Influence of adenylate cyclase in Pleiotropic Drug Resistance Transporter Genes expression after azole exposure and ergosterol content of *Rhizopus microsporus*

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FINAL DEGREE PROJECT

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1. ABSTRACT AND KEYWORDS

Mucormycosis is an opportunistic and angioinvasive infection caused by fungi of the order Mucorales, being those of the genus *Rhizopus* responsible for around 70% of the cases worldwide. One of the most used pharmacological therapies for its treatment is the administration of antifungals from the azole group. However, the innate and acquired resistance of Mucorales to these drugs complicates the treatment of mucormycosis, reaching mortality rates greater than 90% in cases of rhinocerebral or disseminated infection. One of the most prominent acquired resistance mechanisms to azoles is the upregulation of the Pleiotropic Drug Resistance Transporter Genes (*pdr*). They encode efflux pumps that actively pump drugs outside the cell, lowering its concentration below the effective one. In both yeasts and filamentous fungi, an increase in its expression is related to resistance to different azoles. In addition, resistance to azoles and the expression of those genes have also been linked with the enzyme adenylate cyclase and the ergosterol content in the fungal membrane. However, these findings have not been studied in fungi such as *Rhizopus microsporus*, the main species causing mucormycosis. In this work it has been studied the impact of the lack of adenylate cyclase in *R. microsporus* on the ergosterol content, susceptibility to different azoles and the expression of said genes after exposure to itraconazole and posaconazole. The results have shown a higher ergosterol content in the strain without adenylate cyclase, while the susceptibility to azoles, evaluated by the Microdilution Broth method, has not been modified. Besides, cultures of *R. microsporus* strains with and without adenylate cyclase have been passed in increasing concentrations of itraconazole and posaconazole in microdilution plates. In this way, it has been observed a greater adaptation of the strain without adenylate cyclase to high concentrations of itraconazole. Moreover, through semiquantitative PCR, there has been seen upregulated expression patterns of the *pdr* genes after exposure to the drugs in the strain lacking the enzyme. Meanwhile, the pattern is not that clear in the wild-type strain. In conclusion, it seems that adenylate cyclase increases the ergosterol content, azole resistance and *pdr* genes expression. However, it is necessary to carry out more replications and assays to corroborate and consolidate these results.

Keywords: *Rhizopus microsporus*, adenylate cyclase, antifungal resistance, ATP-binding-cassette superfamily proteins, ABC transporters, Pleiotropic Drug Resistance.

2. INTRODUCTION

The kingdom *Fungi* was proposed by Whittaker in 1969 to design a group of eukaryotic unicellular (yeasts), pluricellular (filamentous) or dimorphic organisms, mainly motionless (Sanchis, 2016). Most fungi are saprophytic, nourishing from the organic matter previously decomposed and absorbed present in the substrate where they grow. As they are ubiquitous organisms, their nutrition is also relevant to the rest of the ecosystem, since it plays an important role in the recycling of carbon and energy (Roth *et al*., 2023). Morphologically, fungi are characterized for having a cell membrane rich in ergosterol, essential for its fluidity and transmembrane transport. They are also distinguished for having a cell wall rich in beta-glucans, chitin and chitosan, crucial in cell formation and division, the integrity of spores and resistance to multiple stresses (Sanchis, 2016).

Nowadays, fungi are classified into twelve or twenty phyla depending on the taxonomic methodology used for their identification (Li *et al.*, 2021; Voigt *et al*., 2021). Of them, the majority comprise the so-called group “Basal Fungi”, which encompasses multiple phyla among which is the phylum *Mucoromycota*, highlighting the Mucorales order for its pathogenicity. Even so, Mucorales is one of the least known and studied groups of fungi due to the difficulty in manipulating and genetically analysing its members (Tahiri *et al*., 2023). While is true that the recent use of mRNA sequencing or Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9 (CRISPR-Cas9) has provided greater knowledge in this area (Lax *et al*., 2022), these techniques are still little used in Mucorales research.

Mucorales are fast-growing filamentous fungi with wide aseptate hyphae and present both sexual and asexual reproduction through spores (Alqarihi *et al*., 2023; Sanchis, 2016). They stand out for the opportunistic disease they cause, known as mucormycosis, being those belonging to the genus *Rhizopus* the most common causal agents. They are responsible for more than 70% of mucormycosis cases worldwide, being *Rhizopus microsporus* the most prevalent species along with *Rhizopus arrhizus* (Martín & Salavert, 2021). Infection occurs mainly by inhalation of its asexual spores or by contact with open wounds in susceptible individuals. Depending on the organs affected, they are divided into cutaneous, gastrointestinal, pulmonary, rhinocerebral and disseminated (Martín & Salavert, 2021). Due to the innate and acquired antifungal

resistance of Mucorales, few effective drugs are available for mucormycosis treatment. Consequently, debridements and amputations of affected tissue are common and in rhinocerebral and disseminated infections the mortality rate rises to 90% (Tahiri *et al.*, 2023; Alqarihi *et al.*, 2023). Furthermore, in the last years, there has been an increase in cases due to the higher population with factor risks. The main ones are neutropenia, corticosteroid treatment, organ transplants, severe burns and uncontrolled diabetes mellitus, among others (Martín & Salavert, 2021).

2.1. Mucorales resistance to antifungal compounds

Antifungal compounds are substances whose target is crucial for fungal growth and/or viability, aiding the host-infected immune system to eliminate these pathogens (Odds *et al*., 2003). These drugs are classified into five families depending on their chemical structure: polyenes, azoles, allylamines, echinocandins and fluorinated pyrimidines. Polyenes bind to ergosterol and form pores in the fungal membrane, azoles and allylamines diminish ergosterol content, echinocandins target cell wall compounds as beta-glucans and chitin and fluorinated pyrimidines interfere in nucleic acid synthesis (Campoy & Adrio, 2017).

The objective of evaluating antifungal activity *in vitro* is to determine how effective antifungals are in inhibiting fungal growth or development. Two organizations establish standardized protocols for its determination: the Clinical & Laboratory Standards Institute (CLSI, EE. UU) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, Europe). Both use the Broth Microdilution method to assess the minimal drug concentration that inhibits fungal growth (MIC). This method is based on growing fungal suspensions in microplates or tubes filled with increasing double serial concentrations of the antifungal under study and reading the results after the required incubation time (Espinel-Ingroff & Turnidge, 2016).

At present, the polyene amphotericin B is the most used antifungal for mucormycosis treatment due to its generally high effectiveness and the low rate of appearance of Mucorales fungi resistant to it (Cornely *et al*., 2019). It binds to fungal cell membrane ergosterol, creating pores that alter the membrane permeability causing cell death (Campoy & Adrio, 2017). Nevertheless, its relative toxicity (the greatest risk being kidney involvement) has led to the use of other compounds as primary treatments,

mainly azoles. Of them, the most used today are voriconazole, posaconazole and isavuconazole (Martín & Salavert, 2021). However, this group of drugs also has serious side effects (mainly liver damage) and is heavily susceptible to be ineffective against Mucorales fungi because of their target, the lanosterol 14-α demethylase. This enzyme is encoded by the *cyp51* gene (also known as *erg11*) and involved in the biosynthesis of ergosterol. As in other fungi, there have been identified in Mucorales amino acid substitutions which produce an enzyme inefficient in azole drug binding (Figure 1A) or an increased amount of it (Figure 1B) (Caramalho *et al*., 2017; Paul *et al*., 2019). Consequently, functional enzymes remain despite the administration of azoles, impeding the alteration of the ergosterol pathway and the fungal death. Among these, in both filamentous fungi and yeasts there have been found other mutations related to azole resistance, such as alterations in the ergosterol pathway which avoid the formation of toxic products due to azole administration (Figure 1C). They are mostly acquired due to previous exposure to the antifungals through long-term azole patient therapy or the application of azole compounds in the environment for crop protection or material preservation (Chowdhary *et al*., 2013; Rosam *et al*., 2020; Verweij *et al.,* 2016). Of these mutations, one of the main resistance mechanisms is the acquired alteration of ejection pumps as ATP-binding-cassette (ABC), localized in the cell membrane (Canuto & Rodero, 2002) (Figure 1D). They actively pump drugs outside the cell lowering its concentration below the effective one, permitting fungal survival and leading to the named Multidrug Resistance phenomenon, a concern in public health (Catalano *et al*., 2022).

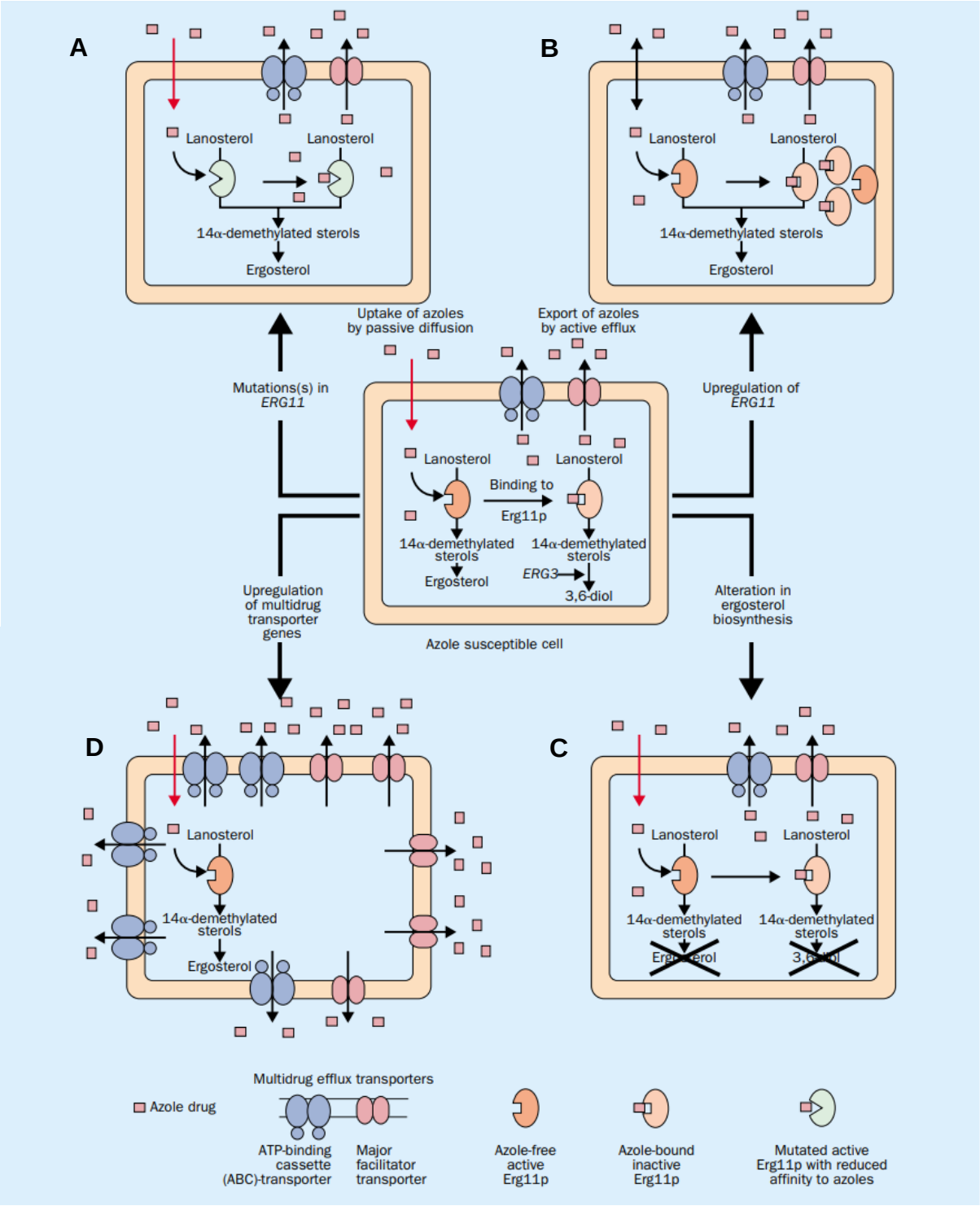

Figure 1. Main known azole resistance mechanisms. **A)** Inefficient azole binding enzyme. **B)** Upregulation of *erg11*. **C)** Alteration of the ergosterol biosynthetic pathway preventing the formation of toxic products such as 3,6-diol. **D)** Upregulation of ABC ejection pumps encoding genes. Adapted from Sanglard & Odds (2002).

2.1.1. ATP-binding-cassette superfamily proteins and Pleiotropic Drug Resistance

The ABC superfamily proteins include a huge number of molecules whose main function is related to the compound transport across biological membranes through ATP energy. The wide substrate repertoire ranges from natural metabolites to xenobiotics, being the last ones the reason which makes some ABC transporters key players in antifungal resistance. Nowadays, these proteins have only been widely studied and functionally characterized in yeasts such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* or *Candida albicans*. Except for these fungi, most of the current knowledge about ABC transporters comes from bioinformatic analysis (Víglas & Olejníková, 2021).

Fungal ABC transporters superfamily is divided into seven subfamilies: ABCA, ABCB/MDR, ABCC/MRP, ABCD/ALD, ABCF, ABCG/PDR and ABCI, being ABCB, ABCC and ABCG the most relevant groups in Multidrug Resistance occurrence (Víglas & Olejníková, 2021). All of them have characteristic features, but it is ABCG/PDR, encoded by Pleiotropic Drug Resistance Transporter Genes (*pdr*), the one involved in the transport of the greatest number of xenobiotics. Nevertheless, ABCG/PDR can also transport physiological substrates, mainly sterols, steroids and phospholipids. Since ergosterol is the target of azole antifungal compounds, the contribution of these transporters to azole resistance seems to be a natural response to changes in lipid homeostasis. This hypothesis is supported by common regulation of ergosterol biosynthesis genes and genes encoding ABC transporters (Víglas & Olejníková, 2021). In this regard, a relationship has been found between the ABCG/PDR transporter encoding gene *abcG1*, an important determinant of azole resistance in *Aspergillus fumigatus,* and *cyp51* in that fungus. There's an ABC transporter regulator factor (AtrR) which binds to both *cyp51* and *abcG1* promoter regions and enables their normal transcription (Paul *et al*., 2019). Further work showed that AtrR controls the expression of multiple genes in the ergosterol biosynthesis pathway, and its paper in azole resistance has also been found in other fungi like *Fusarium graminearum* (James *et al.,* 2022). AtrR target genes also overlap largely with the target genes of the sterol-responsive transcription factor SrbA and itself (Moye-Rowley, 2020). SrbA is a direct transcriptional regulator of *cyp51* and other genes encoding key enzymes in ergosterol biosynthesis. It also plays a significant role in antifungal drug responses, since its lack in fungi such as *A. fumigatus* is correlated

with an increased azole susceptibility although through a still unknown mechanism (Blosser *et al*., 2014; Camps *et al*., 2012). Taken together, this suggests that modifications in *pdr* genes can impact the ergosterol content and azole resistance of fungi and vice versa.

All of this makes ABCG/PDR the subfamily of ABC transporters most closely associated with drug resistance, being the *pdr* genes reported as one of the main ones involved in resistance to azoles. In clinical strains with azole resistance of *Candida spp.* the genes *cdr1* and *cdr2*, which belong to the PDR protein class of ABC transporters, have been found upregulated (Rocha *et al.,* 2016; Khosravi *et al*., 2016). In *Cryptococcus neoformans* and *Saccharomyces cerevisiae* overexpression of *pdr* genes led to fluconazole resistance (Chang *et al.,* 2018; Rogers *et al*., 2001). In filamentous fungi such as *A. fumigatus*, *Fusarium keratoplasticum* and *Mucor lusitanicus* upregulation of *pdr* genes after azole treatment has been found (Fraczek *et al*., 2013; Hagiwara *et al*., 2017; James *et al.,* 2021; Lopez-Ribot *et al.,* 2017; Nagy *et al*., 2021). However, the expression of these genes and azole resistance in Mucorales have only been addressed in the last study mentioned above (Nagy *et al*., 2021). Except for this one, in the currently available literature, there are no studies in this area about *R. microsporus* or other Mucorales.

2.1.2. Adenylate cyclase function and impact of its deletion in *pdr* genes, azole resistance and ergosterol biosynthesis

Adenylate cyclase, known as well as adenylyl cyclase, is the enzyme responsible to produce cyclic adenosine 3 '5' monophosphate (cAMP). In fungi, it impacts growth, development and virulence, among others (Yu *et al*., 2023), and the cAMP pathway is generally required for the pathogenicity in humans (Kim *et al*., 2021). In response to diverse extracellular stimuli, adenylate cyclase is activated by monomeric or heterotrimeric guanine nucleotide-binding proteins (G proteins) and a G protein-coupled receptor. Then, the enzyme converts ATP to cAMP, which binds to the regulatory subunit of protein kinase A (PKA), releasing catalytic subunits that activate downstream transcription factors and other effectors involved in multiple biological reactions (Kim *et al*., 2021).

Since adenylate cyclase impacts in most significant physiological processes, there are various studies analysing the effects derived from its elimination in different fungi, mostly yeasts. In *Candida spp.* cAMP is involved in the expression of genes belonging to the ABC transporters superfamily and ergosterol biosynthesis (Kim *et al*., 2021). Specifically, in *Candida auris* the lack of adenylate cyclase increases susceptibility to azole family drugs due to or together with an increase in *cdr1* expression and a decrease in *cdr2* expression (Kim *et al*., 2021). This increased susceptibility has also been seen in *Candida albicans*, *Saccharomyces cerevisiae* and *Cryptococcus neoformans*, being the strains lacking adenylyl cyclase hypersusceptible to azoles such as fluconazole, itraconazole and miconazole (Jain *et al.*, 2003; Maeng *et al*., 2010). Nevertheless, contrary to what Kim *et al*. (2021) reported, the increased azole susceptibility in *C. albicans* strains without adenylate cyclase was suggested to be related to a deficiency in the upregulation of *cdr1* (Jain *et al*., 2003). Additionally, in this last study, the increased susceptibility to azoles was partially reverted when cAMP was added to the culture medium, establishing a stronger relationship between adenylate cyclase function and azole resistance. In the same way, when an inhibitor of the enzyme was tested in combination with azoles against *Candida spp.* strains their resistance decreased, suggesting a useful adjuvant role of adenylate cyclase inhibitors in azole therapy. Collectively, these data suggest that cAMP increases azole susceptibility through modulation of drug targets, including the expression of *pdr* genes (Kim *et al*., 2021).

Regarding ergosterol, studies in *C. auris* have reported a decrease in the expression of *erg11* and *erg3* genes when the adenylate cyclase encoding gene was deleted (Kim *et al*., 2021). Nonetheless, in *C. albicans* an upregulation in *erg11* expression was observed in the strain lacking adenylyl cyclase (Jain *et al*., 2003) and in *C. neoformans* mutant strains lacking the enzyme the expression of genes involved in ergosterol biosynthesis did not change concerning the wild-type strain (Maeng *et al*., 2010). These genes are essential for ergosterol synthesis, and its known that the alteration of the ergosterol pathway such as deletion of *erg3* contribute to azole resistance (Hirayama *et al*., 2021). However, those strains lacking adenylate cyclase were hypersusceptible to azoles. In summary, knowing that changes in ergosterol content can modulate azole resistance, although sometimes the results obtained are opposite,

there seems to be an interrelated relationship between adenylate cyclase, ergosterol, *pdr* genes and azole susceptibility.

3. WORK JUSTIFICATION

Over time, the number of azoles that are not effective against Mucorales is increasing. In some fungi, there has been observed a relationship between adenylate cyclase, *pdr* genes, ergosterol content and azole resistance. Even so, this line of research has not yet been studied in *R. microsporus*, being the main causative agent of mucormycosis around the world (Martín & Salavert, 2021). Thus, in this work, it has been evaluated the impact of the lack of the two adenylate cyclases of *R. microsporus* in ergosterol content, azole susceptibility and the expression of its six *pdr* genes after exposure to posaconazole and itraconazole antifungals.

4. HYPOTHESIS AND OBJECTIVES

Hypothesis: The lack of adenylate cyclase in *R. microsporus* modifies its ergosterol content and causes lower resistance to azoles due to a decrease in the expression of its *pdr* genes.

General objective: To evaluate the impact of adenylate cyclase on the amount of ergosterol of *R. microsporus* and assess the expression of its six *pdr* genes before and after exposure to itraconazole and posaconazole.

Specific objectives:

- Quantify the amount of ergosterol of *R. microsporus* strains with and without adenylyl cyclases.

- To evaluate the susceptibility to azoles of *R. microsporus* strains with and without adenylate cyclases through the Microdilution Broth method and generate itraconazole- and posaconazole-resistant colonies through increasing exposures to the antifungals.

- Determine the expression level of the six *pdr* genes of *R. microsporus* strains with and without adenylate cyclases by semiquantitative PCR before and after posaconazole and itraconazole exposure.

5. MATERIALS AND METHODS

5.1. Strains and culture conditions

Two *R. microsporus* strains were used in this work, cultured as standard at 30 ºC in Yeast Nitrogen Base (YNB) medium:

- ATCC 11559: obtained from the American Type Culture Collection (ATCC), with its two adenylate cyclase enzymes (wild-type).
- UM 161: provided by the Departamento de Genética y Microbiología from the Universidad de Murcia, mutant for the two adenylate cyclase enzymes. It's generated from the *R. microsporus* ATCC 11559 strain through CRISPR-Cas9 genetic edition. Nevertheless, since the lack of both adenylate cyclases is lethal for the fungus, the strain is a heterokaryon. It has nuclei lacking both adenylate cyclase enzymes and nuclei auxotrophic for leucine lacking only the first adenylate cyclase. In this way, culturing the strain in media without leucine as YNB makes the expression of the double mutated nuclei necessary for the survival of the fungus. Meanwhile, the nuclei with the second adenylate cyclase prevent its death, impeding the lack of enzyme from being total.

5.2. Spore collection

Spore collection was carried out by washing the cultured plates with 0,01% Tween 20 distilled water solution (CLSI, 2017). The obtained suspensions were diluted until an adequate number of spores was achieved to perform a count in the Neubauer chamber and 5×10^6 spores/mL suspensions were made as stock for the corresponding assays.

5.3. Ergosterol quantification

To obtain biomass of the two strains and carry out the ergosterol quantification, 50 µL of spore suspension at a concentration of 5×10^5 spores/mL were inoculated in 250 mL of liquid YNB. The cultures were incubated with shaking at 125 rpm and 30 ºC for 5 days and the biomass obtained was subsequently filtered through monodur filters, washed with sterile distilled water, dried with filter paper and lyophilized to eliminate all moisture. Finally, the dry biomass was pulverized with the help of a sterile mortar (Pérez, 2020).

The determination of ergosterol content was carried out according to the method established by Arthington-Skaggs *et al*. (1999). Of the powdered homogenate

obtained from each strain, 60 mg were weighed in eppendorfs and transferred to 10 mL glass tubes, to which 6 mL of 25% KOH/EtOH solution were added. The samples were vortexed and incubated at 85 °C in a water bath for 1 h, shaking every 15 minutes. Then, they were cooled to room temperature for 10 minutes and 2 mL of sterile distilled water and 6 mL of n-heptane were added. The samples were homogenized for 3 minutes to allow the interaction between ergosterol and n-heptane and the upper layer generated by said compounds was transferred to glass tubes. Finally, quantification was carried out by measuring the absorbance at 281.5 nm and 230 nm of the samples and a blank (n-heptane) using a NanoDrop spectrophotometer and applying the formulas detailed in the referenced methodology (Arthington-Skaggs *et al*., 1999).

5.4. Antifungal susceptibility assay

To evaluate the antifungal susceptibility of the strains depending on the presence or absence of adenylate cyclase, the Broth Microdilution method was carried out as described in the CLSI M38 Guide (2017). Nonetheless, it was necessary to vary the culture medium, since standard Roswell Park Memorial Institute (RPMI), used in the CLSI protocol, has leucine, and to obtain a double-mutant phenotype of the UM 161 strain the culture medium mustn't contain that amino acid. The assayed compounds were fluconazole, itraconazole, posaconazole and voriconazole, all of them from the commercial company Sigma-Aldrich (Saint Louis, USA), with a purity ≥ 98% and soluble in DMSO except for fluconazole, soluble in water. Serial double dilutions were made from stock solutions using DMSO or sterile distilled water as a solvent depending on the drug as shown in Figure 2.

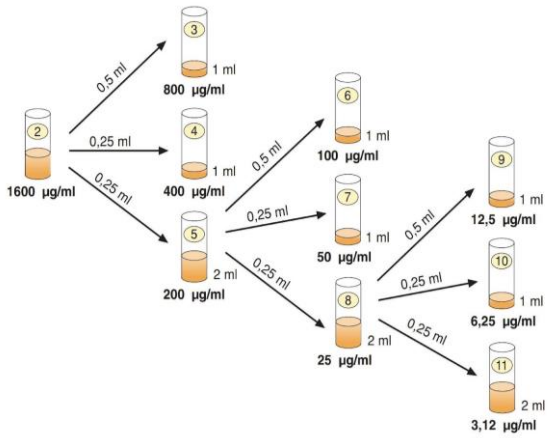

Figure 2. Scheme of the procedure followed for dilutions of the antifungal compounds (CLSI, 2017).

The inoculum was obtained by collecting spores as specified in section 5.2 from 5-day cultures in YNB plates and adjusting the obtained suspensions to 5×10^4 spores/mL in liquid YNB. As a quality control of the procedure and the status of the antifungals, the reference strain ATCC 204304 of *Aspergillus flavus*, whose MICs are standardized by the CLSI, was included in the test. Subsequently, 100 µL of each spore suspension were inoculated into concave-bottom 96-well microdilution plates wells, where 100 µL of the antifungal dilutions were also added. The range of final concentrations was from 16 µg/mL to 0,0312 µg/mL and the microplates were incubated 24 h at 35 ºC. Then, the results were read as described in the CLSI M38 guide (2017) using an inverted mirror and the drugs with the lowest MIC were chosen for the next assay.

To assess the assay reliability, the viability of the spore suspensions was determined to verify that it was within the range stipulated by the CLSI M38 guideline (2017). YNB agar plates were inoculated with 100 µL of a 5×10^2 spores/mL solution to obtain a maximum of 50 colony forming units (CFUs) per plate and after 24 h of incubation at 30 ºC CFUs were counted.

5.5. Antifungal resistant emergence rate assay

To evaluate the existence of a possible better adaptation to a high drug concentration depending on the presence or absence of adenylate cyclase a resistant emergence rate assay was carried out. The tested azoles were posaconazole and itraconazole, the two compounds to which the strains were not resistant according to the Microdilution Broth method results. As in the antifungal susceptibility assay, 5×10^4 spores/mL solutions in liquid YNB medium were incubated in microdilution plates filled with itraconazole and posaconazole solutions at two-fold subinhibitory concentration respect the MIC obtained in the antifungal susceptibility assay (0,5 µg/mL for itraconazole and 0,25 µg/mL for posaconazole). After 48 h at 35 °C the obtained mycelia were discarded and 100 µL from the suspension of the wells were transferred to microdilution plates with the MIC concentration of posaconazole and itraconazole (1 µg/mL and 2 µg/mL respectively). Even without being able to control the concentration of the inoculum, the assay was carried out using the medium from the wells because exposing the strains directly at inhibitory concentrations using spore suspensions of known concentration didn't allow their growth. Regarding the type of sample, using the obtained mycelia was not an option because they filled the entire

bottom of the new wells and was not possible to distinguish which colonies had increased their growth and which had not. Finally, as in the MIC assay, the microplates were incubated 24 h at 35 °C and the resistance ratio was calculated by counting the wells with fungal growth concerning the total number of cultured wells.

5.2. Search of *pdr* genes in *Rhizopus microsporus*

Basic Local Alignment Search Tool (BLAST) from the Joint Genome Institute (JGI) database was used to align the predicted amino acid sequence from the eight *pdr* genes sequences found in *M. lusitanicus* CBS277.49v2.0 genome database by Nagy *et al*. (2021) with the *R. microsporus* ATCC11559 v2.0 genome (Lax *et al*., 2021). *M. lusitanicus pdr* gene sequences were chosen as reference because it is the only Mucoral such as *R. microsporus* in which these genes have been studied.

5.3. Primer design

Specific primers were designed after identifying the six *R. microsporus* genes homologous to the *pdr* genes of *M. lusitanicus*. The obtained sequences were introduced in the softwares Primer3Web and PrimerBLAST together with some parameters related to the primers and PCR conditions: a G+C content between 40 % and 60 %, an optimum annealing temperature of 60 ºC and a product length of around 200 bp nucleotides. Of the resultant sequences, those with a lower probability of giving rise to pairings or secondary structures among themselves were selected. Then, their characteristics and quality were checked again using the PCR Primer Stats tool (https://www.bioinformatics.org/sms2/pcr_primer_stats.html). In this way, two pairs of primers were obtained for each gene to have a greater probability of achieving gene amplification. The primers for the constitutive expression gene (*mitC*) used to normalize the results had already been designed in previous assays (Table 1).

Table 1. Primers sequences for the amplification of the six *pdr* and the housekeeping genes from *R. microsporus*. Subscript numbers indicate different pairs of primers.

Gene name	Protein ID	Primer sequence (5' → 3')		Product length
pdr1	1956262	Fw_1	CACTGATGTTGAGGGCGATG	238 bp
		Rv_1	AAGACCCAGCATGTTACCGA	
		Fw_2	CTCTCGTGGTGGTGCTCTTT	183 bp
		Rv_2	ACTTGGACCACGGCATAAGG	
pdr2	1849421	Fw_1	TACAACATGGGCCGTCTCTT	239 bp
		Rv_1	CAAGAGAGTGCGAATGGAGC	
		Fw_2	AAGGGTGCTTTGGTCTCTCG	191 bp
		Rv_2	AAGACACGACGACCTTGCAT	
pdr3	1890402	Fw_1	TGGGGTCTATCGGCCATTTT	177 bp
		Rv_1	TGCAATCACAAAACCGAGGG	
		Fw_2	CGGTCTATCAGGAGGCGAAC	274 bp
		Rv_2	TTTTGCTTGGCTGATTGGGC	
pdr4	1830855	Fw_1	AACCTACCTCTGGCTTGGAC	158 bp
		Rv_1	TCCACCACGAACCAACAGTA	
		Fw_2	GGTACTGCTGGCAAAGCAAC	123 bp
		Rv_2	CTTGCGGTTAGGGTTCTGGT	
pdr5	1904058	Fw_1	TTGTGGACTGATGCAAACCG	175 bp
		Rv_1	AGTCTGATTTGGCGGAGGAA	
		Fw_2	AGCGGAGAAGGGTCAAACTG	107 bp
		Rv_2	CTCCCAGACGCTTGGTCTTT	
pdr6	1614348	Fw_1	CCCCTATGGCCCTGGATATG	222 bp
		Rv_1	GCTTTGCTTATACCACCCCG	
		Fw_2	GGAAGTCGTTGGTGCTGGTA	136 bp
		Rv_2	ACGGCTAGGGTTCTTGTTGG	
mitC	1927665	Fw	TAACTTGGCTTCCGGTGGTG	149 bp
		Rv	GCAGACTTGGCATCGTTAGC	

5.8. RNA extraction

After identifying the *pdr* genes of *R. microsporus*, an RNA extraction was made to subsequently compare the basal expression of those genes with its expression in colonies which could resist the MIC concentration of posaconazole and itraconazole. With this aim, they were used colonies that had never been exposed to antifungals as a control and the obtained colonies in the antifungal resistance emergence rate assay grown at 2 µg/mL of itraconazole and 1 µg/mL of posaconazole. Nevertheless, to achieve an adequate amount of biomass for the RNA extraction, after 24 h of incubation in the microdilution plates the content of twenty wells (~ 4 mL) was recovered and transferred to falcons with 6 mL of liquid YNB. They were incubated for 24 h at 30 ºC and 125 rpm in aerobic conditions, allowing to obtain enough biomass. Then, to replicate the microdilution plates conditions, the biomass from colonies previous exposed to antifungals were transferred to new falcons filled with 10 mL of liquid YNB with 1 µg/mL of posaconazole and 2 µg/mL of itraconazole (separately). The biomass obtained from colonies that had never been exposed to antifungals were incubated as the exposed ones, replacing the medium with antifungal with only liquid YNB. All the samples were incubated for 16 h at the same conditions as before (Nagy *et al*., 2021) and the obtained biomass was filtered, washed with sterile distilled water and drained with sterile paper. The dry mycelium was then frozen and powdered with liquid nitrogen in a mortar and 100 mg of it was weighed in a 15 mL tube. Once the liquid nitrogen was consumed 1,5 mL of cold Trizol (4 ºC) was added. The mixture was homogenized with a vortex and was left on ice until this procedure was carried out with all the samples. The content of the falcons was then transferred to 2 mL RNase-free eppendorfs and centrifuged for 10 minutes at 10000 rpm and 4 ºC. The supernatants were transferred to a new eppendorf and incubated for 5 minutes at room temperature before adding 300 µL of chloroform RNase-free without isoamyl. The samples were then incubated for 3 minutes at room temperature, centrifuged for 15 minutes at the same conditions as before and the aqueous phase was recovered in another eppendorfs. RNA was precipitated by adding 750 µL of Isopropanol RNase-free and incubating the samples at -20 ºC for half an hour. After centrifugation for 10 minutes at 10000 rpm and 4 ºC the supernatants were removed and 1,5 mL ethanol 70 % RNase-free was added to the pellets. Another centrifugation was carried out, the supernatants were removed and the pellets were left to dry at room temperature until they became translucent. Finally, they were resuspended in 50 µL miliQ sterile water,

incubated for 10 minutes at 65 ºC and the samples were quantified using a Nanodrop. Their quality was checked corroborating that the 260/280 and 230/260 ratios were within the stipulated optimal ranges and carrying out a 1,5 % agarose gel (125 volts, 400 milliamps, 30 minutes) to verify the integrity of the RNA (Figure 3). The samples were stored at -80 ºC until the next step was carried on.

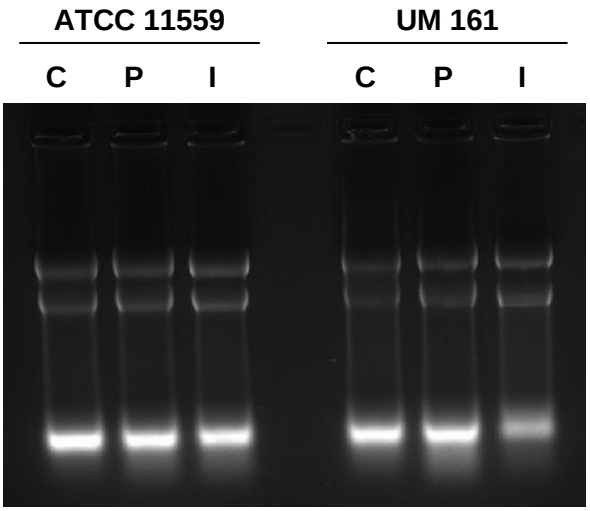

Figure 3. Visualization of the integrity of the extracted RNA. The first band corresponds to the 28s ribosomal RNA (rRNA) fragment and the second one to the 18s rRNA fragment. “C” corresponds to the control samples, “P” to the posaconazole-exposed samples and “I” to the itraconazole-exposed samples.

5.9. Semiquantitative PCR

Once good quality samples of RNA were obtained, a DNase treatment and cDNA synthesis were carried out following the manufacturer’s instructions of the DNase I, RNase-free (Thermo Scientific) kit and the iScript cDNA Synthesis Kit (Bio Rad 708890) respectively. Then, 2 µg of the obtained cDNA were added to eppendorfs with 12,5 µL of EmeraldAmp GT PCR Master Mix (TaKaRa), forward and reverse primers (0,2 µM) of one of the genes and 9,5 µL of miliQ water reaching a final volume of 25 µL. A negative control sample was also made without cDNA adjusting the volume with water. The procedure was repeated for all the other genes and a PCR with the conditions listed in Table 2 was carried out.

Table 2. PCR conditions for the six *pdr* genes from *R. microsporus* strains ATCC 11559 and UM 161 amplification.

Cycles	Temperature	Time
1x	95 ºC	5’
35x	95 ºC	30”
	60 ºC	30”
	72 ºC	30”
1x	72 ºC	5’
1x	4 ºC	-

Then, 10 µL of a 100-1000 bp molecular weight marker (PCR Sizer 100 bp DNA Ladder, Norgen Biotek) and 5 µL of the samples were loaded on a 1,5% agarose gel elaborated with TAE 1X (Tris, acetic acid and ethylenediamine tetraacetic acid (EDTA)) as buffer and GelRed as fluorescent marker. The gel was revealed using a transilluminator after 50 minutes at 100 volts and 140 milliamps. Then, the intensity of the bands corresponding to the fragments of the *pdr* genes was measured with ImageJ software (Schneider *et al*., 2012) and normalized with respect to those corresponding to the housekeeping gene.

5.10. Data analysis

The software used for the statistical analysis of the obtained results is GraphPad prism 8.0 for Windows. Significances have been calculated using a Student’s t-test for the ergosterol quantification assay and a two-way analysis of variance (ANOVA) for the resistance emergence rate assay. The graphical representations have been carried out with the average of the data and the standard error of the sample (SEM). *p* values < 0,05 have been considered statistically significant.

6. RESULTS

6.1. Ergosterol quantification

As shown in Figure 4, the quantification of ergosterol revealed a lower concentration in the wild-type strain compared to the UM 161 strain (*p* value = 0,0002).

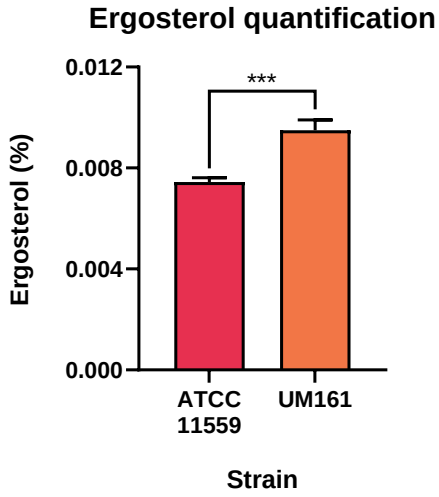

Figure 4. Ergosterol content of the two strains of *R. microsporus* under study. The values represent the mean and the error bars are the SEM. *** *p* = 0,0002 vs. UM 161.

6.2. Antifungal susceptibility assay

The MICs were read after 24 h of incubation as stated in the M38 CLSI guide (2017) (Table 3). Fluconazole and voriconazole did not cause any effect on the growth of the strains meanwhile itraconazole and posaconazole inhibited it at relatively low concentrations, being the second most effective even by a small difference. However, there was no difference between the MICs of the two strains.

Table 3. MICs (µg/mL) from itraconazole, fluconazole, posaconazole and voriconazole for the two strains of *R. microsporus* determined using the CLSI-standardized microdilution method.

Strain	Itraconazole	Fluconazole	Posaconazole	Voriconazole
ATCC 11559	2	>16	1	>16
UM 161	2	>16	1	>16

6.3. Antifungal resistance emergence rate assay

After counting colonies grown in previously determined inhibitory concentrations of posaconazole and itraconazole, the resistance emergence ratio of both strains was calculated as a percentage (Figure 5). There were no statistically significant differences between the growth in posaconazole. Nevertheless, although with a high variation in the data, the number of colonies resistant to itraconazole was statistically higher in strain UM 161 ($p < 0,05$).

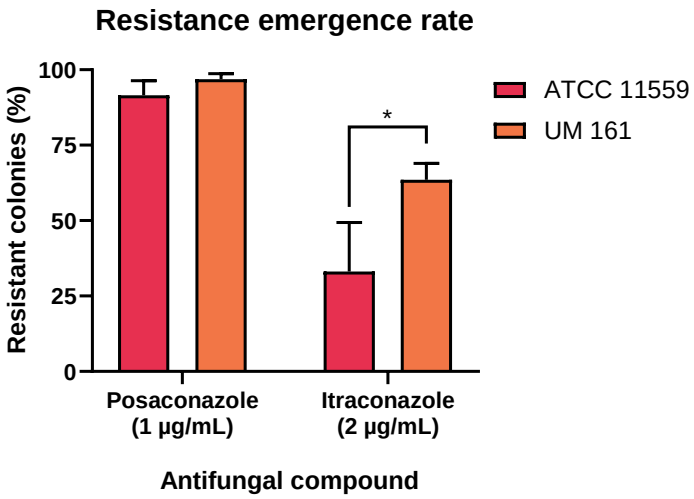

Figure 5. Rate of emergence of resistance to posaconazole (1 µg/mL) and itraconazole (2 µg/mL) of the two strains of *R. microsporus* under study. The values represent the mean and the error bars the SEM. * $p < 0,05$ vs. ATCC 11559 itraconazole-treated cultures.

6.4. *pdr* genes expression

The obtained results differ depending on the strain and the azole compound. Regarding *pdr1*, without drug exposure, the absence of adenylate cyclase barely modified the gene expression (Figure 6A). Exposure to both azoles only increased *pdr1* expression in the UM 161 strain. In ATCC 11559 the difference between the control sample and the one exposed to posaconazole was minimal and the exposure to itraconazole caused a downregulation of the gene expression (Figure 6B).

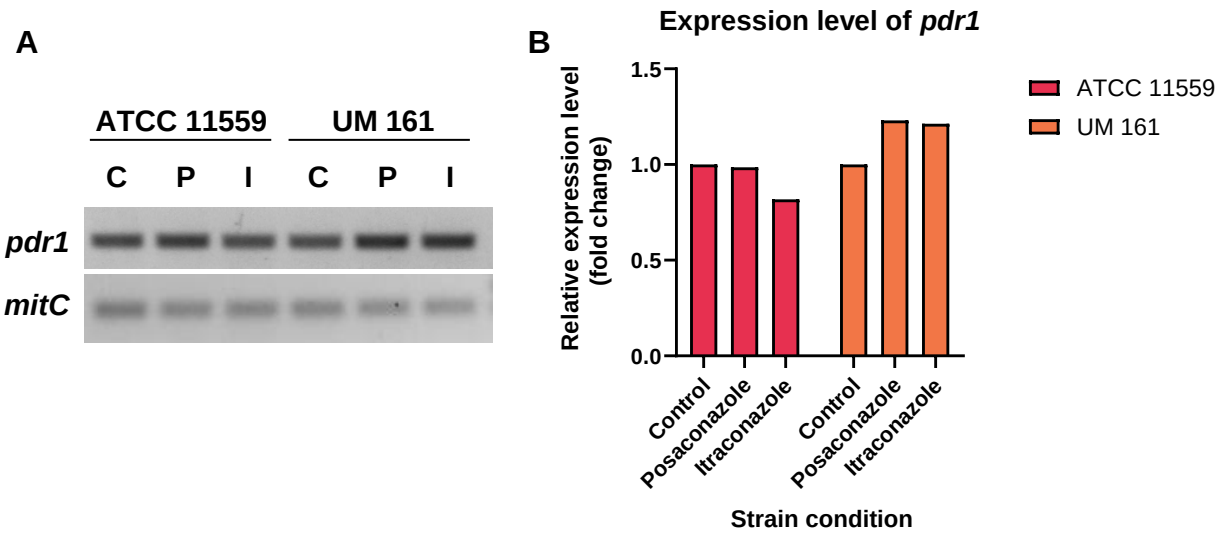

Figure 6. A) Bands of the amplified fragment of *pdr1* and the housekeeping *mitC* in ATCC 11559 and UM 161 strains. **B)** Semiquantitative PCR analysis of the *pdr1* gene expression. The expression level of the gene is normalized with *mitC* as the housekeeping gene and the fold change is calculated relative to the expression level of the control sample.

Concerning *pdr3*, in the control sample, the lack of adenylate cyclase slightly increased the expression of the gene (20% increase) (Figure 7A). On the other hand, *pdr3* expression increased in both strains after exposure to both azole compounds, although slightly higher in strain ATCC 11559 (Figure 7B).

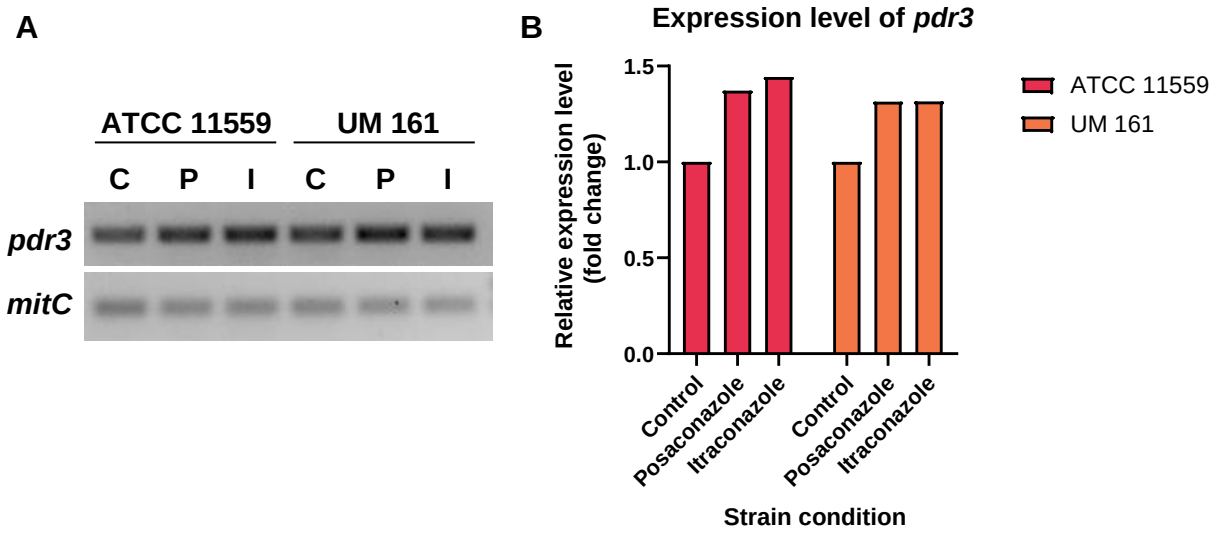

Figure 7. A) Bands of the amplified fragment of *pdr3* and the housekeeping *mitC* in ATCC 11559 and UM 161 strains. **B)** Semiquantitative PCR analysis of the *pdr3* gene expression. The expression level of the gene is normalized with *mitC* as the housekeeping gene and the fold change is calculated relative to the expression level of the control sample.

In the case of the *pdr4* gene, without exposure to the azoles the lack of adenylate cyclase notably increased its expression (83% increase) (Figure 8A). In the ATCC 11559 strain exposure to both drugs led to a slight downregulation of the expression, to a greater extent in the case of posaconazole. However, in the strain UM 161 exposure to posaconazole caused overexpression of *pdr4*, and exposure to itraconazole led to underexpression (Figure 8B).

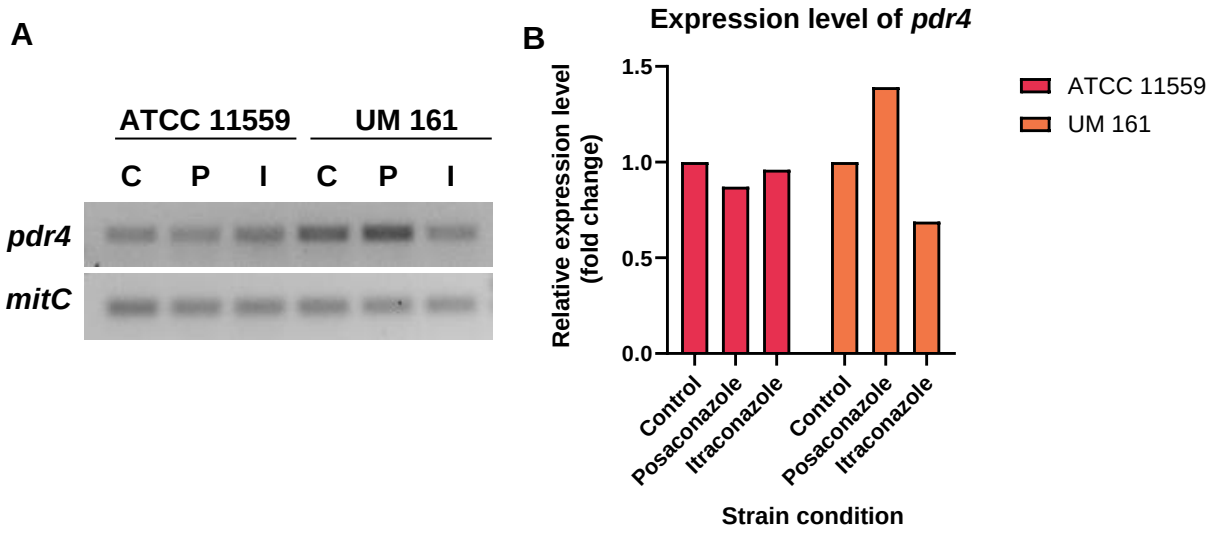

Figure 8. A) Bands of the amplified fragment of *pdr4* and the housekeeping *mitC* in ATCC 11559 and UM 161 strains. **B)** Semiquantitative PCR analysis of the *pdr4* gene expression. The expression level of the gene is normalized with *mitC* as the housekeeping gene and the fold change is calculated relative to the expression level of the control sample.

Finally, the expression of *pdr6* has been the one with the greatest difference between the strains. Unlike what was observed in the expression patterns of the other genes, in the case of *pdr6* the absence of adenylate cyclase led to a drastic decrease in its expression in the control sample (96% reduction) (Figure 9A). Although exposure to both azoles led to an overexpression of the gene expression in both strains, in UM 161 it was higher compared to ATCC 11559, especially in response to Posaconazole (Figure 9B).

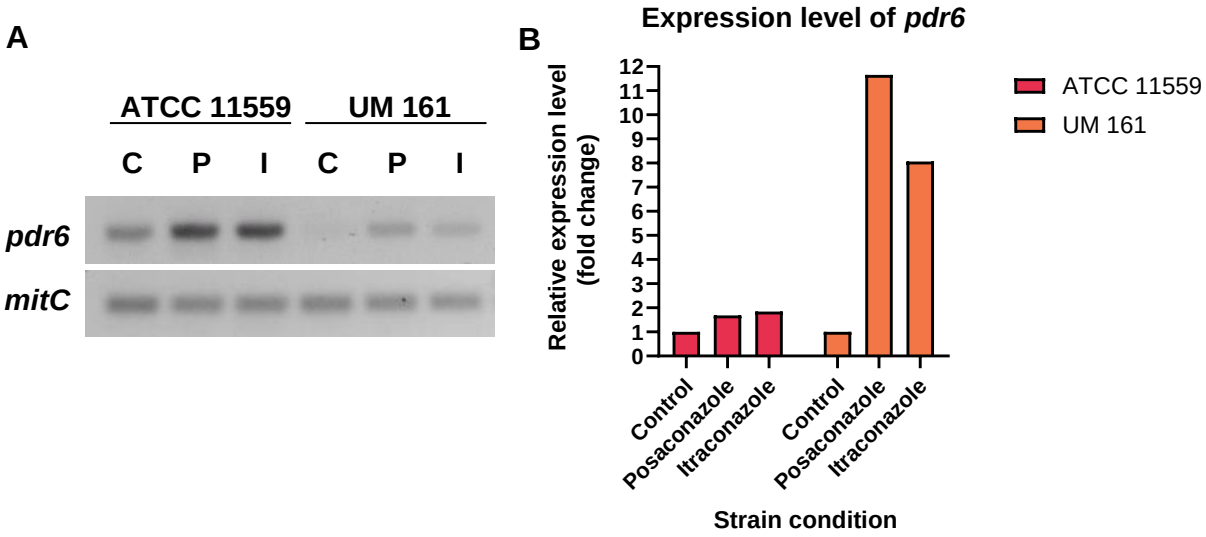

Figure 9. A) Bands of the amplified fragment of *pdr6* and the housekeeping *mitC* in ATCC 11559 and UM 161 strains. **B)** Semiquantitative PCR analysis of the *pdr6* gene expression. The expression level of the gene is normalized with *mitC* as the housekeeping gene and the fold change is calculated relative to the expression level of the control sample.

Unfortunately, the expression of the *pdr2* and *pdr5* genes could not be evaluated due to absence of amplification of the fragments with both pairs of designed primers.

7. DISCUSSION

In different fungi, changes in the enzyme adenylate cyclase, ergosterol and *pdr* genes have been associated with resistance to azoles, often in an interrelated manner (Jain *et al*., 2003; James *et al*., 2022; Kim *et al*., 2021; Víglas & Olejníková, 2021). However, the impact of these factors on *R. microsporus* has not been studied, even though it is the main causal agent of mucormycosis in the world and resistance to azoles is one of the main problems in its treatment (Martín & Salavert, 2021).

Regarding susceptibility to azoles, the lack of adenylate cyclase has not modified the MICs of fluconazole, itraconazole, posaconazole and voriconazole. This differs from most assays performed in *Candida spp.*, *S. cerevisiae* and *C. neoformans* (Kim *et al*., 2021; Jain *et al*., 2003; Jung *et al*., 2010). Nonetheless, it is not surprising that the lack of adenylate cyclase has not impacted drugs such as fluconazole and voriconazole as has been seen in these yeasts since filamentous fungi are intrinsically resistant to said compounds (CLSI, 2017; Lax *et al*., 2024; Nagy *et al*., 2021). Concerning

posaconazole and itraconazole, in both strains the values obtained are within what is expected in *R. microsporus* wild-type strains, with the established MIC being remarkably similar to the average obtained in large trials carried out with more than a hundred strains of *R. microsporus* (Espinel-Ingroff & Turnidge, 2016). This suggests the presence of unknown pathways and/or compensatory mechanisms absent in *Candida spp.*, *S. cerevisiae* and *C. neoformans* which prevent the increase in susceptibility to azoles due to the lack of adenylate cyclase in *R. microsporus*. However, these results agree with those obtained in *A. fumigatus* by Ferreira *et al*. (2006), in which the strains lacking adenylate cyclase showed the same degree of susceptibility (MIC) to itraconazole and other azoles as the wild-type strain.

Given the absence of differences between both strains in the MIC values and knowing that exposure to azoles is a phenomenon associated with the acquisition of resistance to them (Rosam *et al*., 2021; Verweij *et al.,* 2016), a second test was carried out to evaluate the adaptability capacity of the strains to high doses of the two azoles to which they were not resistant. After passing cultures of *R. microsporus* strains in increasing concentrations of itraconazole and posaconazole, growth of colonies of both strains was obtained at concentrations previously established as inhibitory. Firstly, this result corroborates that exposure to azoles can give rise to resistant colonies. Besides, the large percentage of resistance after exposure to posaconazole agrees with the evidence in Mucorales fungi of the ability to develop acquired resistance to this azole (Caramalho *et al*., 2017). In the second place, greater resistance of the strain without adenylate cyclase to high concentrations of itraconazole has been observed, as well as a slight trend of lower susceptibility to posaconazole. Consequently, the higher resistance emergence rate especially to itraconazole by eliminating the adenylate cyclase are results contrary to those reported in assays carried out with other fungi, where lack of adenylate cyclase led to hypersusceptibility to azoles (Jain *et al*., 2003; Kim *et al*., 2021; Maeng *et al*., 2010). This may be due to the type of fungus since Mucorales are one of the most ancient orders, suggesting that they have developed unique characteristics that have allowed them to adapt and survive to multiple conditions through hundred millions of years (Mendoza *et al*., 2015). However, among other mechanisms yet to be elucidated, taking as reference the literature available to date, the impact of adenylate cyclase on

resistance to azoles could be related to the ergosterol content and the expression of the *pdr* genes.

In the present work, the lack of adenylate cyclase in *R. microsporus* has caused an increase in the ergosterol content. These results do not seem to agree with those obtained in trials such as the one performed by Kim *et al*. (2021) in *C. auris*. In this case, the elimination of the enzyme caused a decrease in the *erg3* and *erg11* genes, essential for the synthesis of ergosterol, so a decrease in ergosterol content would have been expected due to the lack of adenylate cyclase in *R. microsporus*. Nevertheless, in other *Candida* species, the lack of the enzyme caused the opposite effect concerning the *erg11* gene (Jain *et* al., 2003) and in *C. neoformans* did not modify any of the studied genes involved in ergosterol synthesis (Maeng *et al*., 2010). The disparity in results between the assays suggests that the mechanism of action of adenylate cyclase and cAMP as a resulting product differs depending on the microorganism. A possible explanation would be the presence of compensatory mechanisms such as genes with redundant functions in those fungi where the ergosterol content is not decreased. This phenomenon has been observed in *Aspergillus flavus*, where ergosterol biosynthesis was unaltered after the deletion of the *erg3* gene, suggesting the presence of other genes that make up for the lack of the absent enzyme (Alcazar-Fuoli *et al*., 2006). Therefore, hypothesizing that the lack of adenylate cyclase could diminish *erg3* expression in *R. microsporus*, its ergosterol content could be not reduced as occurs in *A. flavus.* Furthermore, a decrease in *erg3* expression could explain the trend of greater resistance to posaconazole and itraconazole of the strain without adenylate cyclase. As a mechanism of action, both azoles target the enzyme encoded by the *erg11* gene preventing its functioning (Campoy & Adrio, 2017). In this case, the ergosterol pathway takes an alternative route, where the expression of *erg3* leads to the catalyzing of a reaction that generates 14alfa-metilergost-8,24(28)-dien-3beta,6alfa-diol (3,6-diol) (Figure 10). This compound is toxic to the fungus and promotes its death, so mutations in *erg3* have been associated in multiple fungi with greater resistance to azoles (Hirayama *et al*., 2021; Prasad *et* al., 2011). Hence, the decrease in *erg3* observed in *Candida spp*. after the elimination of the gene encoding for adenylate cyclase could be happening in the UM 161 strain, protecting the fungus from 3,6-diol accumulation caused by azoles through this mechanism. At the same time, other compensatory mechanisms

yet to be discovered would prevent ergosterol synthesis from being negatively affected due to the decrease in *erg3*, as it has been seen in *A. flavus* (Alcazar-Fuoli *et al*., 2006).

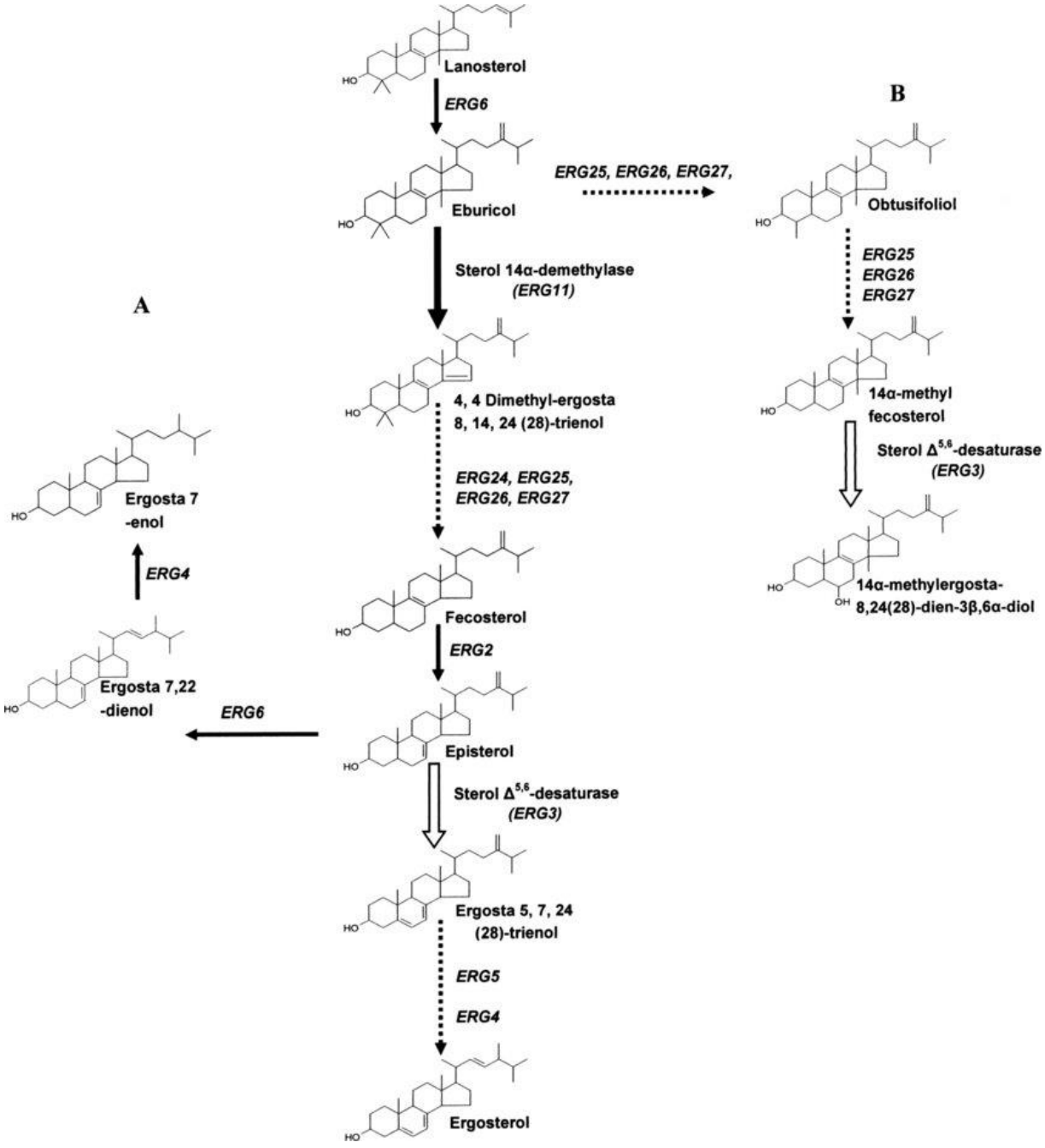

Figure 10. Ergosterol biosynthesis pathway. Adapted from Martel *et al*., 2010.

Another hypothesis to explain the increase in the basal ergosterol content and the trend of greater resistance to azoles after exposure to them in the strain without adenylate cyclase includes the *pdr* genes. It is known that the transcription factor AtrR

controls the expression of multiple genes involved in the synthesis of ergosterol and that of *pdr* genes in fungi such as *A. fumigatus* (Paul et al., 2019), as well as its role in resistance to azoles (James et al., 2022). Consequently, an increase in its expression could enhance the expression of *erg11* and some *pdr* genes, already known mechanisms of resistance to azoles, and, in turn, the synthesis of ergosterol. Besides, the well-known relationship between AtrR and SrbA also gives rise to suggesting an increase in SrbA expression, in turn causing overexpression of *erg11* and leading to the phenomena described above. Furthermore, given that overexpression of *pdr* genes increases the pump of azole drugs from the cell, these are less effective against the enzyme encoded by *erg11* gene and, therefore, prevent a reduction in ergosterol content (López-Ávila *et al*., 2016). However, the relationship between ergosterol and resistance to targeted drugs against it is complex and changes in its quantity do not always imply differences in resistance. Regarding azoles, studies with *A. fumigatus* revealed that resistance to itraconazole, which differs in this work between the two strains, does not seem to be related to the amount of ergosterol (Ferreira *et al*., 2005), suggesting that the observed change lies in other mechanisms yet to be explored. Nevertheless, in fungi such as *Candida lusitaniae* a reduction in ergosterol content was associated with lower susceptibility to amphotericin B (Young *et al*., 2003), so it would be of great interest to perform susceptibility tests to this antifungal compound. Besides, since the decrease in *erg3* and the increase in *erg11* are azole resistance mechanisms, it would also be enriching to carry out ergosterol quantification studies after exposure to azoles. Perform gene expression assays of both genes in the studied strains would also help to elucidate the functioning of the biosynthesis pathway and the possible impact on it of the presence or absence of adenylate cyclase.

Also concerning the *pdr* genes, most of the available literature describes a decrease in its expression in strains of *A. flavus* and *Candida spp.* without adenylate cyclase (Ferreira *et al*., 2006; Jain *et al*., 2003; Kim *et al*., 2021). In the present work, this phenomenon has only been observed notably in the *pdr6* gene, while in *pdr4* the strain without adenylate cyclase not exposed to antifungal presented a notable increase in its expression and in *pdr1* and *pdr3* the difference between strains was minimal. However, in the trial carried out by Ferreira *et al*. (2006) the expression level of one of the four studied *pdr* genes was not dependent on adenylate cyclase and in the assay published by Kim *et al.* (2021) one of the two *pdr* genes of *C. auris* without adenylate

cyclase was overexpressed. Consequently, the obtained results for *pdr1*, *pdr3* and *pdr4* have been previously reported in other fungi. Besides, generally a lesser expression of *pdr* genes implies a higher susceptibility to azoles (Canuto & Rodero, 2002). Therefore, the absence of a decreased expression in most of the *pdr* genes in UM 161 strain concerning the wild-type strain agrees with the absence of differences in the MICs, its trend of lower susceptibility in the resistance test and the possible explanations for the increase in its ergosterol content.

About exposure to inhibitory concentrations of itraconazole and posaconazole, generally both drugs have caused a higher expression of the *pdr* genes compared to the control sample, as expected due to the function of these genes in resistance to azoles (Sanglard & Odds, 2002). In the case of posaconazole, three of the four *pdr* genes, especially *pdr4* and *pdr6*, were more expressed with respect to the control sample in the UM 161 strain, in which a slight trend of greater resistance to the drug was observed, compared to the wild-type strain. Regarding itraconazole, to which the strain without adenylate cyclase also resisted more than the ATCC 11559 strain, the *pdr1* and *pdr6* genes were overexpressed concerning the control sample compared to the wild-type strain. However, in the *pdr3* and *pdr4* genes, the pattern was opposite. Nevertheless, in the trial carried out by Nagy *et al*. (2021) with the Mucoral *M. lusitanicus*, not all *pdr* genes were overexpressed after exposure to azoles. In this last study, exposure to itraconazole only overexpressed five of the eight *pdr* genes, and only one responded significantly to exposure to posaconazole. Furthermore, exposure to itraconazole and posaconazole (among other azoles) caused an underexpression of two of the eight genes. This finding could agree with the decrease in expression observed in this work in *pdr1* in the strain ATCC 11559 exposed to itraconazole and *pdr4* in both strains after exposure to itraconazole and the wild-type strain also to posaconazole. In short, the different impact of the two azoles tested and the lack of a consistent pattern in the expression of the studied *pdr* genes agrees with the results obtained by Nagy *et al.* (2021), who could only establish a solid relationship between resistance to azoles such as posaconazole, ravuconazole and isavuconazole with two of the eight *pdr* genes of *M. lusitanicus*. Therefore, although more replications and other tests are necessary to corroborate and expand the results obtained in this work, it cannot be ignored that the impact of the enzyme adenylate cyclase may go beyond other mechanisms. Literature on this matter is scarce, especially in Mucorales fungi.

Nevertheless, other enzymes involved in the adenylate cyclase signaling pathway can act redundantly or independently with respect to it in multiple important processes. Some of them are cellular growth, mating, virulence factors production, the composition of the cell wall and membrane and the response to different types of stress, which can indirectly impact phenomena such as drug susceptibility (Kim *et al*., 2021; Maeng *et al*., 2010). Moreover, other mechanisms can modify *pdr* and ergosterol-related genes beyond adenylate cyclase, such as some transcription factors and enhancing mutations that increase expression levels of the target genes (Khosravi *et al*., 2016). Despite that, this area of study is still under investigation, and it is necessary to identify and characterize potential regulators of adenylate cyclase, both dependent and independent of other signaling pathway components (Kim *et al*., 2021). Even so, the preliminary data reported in the present work seem to indicate that the enzyme adenylate cyclase is involved in the ergosterol synthesis, itraconazole resistance and *pdr* gene expression of *R. microsporus*.

8. CONCLUSIONS

- The lack of adenylate cyclase increases the ergosterol content in the studied *R. microsporus* strain.

- The absence of adenylate cyclase does not modify the susceptibility to azoles of *R. microsporus* according to the Microdilution Broth method.

- Exposure to subinhibitory concentrations of posaconazole and itraconazole leds to the generation of resistant colonies since they grow at concentrations previously established as inhibitory. This agrees with the previous knowledge that exposure to azoles can led to the development of resistance towards them.

- Contrary as hypothesized, the strain without adenylate cyclase has a better adaptation capacity to high concentrations of itraconazole, although the wide variation in the data would require the study of new protocols to obtain more precise results. This correlates with the lack of a decrease in most of its *pdr* genes expression after exposure to both azoles.

- Without exposure to azoles, in UM 161 strain most *pdr* genes have not experienced a decrease in its expression either, although *pdr6* expression diminished substantially concerning the wild-type strain. Nevertheless, more replicates are necessary to corroborate and consolidate these results.

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