba, Paraná, Brazil) and 0.015–16 for itraconazole (Janssen Pharmaceutica, Beerse, Antwerp, Belgium) and voriconazole (Pfizer, New York, New York, USA). The plates were read after 24 and 48 h of incubation at 35 °C, but the MIC values considered were those obtained after 48 h of growth. MIC for azoles and malathion were the lowest drug concentration capable of inhibiting 50% of growth, when compared to the drug-free growth control. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 grown on potato dextrose agar were included as controls.

Induction of resistance

After performing the antifungal susceptibility assay, the following MIC values were obtained: 8, 8, 2, 0.0625 and 0.03125 μg ml^{-1} for tetraconazole,

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malathion, fluconazole, itraconazole and voriconazole, respectively, against the strain *C. parapsilosis* ATCC 22019. It is important to highlight that the obtained clinical antifungal MICs were within the expected range for this control strain.[25] Afterwards, this strain was subcultured into 12 tubes containing YEPD broth (0.5% yeast extract, 1% peptone and 2% dextrose). These tubes were, then, subjected to four different treatments, each one in three independent replicates: (i) tetraconazole (agricultural azole); (ii) malathion (agricultural organophosphate), (iii) fluconazole (positive control) and (iv) drug-free YEPD broth (negative control). The initial concentration to which the replicates of each treatment group were exposed was equivalent to half the MIC value of each treatment compound, i.e. 4, 4 and 1 $\mu g\ ml^{-1}$ for tetraconazole, malathion and fluconazole respectively. The three replicates of each treatment group were exposed to the same drug concentration for 1 week, during which these replicates were subcultured to YEPD broth containing the drugs, at a 48-h interval. At the end of each week, fluconazole susceptibility of all three independent replicates of each treatment group was assessed. Then, the concentration of each treatment drug was doubled and a new cycle of three subcultures was started. These procedures were performed until fluconazole MICs stabilised, which occurred when the independent replicates were exposed to drug concentrations 32 times higher than the initial MIC value of each tested drug. Additionally, at the end of the induction of resistance procedures, three independent replicates of each treatment group were stored at 4 °C in 10% glycerol for 10 months. After this period, the independent replicates were subcultured in drug-free YEPD broth, during 2 weeks, and the MICs for the antifungal drugs (fluconazole, itraconazole and voriconazole) and the agricultural drugs (tetraconazole and malathion) were reassessed. It is important to highlight that for the weekly evaluation of fluconazole susceptibility, the three replicates from the negative control treatment and *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 grown on potato dextrose agar were included as controls. Moreover, all three independent replicates of each treatment group were tested in duplicate throughout this research.

Investigation of resistance mechanisms

Antifungal susceptibility with and without efflux pump inhibitor

Fluconazole, itraconazole and voriconazole MICs, with and without an efflux pump inhibitor, against the three independent replicates of the groups exposed to tetraconazole and fluconazole, and the drug-free group (negative control) were determined according to the document M27-A3,[25] after finishing the protocol of inducing resistance, in order to verify the contribution of these pumps to the resistant phenotype. For such, MIC values were determined in the presence of a subinhibitory concentration (i.e. 32 $\mu g\ ml^{-1}$) of promethazine, a CDR1p and CDR2p inhibitor.[26] The obtained antifungal MICs before and after the addition of promethazine were observed. Malathion-treated group was not included in this analysis because this compound was not able to induce fluconazole resistance.

Efflux of rhodamine 6G

Since rhodamine 6G (R6G) acts as a substrate for CDR pumps,[21,26] this fluorescent substance is used as a tool for the evaluation of their efflux activity. Here, the test was performed according to the protocol described by Ivnitski-Steele *et al.* [26] and Vale-Silva *et al.* [21] with modifications. Briefly, each of the three independent replicates of the groups exposed to tetraconazole and fluconazole, and the drug-free group (negative control) were grown in triplicate (nine replicates for each treatment group), overnight in 5 ml of YEPD broth at 37 °C for 24 h. Afterwards, the replicates were transferred to 5 ml of fresh YEPD broth at 37 °C, under constant agitation, allowing growth until reaching a concentration 2×10^7 cells ml^{-1}. Cells were centrifuged at 4500 ***g*** for 5 min, the supernatant was discarded, and the pellet was washed twice in 2 ml of PBS (pH 7.0). Subsequently, cells were deprived of energy by incubation under constant agitation in 2 ml of PBS at 37 °C, for 1 h. Then, R6G was added to obtain a final concentration of 15 $\mu mol\ l^{-1}$ and the suspension was incubated at 37 °C, for 1 h, in the dark, under constant agitation, in order to allow the influx of R6G into the yeast cells.

After incubation, the cells were washed twice in 2 ml of PBS, at 4 °C, and suspended in 300 μL of PBS. Then, 12.5 μL of the suspension were added to 32.5 μl of PBS in a 96-well plate (Hard-Shell® Low-Profile Thin-Wall) and left to settle for 5 min. Subsequently, glucose was added to reach a final concentration of 1% and the relative fluorescence units (RFU) were recorded at 1 min intervals, during 60 min at 37 °C.[27] The fluorescent signal was measured with a CFX96 Touch™ Real-Time PCR Detection System (Bio Rad, Hercules, California, USA), using 610-650 nm filtre. All replicates of the different treatment groups were read in the presence and in the

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absence of glucose. Negative controls were prepared with PBS instead of glucose. Replicates of the malathion-treated group were not included in the R6G assay because this compound did not induce fluconazole resistance.

Ergosterol content

The amount of cellular sterols within the treated yeast cells was quantified, in order to detect phenotypical alterations in ergosterol biosynthesis that could lead to azole resistance. For such, the extraction of total sterols was carried out, as described by Cordeiro *et al.* [28] The three independent replicates of each treatment group were grown overnight in YEPD broth at 37 °C and centrifuged at 9660 ***g*** for 3 min. Pellets were washed in PBS and the turbidity was adjusted to 0.5 on McFarland scale (i.e. approximately 1.0–5.0×10^6 cells ml^{-1}). Then, 1 ml of this suspension was centrifuged at 9,660 × *g* for 3 min and the pellet was resuspended in 0.5 ml of KOH alcoholic solution (0.7 mol l^{-1} KOH in 60% ethanol) and incubated for 1 h at 95 °C, in a water bath. After cooling, 0.6 ml of n-hexane was added to the tubes, which were vigorously vortexed for 5 min. Then, the tubes were centrifuged at 13,416 × *g* for 1 min and the top organic layer was transferred to a new tube and mixed with 1 ml of n-hexane. The absorbance of this solution was read at 295 nm, in a spectrophotometer. The optical density data were compared to those obtained in a standard curve with ergosterol (Sigma-Aldrich, St. Louis, Missouri, USA). Readings were performed in duplicate for each of the three independent replicates of the treatment groups. The data obtained from replicates exposed to tetraconazole and fluconazole were compared to the negative control. Malathion-treated replicates were not included in this analysis.

CDR, MDR and ERG11 gene expression

The expression of *CDR* and *MDR* (efflux pump genes) and *ERG11* (gene that encodes 14-alpha-demethylase, the target enzyme for azoles) was also assessed in treated groups, since the overexpression of these genes is the most common mechanism of azole resistance. For such, total RNA was extracted, in triplicate, from each of the three independent replicates of the groups exposed to tetraconazole and fluconazole and the drug-free group (negative control), using the RNeasy mini kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. Briefly, the independent replicates were grown in YEPD broth at 37 °C, for 24 h, until reaching the mid-log growth phase. Then, 1 ml of each independent replicate, containing approximately 5×10^7 cells was centrifuged at 4500 ***g*** and the pellet was lysed with RLT buffer and 0.45–0.55 mm glass beads, using Precellys 24 disrupter (Bertin Technologies, Montigny le Bretonneux, Yvelines, France). The lysate was diluted 1 : 1 with 70% ethanol and transferred to a spin column. Genomic DNA was degraded using RNase-free DNase for 15 min at room temperature. After three washes, the RNA was eluted in RNase-free water and the concentration was measured using Qubit fluorometer and RNA Assay Kit (Invitrogen, Paisley, UK). Reverse transcription was performed with 1 µg of RNA combined with 1 µL of Improm II (Promega, Madison, Wisconsin, USA), 0.5 mmol l^{-1} of each dNTP, 40 U of RNaseOUT, 0.5 µg of random primers and RNase-free water to a final volume of 20 µL. Reverse transcription was performed at 42 °C, for 60 min, followed by 70 °C, for 15 min. The first strand cDNA products were stored at –80 °C for later use as templates for quantitative real-time PCR (qPCR). Negative controls and RT blanks were prepared under the same conditions, but without inclusion of RNA or reverse transcriptase respectively.

Expression levels of *CDR*, *MDR* and *ERG11* and reference endogenous *ACT1* and *18S* genes were assessed through qPCR. The reaction mix contained 0.5 µL of cDNA, 1.5 µL of primer (2 µmol l^{-1}) and 7.5 µL of 2 × FastStart Universal SYBR Green Master (Roche, Indianapolis, IN, USA), in a final volume of 15 µL per reaction. The reactions were carried out in a CFX96 Touch™ Real-Time PCR Detection System (Bio Rad) with the following amplification conditions: enzyme activation at 95 °C for 5 min, followed by 40 cycles, each consisting of denaturation at 95 °C for 30 sec, annealing at a specific temperature for each primer pair for 30 sec (Table S1) and extension at 72 °C for 30 s.[29] Fluorescence data were acquired at the end of each extension step. After the final extension, the specificity was ascertained by melting of the amplicons, detecting the fluorescence obtained at the annealing temperature of each primer, at an increase of 0.5 °C every 5 s, until the temperature reached 95 °C. To determine the linearity and reaction efficiency for each primer, the standard curve was plotted using serial dilutions of cDNA with qPCR conditions identical to those established by the experimental protocol. Negative control and RT blank were used. The relative quantification of the gene expression was performed using the $2^{-\Delta\Delta Cq}$ method.[30] Target gene expression was normalised against *ACT1* and *18S* mean expression. Overexpression occurred when the

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levels obtained for the target genes from the replicates of the treated groups (tetraconazole or fluconazole) were statistically higher than those obtained from the negative group. Threshold and Cq (threshold cycle) values were automatically determined by the BioRad CFX manager 3.0 software (BioRad), using default parameters. Replicates of the malathion-treated group were not included in this analysis.

Statistical analysis

The correlation between the exposure to each treatment group and fluconazole MICs was compared by using Pearson's correlation coefficient (r^2). The efflux pump activity of different groups was compared through the analysis of R6G efflux with and without glucose, using the Kruskal–Wallis test with Dunn's multiple comparison test for *post hoc* comparisons. The gene expression levels were compared using one-way analysis of variance (ANOVA), with Bonferroni's *post hoc* test for comparisons. In all tests, $P < 0.05$ was considered statistically significant.

Results

Induction of resistance

The three independent replicates of *C. parapsilosis* ATCC 22019 of each treatment group, exposed to increasing concentrations of tetraconazole, malathion and fluconazole (positive control) evolved to present higher tolerance to the compound they were exposed. At the end of the experiment, after storage in 10% glycerol, at 4 °C, for 10 months, the replicates were grown in drug-free YEPD broth, for 2 weeks, and reassessed for their susceptibility to the compounds to which they were previously exposed. All tested compounds (tetraconazole, malathion and fluconazole) presented increased MICs against the set of replicates exposed to the respective compound. Tetraconazole MICs increased from 4 to 8 times, malathion MICs increased from 128 to 512 times, and fluconazole MICs increased from 64 to 128.

In the tetraconazole-treated group, a positive correlation ($r^2 = 0.97$) between the exposure concentration of tetraconazole and fluconazole MICs was observed for all the three independent replicates. Resistance to this clinical azole was rapidly induced by exposure to increasing concentrations of the agricultural drug. In fact, fluconazole resistance started after exposure to tetraconazole concentrations of 2 × MIC (Fig. 1). Similar results were observed for the replicates that were

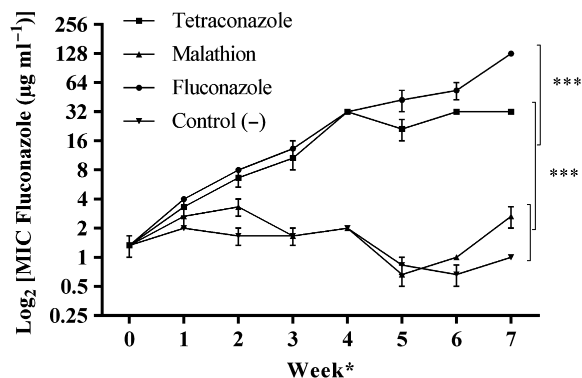

Figure 1 Dynamics of fluconazole susceptibility of independent replicates of *C. parapsilosis* ATCC 22019 exposed to increasing concentrations of tetraconazole ($n = 3$), malathion ($n = 3$) and fluconazole ($n = 3$). Data are expressed as geometric mean ± standard deviation (SD) of each treatment group. Unexposed group: control (–) ($n = 3$). '0' represents the beginning of treatment, with the MICs obtained prior to resistance induction assay. *Drug concentration was doubled weekly and fluconazole susceptibility was tested at the end of each week. Data obtained for each treatment group were compared to drug-free group (negative control). ***$P < 0.001$. In the Y-axis, antifungal concentration ranges from 0.5 μg ml^{-1} [$\log_2(0.5) = -1$] to 256 μg ml^{-1} [$\log_2(256) = 8$].

exposed to fluconazole (positive control). On the other hand, susceptibility to fluconazole did not change in the three replicates exposed to malathion or the negative control replicates. Therefore, the malathion-treated group was not included in the subsequent assays, since exposure to this compound did not interfere with fluconazole MICs.

Antifungal susceptibility testing with and without efflux pump inhibitor

After the induction of resistance, the independent replicates of the groups exposed to tetraconazole and fluconazole, and the drug-free group (negative control) were evaluated for their susceptibility to antifungal agents used in medical practice. In addition to fluconazole resistance, both itraconazole and voriconazole presented increased MIC values against the three replicates exposed to tetraconazole, when compared to the negative control, and similar results were obtained for the positive control drug (fluconazole) (Fig. 2 and Table S2).

The antifungal susceptibility testing showed that efflux pump inhibition in the three tetraconazole replicates promoted 3-dilution and 4-dilution decrease ($P < 0.001$), in MIC for itraconazole and voriconazole respectively (Fig. 2). However, even though *CDR* efflux

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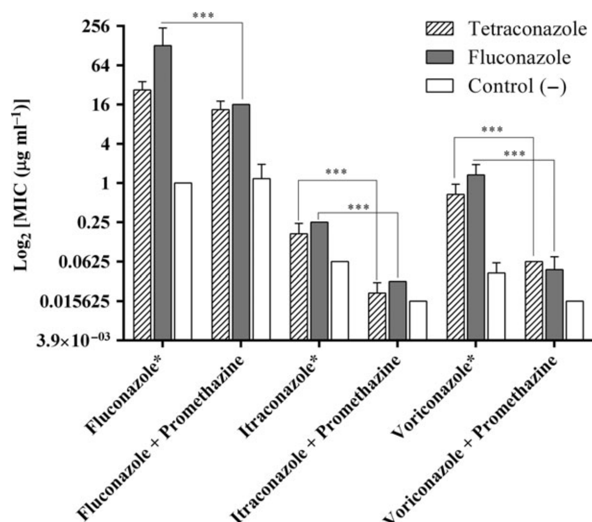

Figure 2 Antifungal susceptibility of the three independent replicates of tetraconazole-treated and fluconazole-treated groups and negative control group, with and without efflux pump inhibitor, after inducing antifungal resistance. Data are expressed as geometric mean ± SD of each treatment group. Unexposed group: Control (−). Data are compared between replicates of the same treatment group, with and without the addition of promethazine. *Indicates a significant increase ($P < 0.05$) in MIC values of fluconazole, itraconazole and voriconazole, after exposure of replicates to increasing concentrations of tetraconazole ($n = 3$) and fluconazole ($n = 3$) compared to the unexposed group ($n = 3$). ***$P < 0.001$. In the *Y*-axis, antifungal concentration ranges from 0.015 μg ml^{-1} [$\log_2(0.015) = -6$] to 256 μg ml^{-1} [$\log_2(256) = 8$].

pump inhibition with promethazine caused a 1-dilution decrease in fluconazole MICs against the three tetraconazole-treated replicates, the resulting MIC values were not statistically different from the fluconazole MICs obtained without promethazine. This finding demonstrates that other mechanisms contribute to fluconazole resistance in the three tetraconazole-treated independent replicates. Similar results were also observed for the three fluconazole-treated replicates (positive control), against which the three tested azole drugs (fluconazole, itraconazole and voriconazole) also presented decreased ($P < 0.001$) MIC values when promethazine was added (Fig. 2).

Efflux of rhodamine 6G

The three tetraconazole-independent replicates presented increased efflux of R6G, when glucose was added to give the energetic support for the activity of *CDR* efflux pumps. The efflux of R6G in these replicates was similar to that observed in the fluconazole-treated replicates (positive control), demonstrating the increased activity of the energy-dependent efflux pumps (Fig. 3).

Ergosterol content

The ergosterol concentrations (mean ± SE) extracted from the three independent replicates of the groups exposed to tetraconazole and fluconazole, and the drug-free group (negative control) were 4.13 ± 0.67, 3.83 ± 0.35 and 3.58 ± 0.46 μmol l^{-1} respectively. Thus, previous exposure to agricultural or clinical azole was not able to interfere with the ergosterol concentration of yeast cells.

CDR, *MDR* and *ERG11* gene expression

In the three tetraconazole-treated replicates, the expression of the *CDR* gene was increased, while the expression of *ERG11* gene was not altered, when compared to the negative control. These results were similar to those found for the three fluconazole-treated

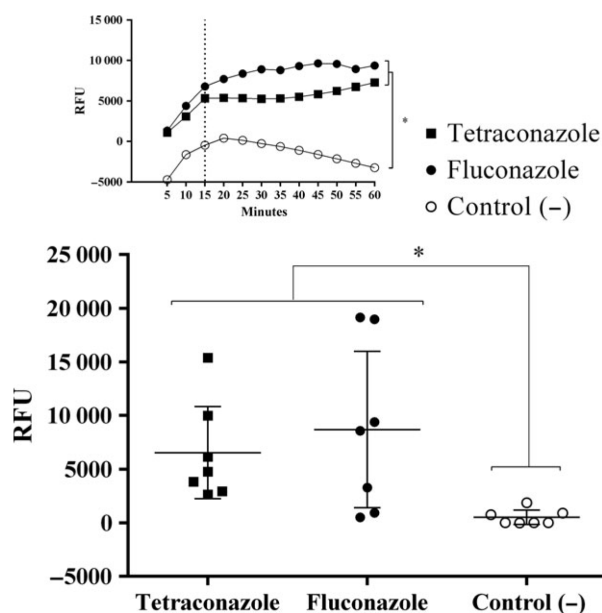

Figure 3 Efflux of rhodamine 6G by the three independent replicates of tetraconazole-treated and fluconazole-treated groups and negative control group, after induction of antifungal resistance. Data are expressed as relative fluorescence units (RFU). The top graph shows the RFU curve of rhodamine 6G efflux, demonstrating a plateau starting at 15 min after the addition of glucose. Each point on the curve represents the mean RFU of replicates with glucose minus the mean RFU of replicates without glucose. The lower graph depicts the median with interquartile range of the difference in RFU of the replicates with and without glucose, 15 min after the addition of glucose. *$P < 0.05$.

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replicates (Fig. 4). The *MDR1* gene reached detectable levels only in the replicates exposed to tetraconazole and fluconazole, but not in those submitted to the negative control. Thus, *MDR1* expression level was not calculated. It is important to highlight that neither primer-dimers nor unspecific products were detected in qPCR amplifications.

Discussion

Candida parapsilosis ATCC 22019 was chosen due to its phenotypical stability, as it is a quality control strain for antifungal susceptibility testing, according to the recommendations of CLSI, which were followed during the present research.[25] In addition, this particular strain was originally recovered from a human case of sprue and the species *C. parapsilosis* is commonly isolated from the microbiota of humans and other animals and from terrestrial and aquatic environmental sources, often presenting reduced azole susceptibility.[4,5,7,31] These characteristics supported the choice of this strain as a model for inducing antifungal resistance through the chronic exposure to compounds used in agricultural practices.

The effect of the *in vitro* exposure of an azole-susceptible *Candida* strain to the azole tetraconazole and the organophosphate malathion, both widely used in farming activities, and its consequence on the susceptibility of microorganisms to azoles of clinical use were evaluated. The obtained results indicate that exposure of *C. parapsilosis* to tetraconazole decreases susceptibility to fluconazole, itraconazole and voriconazole, similar to what was seen in the positive control group exposed to fluconazole. However, exposure to malathion did not change the susceptibility to the tested antifungal drugs; hence, the three malathion-treated independent replicates were not included in the analyses to investigate the resistance mechanisms.

The stress caused by tetraconazole exposure, as demonstrated in this study, most likely promotes cellular mechanisms to escape from the effects of this agricultural antifungal. This allows the development of important characteristics that also promote fungal protection against antifungal agents with similar mechanisms of action. The induction of azole resistance in *Candida* species has previously been observed due to *in vitro*[18,32–34] and *in vivo*[35] continuous exposure to azoles of medical use. In some researches, the MIC values of several azole drugs were simultaneously increased and not only the MIC of the drug used in the induction assay.[33,34] Similarly, in this study, the MIC values of fluconazole, itraconazole and voriconazole were significantly increased, after the continuous *in vitro* exposure of three *C. parapsilosis* ATCC 22019 independent replicates to increasing concentrations of tetraconazole. These results are equivalent to those obtained for the positive control drug (fluconazole), demonstrating that exposure to clinical azole,[18,32–35] as well as to agricultural drugs, may be responsible for the development of cross-resistance to several azoles in strains of *Candida* spp. and other fungal species.[36–38]

There are several reports of resistance in fungal species, which involves common mechanisms, regardless of the triggering factor, i.e. azoles used in human and animal medical practice, or in agricultural practice. These indications associated with the recovery of resistant fungal strains from the microbiota of animals without prior history of antifungal treatment[4,5,7] reinforce the assumption of the existence of a selective pressure for the development of antifungal-resistant strains within the rural and/or wild environment. This environmental adaptation is crucial for the

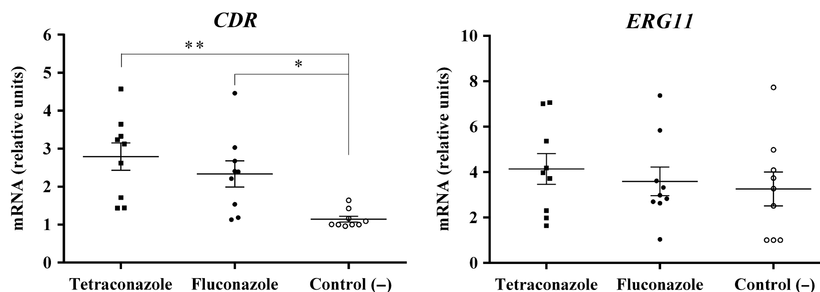

Figure 4 Expression of *CDR* and *ERG11* genes in the three independent replicates of tetraconazole-treated and fluconazole-treated groups and negative control group, after induction of antifungal resistance. Data are normalised to mean signal of *ACT1* and *18S* genes. The expression of one replicate of the negative control group was used as calibrator and their transcription level was set as '1 relative unit'. Data are expressed as dispersion and mean ± SE of the replicates. Data of each treatment are compared to the negative control group. **P* < 0.05; ***P* < 0.01.

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development of commensal and pathogenic characteristics of *Candida* spp.[39] In this sense, Hube [39] proposed the existence of 'commensal virulence schools' in which the microorganism develops certain characteristics to adapt or successfully infect the host. Similarly, the stress caused by the exposure to azoles in agriculture[36] (e.g. tetraconazole) could be an 'environmental resistance school', promoting the development of important features in microorganisms to enhance their survivability in the environment. Finally, the development of these features seems to induce cross-resistance to medical azoles.

The knowledge on azole resistance in *C. parapsilosis* is still incipient. Even though this phenomenon probably results from a combination of classical mechanisms studied in other *Candida* species,[14,35,40,41] the high MIC values of azoles against *C. parapsilosis* may also be associated with other molecular mechanisms that still need to be elucidated.[40] However, it has been show that in fluconazole-resistant *C. parapsilosis*, *CDR* overexpression is more common than the overexpression of *MDR1*, and both are more common than overexpression of *ERG11*.[14,35,40,41] Thus, in this study, we primarily decided to investigate the drug-induced efflux-mediated mechanisms of resistance in *C. parapsilosis*. In this context, it was observed that tetraconazole exposure increased the activity of efflux pumps in *C. parapsilosis*, suggesting that this induced resistance is mainly associated with increased drug efflux through ATP-dependent pumps. These results are similar to those obtained for the fluconazole-treated (positive control) replicates in this study, and in others.[18,32–34,40,41] One of the evidences for the involvement of ATP-dependent pumps is the reduction of the antifungal MICs, especially those of itraconazole and voriconazole, against the azole-resistant tetraconazole-treated replicates, when these efflux pumps are inhibited by promethazine.[42] Other evidence for the involvement of these pumps in the development of antifungal resistance after exposure to tetraconazole is the increase in R6G efflux, which presented clonal difference between the independent replicates, based on the observed efflux outliers and the relative stability in efflux activity among the replicates from the negative control group. These findings demonstrated that the CDR efflux pumps, among other ATP-dependent pumps, are primarily involved in the development of azole resistance. Moreover, this observation was confirmed by the increased *CDR* gene expression in tetraconazole-treated replicates, similar to what was seen for the fluconazole-treated replicates.

The inhibition of the CDR efflux pumps reduced the azole MICs against the tetraconazole and fluconazole-treated replicates, but these MICs were still higher than those obtained against the negative control group. Moreover, CDR efflux pump inhibition did not cause significant reduction in fluconazole MICs against tetraconazole-treated replicates, which suggests the involvement of MDR proton pumps in this azole-induced resistance.[14,43] Additionally, the detection of *MDR1* transcripts only in tetraconazole and fluconazole-treated replicates, but not in negative control, strengthens the assumption that these proton pumps are also involved in the development of tetraconazole-induced fluconazole resistance,[43] similar to what has been described for clinical strains.[14,35,40] The maintenance of the amount of ergosterol extracted from the tetraconazole and fluconazole-treated *C. parapsilosis* ATCC 22019 replicates, when compared to the negative control, and the undifferentiated *ERG11* expression between treated replicates and the negative control, showed that, in this study, increased ergosterol biosynthesis was not involved with the development of resistance.[43] However, mutation in genes involved in ergosterol biosynthesis cannot be ruled out, especially, considering the possibility that more than one resistance mechanism may be acting at the same time. Even though we did not perform a sequence analysis of the genes involved in the biosynthesis of ergosterol, it is known that the single-nucleotide polymorphism (SNP) Y132F in *ERG11* nucleotide sequence is the most common alteration in strains of fluconazole-resistant *C. parapsilosis*.[14,40]

The investigation of the possible resistance mechanisms demonstrated that the tetraconazole-treated replicates presented similar results to those treated with fluconazole. Thus, we have demonstrated that resistance to medical azoles may be a consequence of the exposure to agricultural azoles. In *C. parapsilosis* ATCC 22019, for instance, this phenomenon seems to be mainly associated with the increased activity of *CDR* efflux pumps, as a response to the continuous exposure to a drug-rich environment. These adaptive changes decrease azole susceptibility through unspecific pathways and seem to be phenotypically stable, after the storage of the strains at 4 °C for 10 months in glycerol. Thus, we believe that the misuse or prolonged use of azoles in humans[8,9,16] and also in the environment may be the cause of resistance to these drugs in strains from diverse origins. The selection of *Candida* strains with reduced antifungal susceptibility in environments of agricultural production is relevant because these strains may colonise cultivated food items, and, subsequently, they may colonise the gastrointestinal tract of humans and other animals. In

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addition, considering that these agricultural drugs have long residual effect,[44] their direct action on the yeast microbiota of humans and animals that feed on products with drug residues may also be speculated. Either or both mechanisms may be involved in the recovery of azole resistant *Candida* strains from several animal species, such as prawns, porcupine, raptors, rheas and tortoises, as reported in previous works of our group.[4,5,7,45,46]

The occurrence of azole resistance in strains from veterinary and environmental sources suggests that these strains can acquire such features in the environment,[36,38,47,48] as a response to stress.[24] Thus, in allusion to what was described by Hube [39] who defended the existence of 'commensal virulence schools', the presence of agricultural azoles in the environment would function as an 'environmental resistance school', and, in this case, tetraconazole would act as an intensive school, since it acts on environmental microbial communities for periods longer than 90 days.[44] Thus, these data bring perspectives for further studies on the impact of drugs and other chemical compounds used in agriculture on microbial populations and on the induction of azole resistance in the environment.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Primers used for gene expression analysis of *Candida parapsilosis* ATCC 22019 subjected to resistance induction assay through qPCR.

Table S2. Antifungal susceptibility of *Candida parapsilosis* ATCC 22019 independent replicates to fluconazole, itraconazole or voriconazole after resistance induction assay through the exposure to increasing concentrations of tetraconazole, malathion or fluconazole, during 7 weeks

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