STUDY AND CHARACTERIZATION OF AZOLE RESISTANCE IN ASPERGILLUS SECTION NIGRI

Alba Pérez Cantero

UNIVERSITAT ROVIRA i VIRGILI

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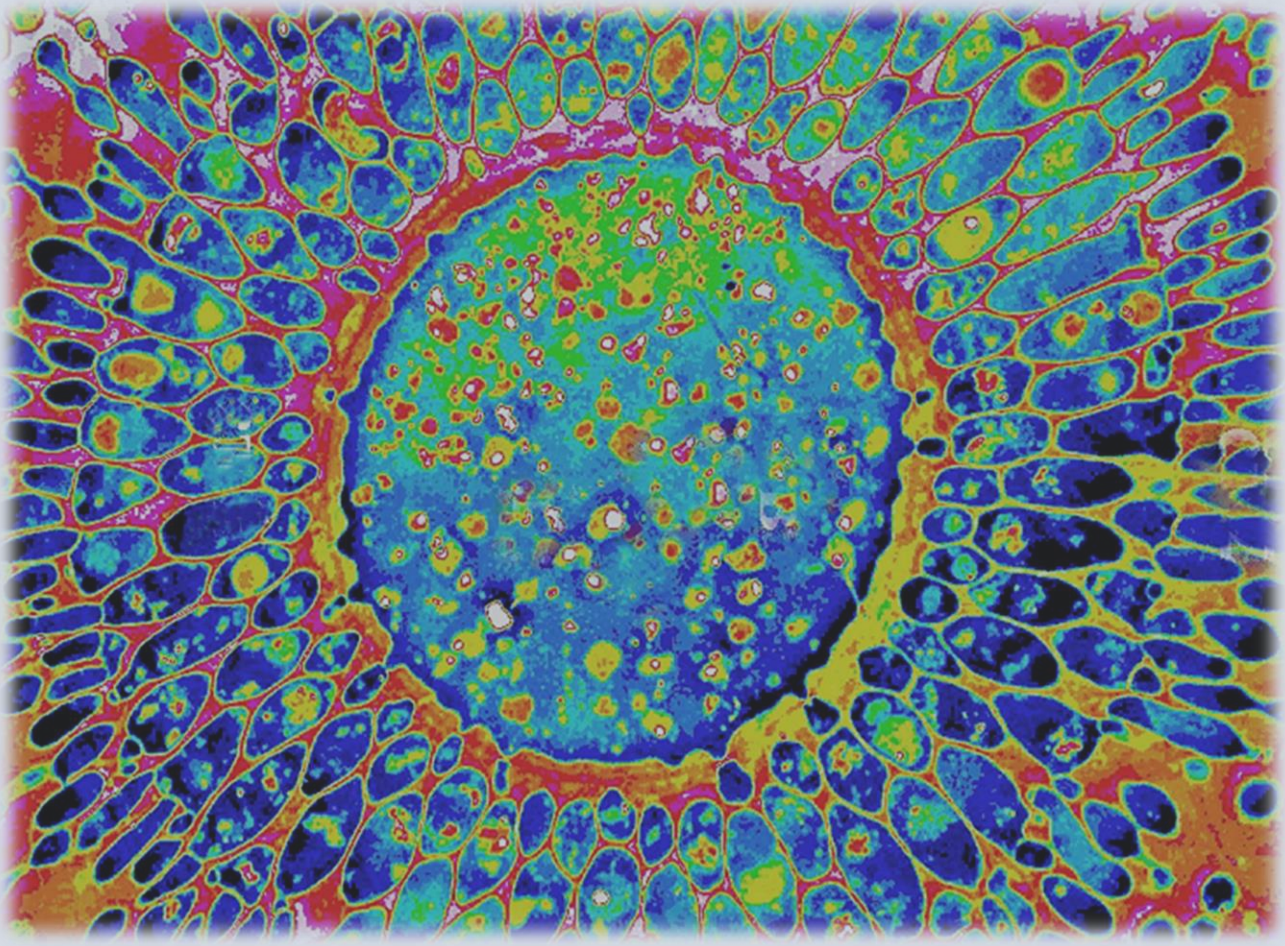

Doctoral Thesis 2020

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Doctoral Thesis 2020

Supervised by Drs. Javier Capilla Luque, Loida López Fernández and Josep Guarro Artigas

Department of Basic Health Sciences
Faculty of Medicine and Health Sciences

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Alba Pérez Cantero

Drs. Javier Capilla Luque, Loida López Fernández and Josep Guarro Artigas, researchers of the Department of Basic Health Sciences at Universitat Rovira i Virgili,

STATE that the present study, entitled "**Study and characterization of azole resistance in *Aspergillus* section *Nigri***", presented by **Alba Pérez Cantero** for the award of the degree of Doctor, has been carried out under our supervision at the Department of Basic Health Sciences and that it fulfils the requirements to obtain the International Doctorate Mention.

Reus, August 2020.

Doctoral thesis supervisors:

Javier Capilla Luque Loida López Fernández Josep Guarro Artigas

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A mi madre

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LIST OF ABBREVIATIONS

24(28)DHE	24(28) Dehydroergosterol
5-FC	Flucytosine
ABC	ATP-Binding Cassette
ABLC	AMB Lipid Complex
ABPA	Allergic Bronchopulmonary Aspergillosis
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
actA	Actin gene
AMB	Amphotericin B
AMM	*Aspergillus* Minimal Medium
AND	Anidulafungin
AspGD	Aspergillus Genome Database
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
a_w	Water Activity
benA	β-tubulin gene
BID	Twice a day
BLASTn	Basic Local Alignment Search Tool (nucleotide BLAST)
BLASTp	Basic Local Alignment Search Tool (protein BLAST)
BSA	Bovine Serum Albumin
CAFF	Caffeine
caM	Calmodulin gene
CAS	Caspofungin
Cas9	CRISPR Associated Protein 9 (DNA endonuclease enzyme)
CBP	Clinical Breakpoint
CBS	CBS-KNAW Fungal Biodiversity Center
cDNA	Complementary DNA
CFW	Calcofluor White
CLSI	Clinical Laboratory Standards Institute

CPA	Chronic Pulmonary Aspergillosis
CR	Congo Red
CREA	Creatine Sucrose Agar
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
Ct	Cycle Threshold
CTAB	DNA extraction buffer (cetyl trimethylammonium bromide)
CYA	Czapek Yeast Autolyzed Agar
Cyp51	Cytochrome P450 sterol 14 α-demethylase enzyme
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
E	Efficiency
ECM	Extracellular Cell Matrix
ECV	Epidemiological Cutoff Value
EDTA	Ethylenediaminetetraacetic Acid
ERG11	Lanosterol 14α-demethylase enzyme
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FLC	Fluconazole
FMR	Facultat de Medicina i ciències de la salut de Reus
Fw (F)	Forward
gDNA	Genomic DNA
gRNA	Guide RNA
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
IA	Invasive Aspergillosis
IDT	Integrated DNA Technologies
ISA	Isavuconazole

ITC	Itraconazole
ITS	Internal Transcribed Spacer
IV	Intravenous
JGI	Joint Genome Institute
KO	Knock-out
LAmB	Liposomal AMB
MCF	Micafungin
MCO	Multicopper Oxidase
MDR	Multidrug Resistance
MEA	Malt Extract Agar
MFS	Major Facilitator Superfamily
MIC	Minimal Inhibitory Concentration
MOI	Multiplicity Of Infection
MOPS	[3-(N-morpholino)propanesulfonic acid]
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
NE	Not Established
OD	Optical Density
OM	Osmotic Media
ORF	Open Reading Frame
PAM	Protospacer-Adjacent Motif
PAMP	Pathogen-Associated Molecular Pattern
PCA	Primary Cutaneous Aspergillosis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PDR	Pleiotropic Drug Resistance
PEG	Polyethylene Glycol
PMN	Polymorphonuclear leukocyte

PMSF	Phenylmethylsulfonyl fluoride
PRR	Pattern Recognition Receptor
PSC (POS)	Posaconazole
qPCR	Quantitative Real Time PCR
Rev (R)	Reverse
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcription
SDS	Sodium Dodecyl Sulfate
siRNA	Small Interfering RNA
SM	Minimal Medium
SMM	Stabilized Minimal Medium
SREBP	Sterol Regulatory Element Binding Protein
STC	Buffer (Sorbitol, Tris-HCl and $CaCl_2$)
TENS	Lysis buffer (Tris-HCl, EDTA, NaCl and SDS)
TF	Transcription Factor
TID	Three times a day
Tm	Melting Temperature
TORC	Target Rapamycin Complex
TR	Tandem Repeat
tracrRNA	Transactivating CRISPR RNA
UTR	Untranslated Region
VRC	Voriconazole
wt	Wild Type
YG	Yeast Extract and Glucose

ABSTRACT

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Abstract

Several species of *Aspergillus* display clinical relevance, since they are opportunistic pathogens associated to a broad variety of human conditions. Among these, invasive aspergillosis is the most severe in terms of morbidity and mortality. Although *Aspergillus fumigatus* is the most common causal agent of invasive disease, elevated prevalence of species from other sections, such as section *Nigri* members, has been reported lately. Despite the current treatment of choice is voriconazole, azole resistance events have alarmingly increased in the last years, critically impairing disease management. In this context, whereas azole resistance mechanisms in *A. fumigatus* have been extensively studied, azole resistance in non-*fumigatus* species remains poorly characterized. In this work we aimed to characterize azole resistance in species of *Aspergillus* section *Nigri*. With this purpose, we have determined azole susceptibility in section *Nigri* strains, and we have carried out molecular and genetic analyses of azole target proteins Cyp51 encoded by *cyp51* genes. In addition, ergosterol content and properties of the plasma membrane and the cell wall were investigated. Finally, potential resistance mechanisms related to drug degradation and the association between azole resistance and *in vitro* macrophage phagocytosis were evaluated. Our results slightly diverge from those obtained in *A. fumigatus*, which evidence the complexity behind azole resistance, especially in non-*fumigatus* species.

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INTRODUCTION

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1.1. The genus *Aspergillus*

The genus *Aspergillus* is classified in the *Ascomycota* phylum, *Pezizomycotina* subphylum, *Eurotiomycetes* class, *Eurotiales* order and *Aspergillaceae* family (Houbraken *et al.* 2014). It was first described in 1729 by the catholic priest and botanist Pier Antoni Micheli, who reported it as a fungus with a spore-bearing structure that resembled an aspergillum, a device used to sprinkle holy water, and thus named it *Aspergillus* (Micheli 1729).

Aspergillus is a multicellular and heterotrophic organism in which cells are organized as branched filaments known as hyphae. This filamentous fungus displays hyaline and septate hyphae, arranged as a network forming the vegetative mycelium. However, the most distinctive features displayed by the species of the genus are the conidiogenous structures involved in the asexual reproductive stage (anamorph). These comprise an unbranched aseptate conidiophore, which emerges from a foot cell and expands apically with a vesicle at the top. From the vesicle, the conidiogenous cells known as phialides, produce mitotic hydrophobic and usually pigmented conidia arranged in long chains. Phialides can be borne directly or on metulae (the adjacent cells to phialides). When phialide are borne directly from the vesicle, the conidiogenous cells are called uniseriate, while they are biseriate when grown on metulae (Figure 1). In addition, some species are also known to display sexual reproduction by producing fruiting bodies called ascomata, that are spherical structures usually containing asci with 8 one-celled ascospores. Interestingly, while some species of *Aspergillus* regularly produce both, sexual and asexual spores, in other species the

sexual morph is rare or even unknown (Bennett 2010; Guarro *et al.* 2010; Minter *et al.* 1986; Raper & Fennell 1965).

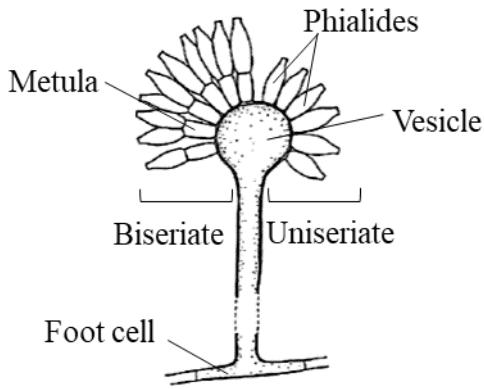

Figure 1. Conidiogenous structures in *Aspergillus* (adapted from Guarro *et al.* 2010).

Currently, the genus comprises over 450 ascomycete species distributed worldwide (Steenwyk *et al.* 2019), which are widespread in the environment and mainly found on vegetal substrates, decaying organic matter, soil, freshwater and marine habitats. They can also be present in indoor environments or in drinking water and dust. This ubiquity is due to the ability of airborne conidia to globally disperse in air currents and their adaptation to germinate in a very wide variety of surfaces and conditions, making *Aspergillus* one of the most common fungi in the planet. Moreover, the genus displays a prominent economic impact in biotechnology, since many of its species are used in the production of metabolites, antibiotics, enzymes or as agents in food fermentations. Additionally, the fundamental studies in the model fungus *Aspergillus nidulans* have extensively contributed to the understanding of eukaryotic cell biology and molecular processes. However, the genus is also responsible for significant economic

loss, since it can negatively affect crops or act as food borne contaminant by food spoilage or mycotoxin production. It is worth mentioning the clinical relevance of a few species within the genus, which are important and frequent human opportunistic pathogens (de Vries *et al.* 2017; Kocsubé *et al.* 2016; Paulussen *et al.* 2017; Samson *et al.* 2014; Steenwyk *et al.* 2019).

During the 20th century, *Aspergillus* species were morphologically organized in different intrageneric "groups" (Raper & Fennell 1965; Thom & Church 1926; Thom & Raper 1945) with no taxonomical status within the formal rules of biological nomenclature. In 1985, the genus was reorganized with a new scheme of classification in which the subgeneric taxonomic hierarchy was based on "sections" (Gams *et al.* 1985). Currently, genetically and morphologically related species have been grouped in subgenera and sections (or species complexes) (Figure 2), and more than 20 sections have been proposed (Bennett 2010; Kocsubé *et al.* 2016; Samson *et al.* 2014). Of these, the most clinically relevant species of the genus belong to sections *Fumigati*, *Flavi*, *Nigri* and *Terrei*, whereas the most biotechnologically and

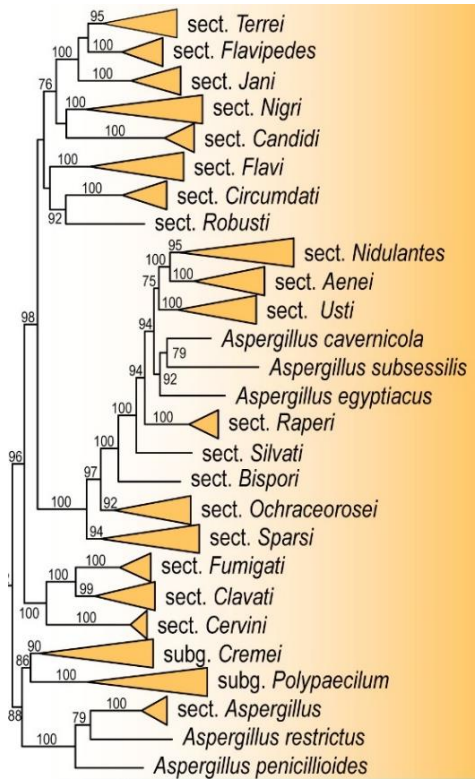

Figure 2. Proposed sections for the genus *Aspergillus* (adapted from Kocsubé *et al*. 2016).

industrially important species are included in sections *Flavi* and *Nigri* (Lass-Flörl & Cuenca-Estrella 2017; Park *et al.* 2017).

1.2. *Aspergillus* section *Nigri*

Aspergillus section *Nigri* corresponds to the *Aspergillus niger* species complex (black aspergilli), which is a group of closely related species commonly found in soil, but also in numerous other environments and conditions. Species of this section display high importance in the food industry, medical mycology and biotechnology. Some of its species are industrially used in fermentation processes and in the production of extracellular and hydrolytic enzymes, such as amylases or lipases, organic acids, such as citric and gluconic acids, vitamins, several biopharmaceuticals and proteins. However, members of the section are also known to spoil food or produce mycotoxins. Their ability to grow under elevated temperatures and relatively low water activity (a_w) also allows them to adapt and colonize a very diverse range of cereals and dried fruits. Along to their economic impact, some species of section *Nigri* also display significant relevance in the medical field, causing non-invasive infections such as otomycosis, keratitis, allergic aspergillosis or aspergillomas, but also invasive mycoses such as pulmonary aspergillosis (Astoreca *et al.* 2010; D'hooge *et al.* 2019; Varga *et al.* 2011; Vesth *et al.* 2018).

Section *Nigri* comprises *A. niger* and other cryptic species, which are morphologically indistinguishable species that can only be properly identified by molecular techniques. Thus, combined phenotypic and phylogenetic approaches with DNA sequencing of the calmodulin (*caM*) and the β-tubulin (*benA*) molecular markers have been proven useful for

species identification within this section (Howard 2014; Varga *et al.* 2011). Recently, it has been shown that the section comprises 28 different species that can be phylogenetically divided in two main clades: the group of biseriate species and that of uniseriate species, which show main differences in their sexual states and the production of secondary metabolites. Significant species included in the section are *A. niger, Aspergillus tubingensis* or *Aspergillus brasiliensis.* (Fungaro *et al.* 2017; Varga *et al.* 2011; Vesth *et al.* 2018).

In general terms, the main morphological characteristic of the species within this section consists of dark brown to black conidia that radiate from conidiogenous cells in chains. Furthermore, the section includes species with uniseriate and biseriate conidiophores, spherical vesicles and hyaline or lightly pigmented hyphae. Maximum temperature of growth varies among species, ranging between 30 ºC and 40 ºC (Guarro *et al.* 2010; Kredics *et al.* 2008; Samson *et al.* 2007; Simões *et al.* 2013).

Regarding the significant species *A. niger, A. tubingensis* and *A. brasiliensis,* subtle differences can be observed. The maximum temperature of growth for these three biseriate species is 40 ºC, and the slight morphological differences among them are mainly found in conidia size. Specifically, conidia size ranges of 3.5 - 5 µm in *A. niger*, 3 - 5 µm in *A. tubingensis* and 3.5 - 4.5 µm in *A. brasiliensis.* Similarly, minor differences can be observed in their vesicles size: *i.e.* 45 - 80 µm in *A. niger*. 40 - 80 µm in *A. tubingensis* and 30 - 45 µm in *A. brasiliensis* (Samson *et al.* 2007). Figure 3 shows macroscopic and microscopic features of these three species.

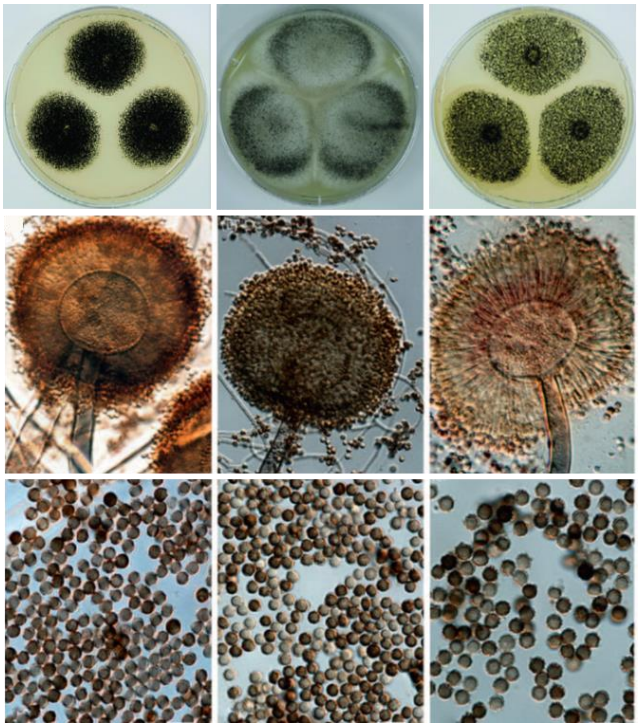

Figure 3. Macro- and micromorphology of *A. niger* (left column), *A. tubingensis* (middle column) and *A. brasiliensis* (right column). Upper row: fungal colonies; middle and bottom rows: microscopic view of conidiophores and conidia, respectively (adapted from Kredics *et al.* 2008 and Simões *et al.* 2013).

In addition, all of them exhibit good growth and sporulation on CYA (Czapek Yeast Autolyzed Agar) with 5 % NaCl and produce a large yellowish halo indicative of acid production in CREA (Creatine Sucrose Agar) media (Samson *et al.* 2007; Varga *et al.* 2007). However, *A. niger* and *A. brasiliensis* do not grow on MEA (Malt Extract Agar) supplemented with 10 µg/mL of boscalid, contrarily to what occurs in the case of *A. tubingensis.* Similarly, the Ehrlich reaction, which is useful for the detection of indole secondary metabolites, shows a positive result for *A.*

niger and *A. brasiliensis* and a negative result for *A. tubingensis*. Extrolites production also differs among these species (Table 1) (Samson *et al.* 2007).

Table 1. Extrolites produced by *A. niger, A. tubingensis* and *A. brasiliensis.*

Species	Extrolites
A. niger	Funalenone, ochratoxin A (some strains), malformins, naphtho-ɣ-pyrones, pyranonigrin A and tensidol A and B
A. tubingensis	Asperazine, funalenone, malformins, naphtho-ɣ-pyrones, pyranonigrin A and tensidol A and B
A. brasiliensis	naphtho-ɣ-pyrones, pyrophen and tensidol A and B

1.3. Clinical spectrum of *Aspergillus* infections

Aspergillus infections comprise a wide range of manifestations and clinical syndromes, from localized colonization of the tissue to devastating invasive disease (invasive aspergillosis; IA). Although *Aspergillus* most frequently affects the lungs, aspergillosis can develop within virtually any organ. This is due to the fact that *Aspergillus* is transmitted through the inhalation of conidia, but also by direct inoculation at a site of an injury or skin break. The incubation period between conidia exposure and clinical manifestations appearance is estimated to range from two days to several months depending on the species pathogenicity and the host susceptibility (Cadena *et al.* 2016; Darling & Milder 2018; Van De Veerdonk *et al.* 2017).

Despite *Aspergillus* constitutes one of the most abundant fungi, only a small number of species of the genus are known to cause human infections (Thornton 2020). In fact, they are typically caused by *A. fumigatus, A. nidulans, A. niger, Aspergillus flavus, Aspergillus terreus* and related species (Paulussen *et al.* 2017).

1.3.1. Pulmonary aspergillosis syndromes

Noninvasive pulmonary aspergillosis includes infections driving hypersensitivity reactions such as allergic *Aspergillus* sinusitis or allergic bronchopulmonary aspergillosis (ABPA), but also aspergillomas. In contrast, other forms of aspergillosis that could be classified as semi-invasive include chronic conditions, such as chronic pulmonary aspergillosis (CPA) (Figure 4).

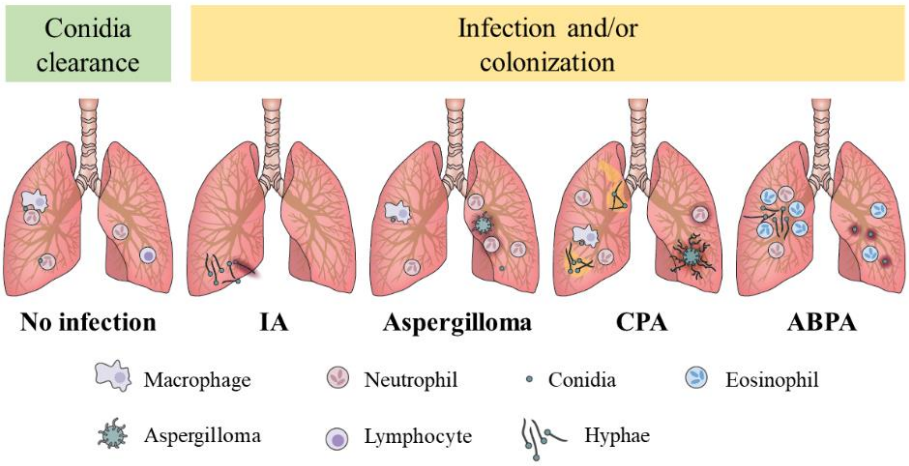

Figure 4. Spectrum of pulmonary aspergillosis (adapted from van de Veerdonk *et al*. 2017).

Characteristic features of allergic *Aspergillus* sinusitis are inflammation in the sinuses and symptoms such as drainage, stuffiness and headache. Presence of eosinophilic mucin and fungal hyphae in paranasal sinuses without invasion into surrounding mucosa are indicative of the condition (Chakrabarti & Kaur 2016).

In contrast, ABPA is characterized by the presence of chronic immune activation, pulmonary infiltrates and asthma. Lungs show inflammation

and affected patients display allergy symptoms such as coughing and wheezing (Cadena *et al.* 2016; Greenberger *et al.* 2014).

Regarding aspergilloma, the condition consists of a fungus ball formed in a pre-existing pulmonary cavity caused by emphysema, malignancy or pulmonary tuberculosis. Specifically, aspergillomas are rounded conglomerates of fungal hyphae, fibrin, mucus and cellular debris. Clinical manifestations of this conditions range from asymptomatic to life-threatening hemoptysis that requires urgent intervention. Aspergillomas usually do not spread to other parts of the body and can be removed by surgical resection (Cadena *et al.* 2016; Kosmidis & Denning 2015).

Finally, CPA is a long-term condition (3 months or more) characterized by symptoms like weight loss, malaise, sweats, anorexia, productive cough, breathlessness, chest discomfort and in some occasions, hemoptysis. Lung cavities with or without aspergillomas, neutrophil infiltrates, nodules and lung or pleural fibrosis can be found in patients suffering from CPA (Kosmidis & Denning 2015).

1.3.2. Non-pulmonary aspergillosis

Primary cutaneous aspergillosis (PCA) is defined as aspergillosis in which an infected skin lesion is the initial source of disease. It is a rare but life-threatening condition caused by conidia entrance through skin breaks due to trauma or surgical wounds. This contrasts with secondary cutaneous aspergillosis, that is the most common form, which involves the hematogenous spread of the *Aspergillus* infection from a distal site, such as the lungs, to the skin (Tatara *et al.* 2016).

1.3.3. Invasive aspergillosis

Invasive aspergillosis (IA) is the most serious condition caused by *Aspergillus* in terms of disease management and outcome. It is a life-threatening condition that mostly affects patients with weakened immune system, such as organ or stem cell transplant recipients, those suffering from cancer and those undergoing chemotherapy and corticosteroid treatment. However, it can also affect immunocompetent patients in some occasions. The clinical presentation of the disease usually varies between neutropenic and non-neutropenic patients. As a matter of fact, while IA in non-neutropenic patients displays few symptoms such as fever, cough and chest pain, neutropenic patients suffering from IA show the highest risk of rapid progression of the disease, with pneumonia and higher fatality rates. Other symptoms of IA include pleuritic pain, dyspnea and hemoptysis. The most commonly involved system, apart from the respiratory tract, is the central nervous system, which is associated to faster progression of the disease and 80 - 90 % mortality. In addition, since dissemination is achieved after blood vessels invasion, thrombosis and catastrophic hemorrhage are possible to occur in some cases. Less frequently involved organs are skin, bones, eyes, heart, spleen, kidney, liver, esophagus and the intestine. (Cadena *et al.* 2016; Darling & Milder 2018; Kanj *et al.* 2018; Tudesq *et al.* 2019).

Regarding the source of the infection, it is estimated that humans inhale between 100 and 1000 conidia every day, some of which reach the lungs, but normally, infection is avoided by the effective action of resident alveolar macrophages and neutrophils (Van De Veerdonk *et al.* 2017). More precisely, after conidia inhalation, macrophages detect the fungal entry into the host by recognizing the pathogen-associated molecular

patterns (PAMPs) displayed on the surface of the fungus, which are mainly fungal cell carbohydrates. This recognition is achieved by macrophage-associated pattern recognition receptors (PRRs) and it stimulates the elimination of inhaled conidia through their engulfment. Subsequently, elimination of conidia is carried out within the maturing phagosome compartments. At this point, if conidia are not properly cleared, invasive hyphae can penetrate the pulmonary tissue, where neutrophils (Polymorphonuclear leukocytes; PMNs) may also target them (Figure 5). In addition, dendritic cells activate the adaptive immune response to assemble the T-cell mediated defence (Erwig & Gow 2016; Feldman *et al.* 2019; Margalit & Kavanagh 2015).

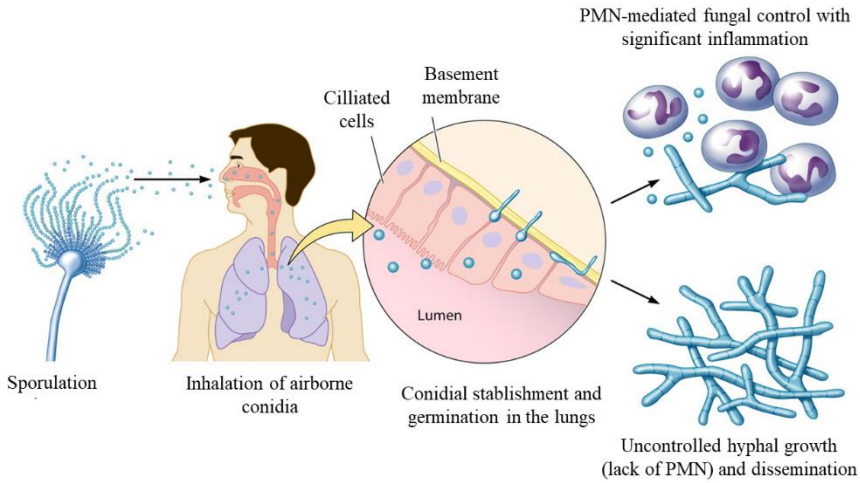

Figure 5. Pulmonary infection by *Aspergillus* after conidia inhalation (adapted from Dagenais & Keller 2009).

Very limited data on the epidemiology of IA is available. It has been estimated that approximately 300,000 cases of IA occur every year worldwide; however, this value may only account for approximately one-half of the actual cases due to misdiagnosis and misidentification.

Additionally, lack of accuracy and delay in diagnosis or treatment strategies contributes to increase IA mortality, which ranges from 50 to > 90 % depending on the extent of the disease, immune status and underlying diseases of the patients as well as the antifungal response displayed by the causal agent of the infection. In fact untreated IA practically shows 100 % of mortality in most patient groups (Brown *et al.* 2012; Thornton 2020). Regarding IA etiology, *Aspergillus fumigatus* is the most frequent causal agent of IA, while the second leading cause of IA are a few cryptic species belonging to sections *Flavi* and *Nigri*, usually isolated from affected patients as well. Nevertheless, infection rates due to non-*fumigatus* species are unclear and probably underestimated because of inaccurate identification (Hagiwara *et al.* 2016b; Lass-Flörl & Cuenca-Estrella 2017).

According to the current guidelines for aspergillosis management (Patterson *et al.* 2016), the treatment of choice for IA is voriconazole (VRC). Guidelines indicate intravenous (IV) VRC doses of 6 mg/kg every 12 h followed by 4 mg/kg every 12 h or oral doses of 200 – 300 mg every 12 h. Alternative treatments consist of IV liposomal amphotericin B (LAmB) at 3-5 mg/kg/day or isavuconazole (ISA) at 200 mg every 8 h for 6 doses and then 200 mg daily. Finally, treatments used as salvage therapy comprise: IV amphotericin B lipid complex (ABLC) at 5 mg/kg/day; IV caspofungin (CAS) at 70 mg/day (1 single dose) followed by 50 mg/day doses; IV micafungin (MCF) at 100-150 mg/day; 200 mg of posaconazole (PSC) in oral suspension three times a day (TID) or 300 mg of oral tablets twice a day (BID) on day 1 followed by 300 mg/day or IV 300 mg BID on day 1 followed by 300 mg/day, and itraconazole (ITC) in oral suspension at 200 mg/day every 12 h. In addition, PSC and VRC are recommended as prophylactic measures for aspergillosis prevention.

1.4. Clinical impact of *Aspergillus* section *Nigri*

A few species belonging to section *Nigri* are commonly isolated from clinical specimens. Specifically, *A. niger, A. tubingensis,* and, to a lesser extent, *A. brasiliensis,* have been frequently described as causal agents of otomycosis, onychomycosis, keratitis, pulmonary aspergillosis and aspergilloma (Hagiwara *et al.* 2016b; Kredics *et al.* 2008; Manikandan *et al.* 2010; Mehta *et al.* 2020). Interestingly, reports on the clinical implication of species other than *A. niger* or *A. tubingensis* are scarce, which might have been contributed by potential misidentification (Abarca *et al.* 2004).

As exemplified by recent studies, members of section *Nigri* are frequently found on clinical samples of diverse origin and nature, despite *A. fumigatus* continues to be the most common species in human infections. As a matter of fact, species of section *Nigri* were the second most prevalent in prospective studies carried out in hospitals from Italy and Japan (Prigitano *et al.* 2020; Toyotome *et al.* 2020). Curiously, the same tendency had been previously observed in Spain with a study involving samples from 29 different hospitals (Alastruey-Izquierdo *et al.* 2013).

Additionally, several reports of IA caused by section *Nigri* can be found in the literature indistinctly affecting male and female with ages ranging from 17 to 80 years old.(Alviar *et al.* 2014; Atchade *et al.* 2017; Bathoorn *et al.* 2013; Ergene *et al.* 2013; Gifford *et al.* 2006; Harada *et al.* 2020; Hollingshead *et al.* 2020; Kimmerling *et al.* 1992; Luce *et al.* 1979; Pozo-Laderas *et al.* 2015; Ramani *et al.* 1994; Shimoni *et al.* 2006; Siraj *et al.* 2005; Vermeulen *et al.* 2014; Workum *et al.* 2018; Xavier *et al.* 2008). These infections showed different manifestations and outcomes, reporting

50 % of deaths and 50 % of therapeutic success, mainly due to treatments with VRC and amphotericin B (AMB).

1.5. Antifungal drugs

Antifungal compounds reduce fungal cell viability or inhibit fungal proliferation, which facilitates the elimination of the fungus by the immune system of the host. Antifungals can be classified according to their chemical structure, nature (natural or synthetic), activity spectrum or their mechanism of action. From this last classification arise four antifungal families that can be used orally, topically or intravenously for the treatment of fungal infections. These are echinocandins, polyenes, pyrimidine analogs (flucytosine) and azoles (Figure 6). A fifth family, allylamines, is used to treat superficial dermathophytic infections (Campoy & Adrio 2017; Reales-Calderón *et al.* 2016).

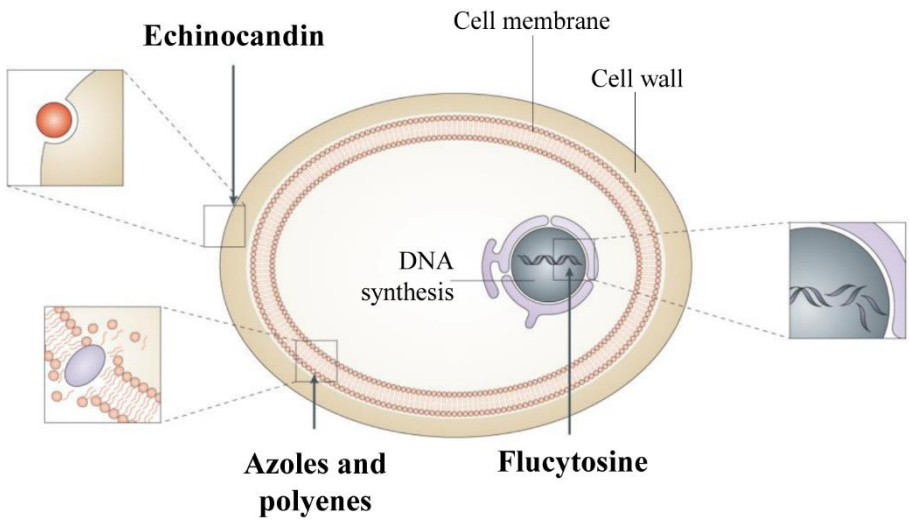

Figure 6. Antifungal targets (adapted from Ostrosky-Zeichner *et al*. 2010).

Although systemic antifungals have been available since the 1950s (polyenes), lack of substantial developments lasted for decades. Azoles were developed within the 1980s and revolutionized fungal infection management because they were available in oral formulations and in addition, they were effective against fungal pathogens refractory to the polyenes. Further azole development and reformulation of amphotericin B with lipid compounds to reduce its toxicity were carried out in the 1990s. Finally, echinocandins were introduced in the 2000s, which still are the newest antifungal family, since antifungal development is challenging due to the metabolic similarities between fungal and mammalian cells (Ostrosky-Zeichner *et al.* 2010).

1.5.1.Echinocandins

Echinocandins are semisynthetic lipopeptides derived from fungal secondary metabolites. Their chemical structure consists of a large molecule composed by a cyclic hexapeptide core with a lipidic side chain (Figure 7).

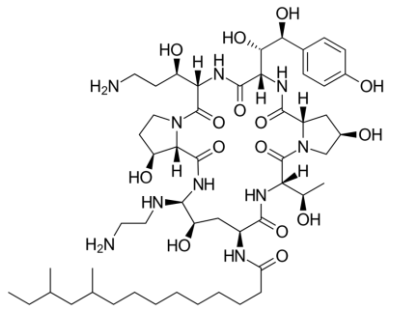

Figure 7. Echinocandin chemical structure (caspofungin).

These antifungal drugs act as noncompetitive inhibitors of the (1,3)-β-D-glucan synthase complexes (Fks proteins), by which a disruption of the (1,3)-β-D-glucan synthesis is achieved. This synthesis inhibition causes cell wall integrity loss as well as severe cell wall stress induction, which is translated into osmotic instability and fungal cell death (Figure 8) (Campoy & Adrio 2017; Odds *et al.* 2003; Shapiro *et al.* 2011).

Echinocandins show very good safety profiles and their toxicity is very low probably due to their unique target, which is a specific component of fungi absent in mammalian cells. Additionally, interactions with other drugs are minimal. Currently, there are three drugs belonging to this class intravenously available for clinical use: CAS, MCF, and anidulafungin (AND). Good antifungal activity of these has been reported against *Candida* and *Aspergillus* (Campoy & Adrio 2017; Odds *et al.* 2003; Shapiro *et al.* 2011).

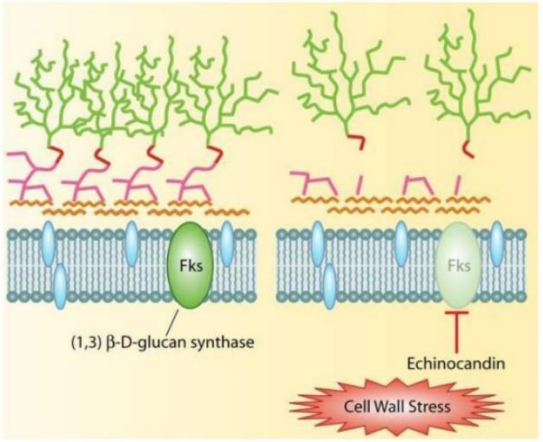

Figure 8. Echinocandin mode of action (Shappiro *et al.* 2011).

1.5.2. Polyenes

Polyenes are macrocyclic organic molecules known as macrolides. Generally, they consist of a carbon macrolactone ring conjugated with a d-mycosimine group and are amphipathic molecules, having both hydrophobic and hydrophilic sides (Figure 9).

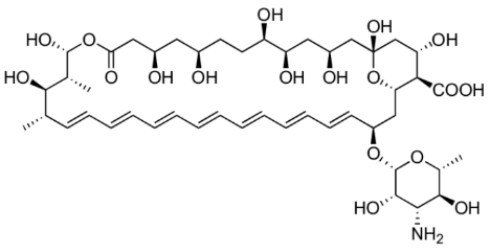

Figure 9. Polyene chemical structure (amphotericin B).

These drugs are natural products obtained from *Streptomyces noursei*, *Streptomyces natalensis* or *Streptomyces nodosum.* Their mode of action consists of a strong binding to the fungal cell membrane-bound ergosterol to form a drug-lipid complex that intercalates into the fungal membrane to form a membrane-spanning channel or pore (Figure 10). This causes its destabilization with ion and cytoplasmic contents leaks into the extracellular space disturbing proton gradient and promoting oxidative damage, which results in fungal cell death.

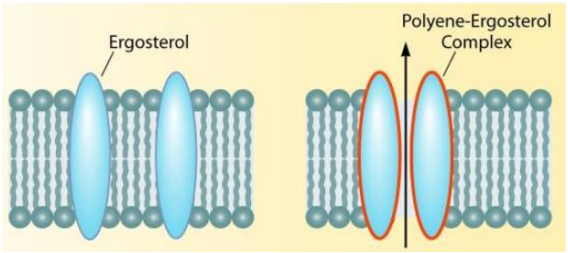

Figure 10. Polyene mode of action (Shappiro *et al.* 2011).

Polyenes are associated to elevated host toxicity and adverse effects that could be due to the structural similarity between the fungal ergosterol and the mammalian cholesterol. In recent years, the introduction of lipid-complexed formulations has reduced the toxicity events. Currently, there are three polyenes in clinical use: AMB, nystatin and natamycin. Good activity of these antifungals has been reported against some species of *Candida*., *Cryptococcus*, *Aspergillus* and *Fusarium* (Campoy & Adrio 2017; Odds *et al.* 2003; Shapiro *et al.* 2011).

1.5.3. Pyrimidine analogs

Flucytosine (5-FC; 5-fluorocytosine) is a fluorinated pyrimidine analog that interferes with pyrimidine metabolism, as well as RNA/DNA and protein synthesis (Figure 11). Its antifungal action is due to several effects:

its conversion to 5-fluorouracil, which becomes incorporated into RNA causing chain termination (protein synthesis impairment), and its effect on thymidylate synthase inhibiting DNA synthesis. For the pyrimidine analog to carry out this inhibition, the target cell needs the cytosine permease enzyme to internalize the flucytosine molecule and the cytosine deaminase enzyme to convert flucytosine into fluorouracil. Since little or no activity of these enzymes is displayed by filamentous fungi, 5-FC has been restricted to pathogenic yeasts such as *Candida* and *Cryptococcus*. It is associated to elevated liver toxicity and bone marrow suppression (Campoy & Adrio 2017; Nett & Andes 2016; Odds *et al.* 2003).

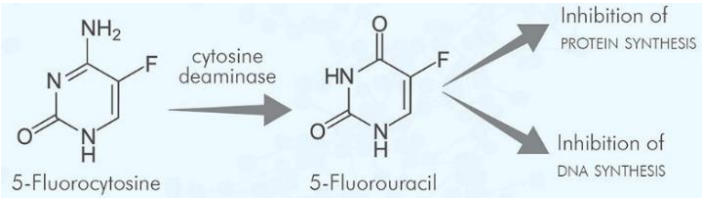

Figure 11. Pyrimidine analog mode of action (Di Mambro *et al.* 2019)

1.5.4. Azoles

Azole antifungals are five-membered heterocyclic organic molecules with a nitrogen atom and, at least, one other non-carbon atom (such as nitrogen, sulfur or oxygen) in their ring (Figure 12). They can be classified into two groups: imidazoles and triazoles, named according to the number of nitrogen atoms they possess in the azole ring. Imidazoles (clotrimazole, miconazole and ketoconazole) were the first developed azoles but they displayed elevated toxicity, side effects and interactions with other drugs, and so, they were

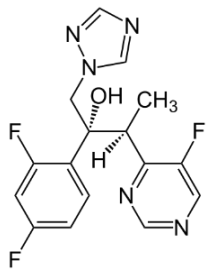

Figure 12. Triazole chemical structure (VRC).

replaced by triazoles, which exhibit broader antifungal activity and improved safety profiles. First generation triazoles include fluconazole (FLC) and ITC, while second generation triazoles include VRC, PSC and ISA (Campoy & Adrio 2017; Nett & Andes 2016).

Azole drugs inhibit the ergosterol biosynthesis by targeting the cytochrome P450 sterol 14 α-demethylase enzyme (Cyp51). This enzyme is bound to the endoplasmic reticulum membrane and catalyzes the conversion from lanosterol to ergosterol by oxidative removal of the 14 α-methyl group in the lanosterol molecule (Figure 13) (Parker *et al.* 2014).

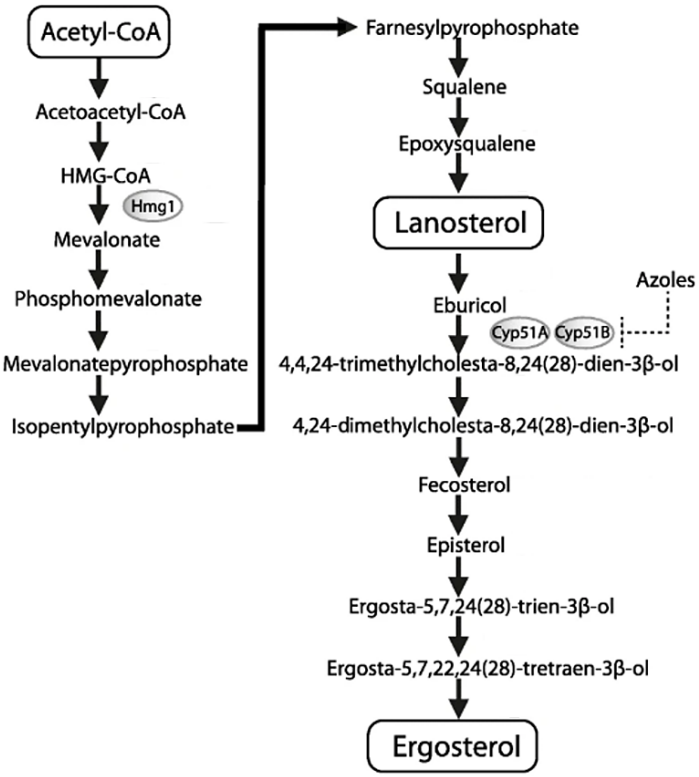

Figure 13. Ergosterol biosynthesis in *A. fumigatus* (adapted from Hokken *et al.* 2019).

The interaction between the Cyp51 enzyme and azoles is considered a non-competitive inhibition. In contrast to Cyp51 substrates, azoles might bind directly through coordination of a nitrogen atom in the azole ring with the heme ferric ion present in the enzyme, and thus the azole and the substrate binding sites might be different (Parker *et al.* 2014). Nevertheless, it has been reported in a few studies that azoles may act as competitive inhibitors of Cyp51 instead, as they might occupy the active site of the Cyp51 protein, and thus impair substrate binding (Conner *et al.* 2012; Hargrove *et al.* 2015). In any case, the Cyp51 inhibition results in a disrupted demethylation and accumulation of 14-methylated sterols, which affect the fungal cell membrane fluidity, reducing membrane-bound enzymes activity and leading to the membrane disruption and inhibition of cell proliferation (Figure 14). (Conner *et al.* 2012; Hargrove *et al.* 2015; Mellado *et al.* 2001; Parker *et al.* 2014). Nevertheless, the precise physiological effects exerted by the azoles within the fungal cell are unclear, and the specifics involved in the fungicidal effect they exhibit against *Aspergillus* are still under study (Geißel *et al.* 2018).

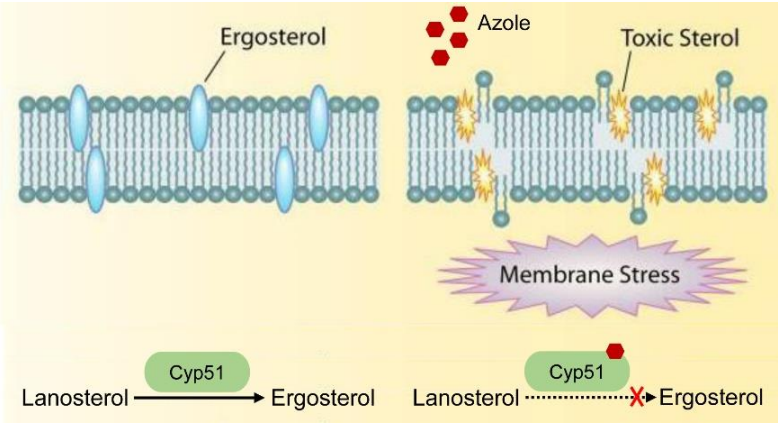

Figure 14. Azole mode of action (adapted from Shappiro *et al*. 2011).

Although the first generation of triazoles displayed important toxicity during systemic administration, the newer triazoles exhibit an improved safety profile and are well tolerated. These antifungals are active against a broad number of diverse fungi, including yeasts such as *Candida* and *Cryptococcus* or filamentous fungi like *Aspergillus* (Campoy & Adrio 2017; Ostrosky-Zeichner *et al.* 2010).

1.6. Antifungal resistance

Antifungal resistance can be defined as the ability displayed by a specific fungus to overcome antifungal drug concentrations that generally show good antifungal activity. In this context, establishing the *in vitro* Minimal Inhibitory Concentration (MIC) distributions of a specific drug along with its *in vivo* data in terms of pharmacokinetics/pharmacodynamics and clinical/microbiological outcomes, allows the setting of Clinical Breakpoints (CBPs), which are used to anticipate the effectiveness of the antifungal treatment, assisting for the selection of the best antifungal treatment in every case (Espinel-Ingroff & Turnidge 2016). With the ultimate aim of acquiring data on the susceptibility profile of an organism to an antifungal agent, available reference methods have been developed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI) (Sanguinetti & Posteraro 2018).

Azole drugs, but also polyene and echinocandin antifungals, generally show good activity against the clinically important *Aspergillus* species, and thus represent good options for IA treatment (see section 1.3.3). Nevertheless, intrinsic and acquired resistance mechanisms correlate to

poor or non-existent activity of one or diverse antifungals, which contribute to therapeutic failure and impairs patient outcomes (Delarze & Sanglard 2015; Perlin *et al.* 2017). While intrinsic resistance is defined as the absence of antifungal activity against not pre-exposed fungi to a drug, acquired resistance is the result of genetic adaptation to antifungal exposure. In the case of *Aspergillus,* all clinically important species show intrinsic resistance to FLC and 5-FC, while *A. terreus* and *A. flavus* display intrinsic resistance to AMB (Table 2) (Delarze & Sanglard 2015; Sharma & Chowdhary 2017; Wiederhold 2017).

Table 2. Overview on the activity of antifungals against several species of *Aspergillus* (adapted from: Delarze & Sanglard 2015).

Antifungal	*A .fumigatus*	*A. niger*	*A. flavus*	*A. terreus*
AMB	+	+	±	-
FLC	-	-	-	-
ITC	+	+	+	+
VRC	+	+	+	+
PSC	+	+	+	+
AND	+	+	+	+
CAS	+	+	+	+
MCF	+	+	+	+
5-FLC	-	-	-	-

Symbols: + , active; - , not active; ± , variable activity.

Acquired resistance to antifungals other than azoles in *Aspergillus* is rare and poorly characterized, since due to their absence of activity (pyrimidine analogs), oral unavailability (echinocandins), adverse effects or toxicity events (polyenes) (see section 1.5) they are not as widely used as azoles to treat aspergillosis.

As to echinocandins, the main mechanism of resistance consists of amino acid substitutions in the Fks1p protein *(fks1* gene), which is the major subunit of the (1,3)-β-D-glucan synthase complex (Rocha *et al.* 2007; Shishodia *et al.* 2019). Nevertheless, up to date only one clinical strain harboring a mutation on *fks1* has been identified. Specifically, it consisted of an *A. fumigatus* strain that had been isolated from a CPA patient subjected to prolonged MCF therapy (Jiménez-Ortigosa *et al.* 2017). As it has been hypothesized, the reason why echinocandin resistance is such a rare phenomenon in *Aspergillus* might be the loss of fitness resulting from *fks1* mutations (Aruanno *et al.* 2019). Another mechanism involved in *Aspergillus* echinocandin resistance comprises the heat shock protein Hsp90, since it can activate cellular signalling pathways required for cell survival against antifungal stress (Sharma & Chowdhary 2017).

In the case of polyenes, acquired resistance among *Aspergillus* species is very uncommon. Although the underlying mechanisms are unclear, it appears that they are related to a reduction in the ergosterol content within the fungal membrane. It has been suggested that an increased production of enzymes such hyperoxide dismutase and catalases might induce AMB resistance by compensation of the oxidizing action exerted by the antifungal (Perlin *et al.* 2017; Sharma & Chowdhary 2017; Shishodia *et al.* 2019).

1.6.1. Azole resistance

Regarding azoles and *Aspergillus*, susceptibility testing categorizing is based on available reference methods developed by the EUCAST and the CLSI. In this case, CBPs have been established for a few antifungals by the EUCAST protocols (EUCAST 2018), but no CBPs have been proposed by

the CLSI, which provides Epidemiological Cutoff Values (ECVs) for some *Aspergillus* sections instead (CLSI 2018) (Table 3). ECVs are another type of susceptibility threshold based on MIC distributions that differentiate wild type (wt) from non-wild type (non-wt) strains. In this case, non-wt strains are defined as those that potentially harbor acquired resistance mechanisms. It must be taken into account, though, that ECVs do not categorize fungal isolates as susceptible or resistant as CBPs do, since ECVs do not account for the pharmacology or the clinical outcome data of the antifungal agent (Espinel-Ingroff & Turnidge 2016).

Table 3. Azole ECVs (CLSI) and CBPs (EUCAST) for clinically relevant *Aspergillus* sections. Values are expressed in µg/mL.

Azole	Section	CLSI ECVs	EUCAST CBPs
ISA	*Flavi*	1	NE[1]
	Fumigati	1	1
	Nigri	4	NE
	Terrei	1	1
ITC	*Flavi*	1	1 – 2
	Fumigati	1	1 – 2
	Nigri	4	NE
	Terrei	2	1 – 2
PSC	*Flavi*	0.5	NE
	Fumigati	2	0.125 – 0.25
	Nigri	1	NE
	Terrei	-	0.125 – 0.25
VRC	*Flavi*	2	NE
	Fumigati	1	1 – 2
	Nigri	2	NE
	Terrei	2	NE

[1] Not Established

Since azoles target the Cyp51 proteins, this enzyme is the major candidate responsible for azole resistance at the molecular level. Alterations in the Cyp51 proteins, that reduce the affinity between the azole drug and its target; and overexpression of the target gene, which increases the azole concentration necessary to inhibit fungal growth, have been described to occur in *A. fumigatus.* Another well-described mechanism (non-Cyp51 mediated) consists of the upregulation of efflux pumps, which decreases the intracellular drug concentration. Nevertheless, there have been proposed several other mechanisms that might contribute to antifungal resistance, such as biofilm formation, cellular stress response, drug enzymatic degradation or activation of alternative pathways to bypass the effects of the drug (Figure 15) (Hagiwara *et al.* 2016b; Wei *et al.* 2015).

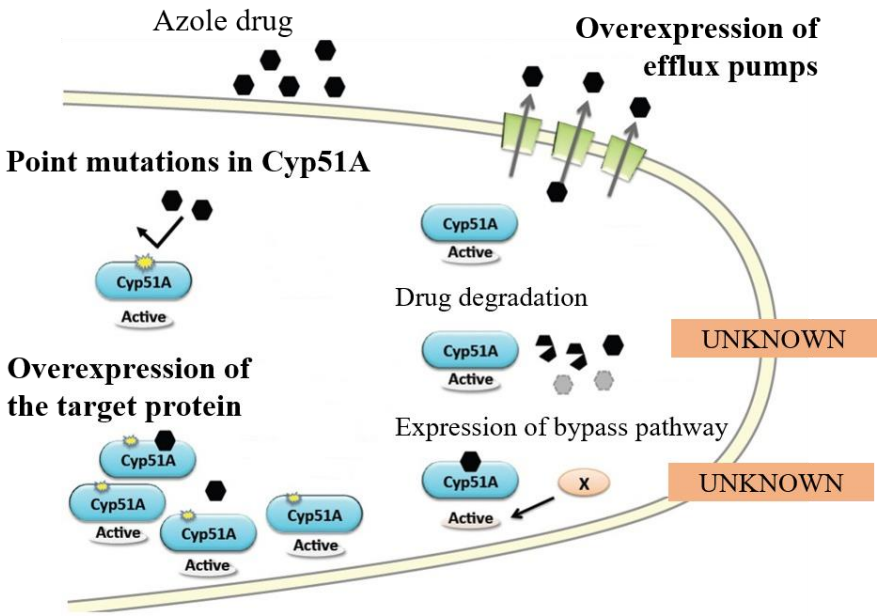

Figure 15. Azole resistance mechanisms described in *A. fumigatus*. Unknown mechanisms such as drug degradation by fungal enzymes or alternative ergosterol synthesis pathways are potentially involved in azole resistance but have not been characterized (adapted from Hagiwara *et al.* 2016b).

A. Azole resistance mediated by Cyp51

The Cyp51 proteins are encoded by *cyp51* genes (*ERG11* in yeasts). In the case of *Aspergillus*, varying number of *cyp51* paralogues can be found located on different chromosomes within species, as it occurs in many filamentous ascomycetes. Specifically, species such as *A. fumigatus, A. nidulans* and *A. niger* contain two *cyp51* paralogues in their genome (*cyp51A* and *cyp51B*), whereas *A. flavus* and *A. oryzae* display three of them (*cyp51A, cyp51B* and *cyp51C*) (Hagiwara *et al.* 2016b; Mellado *et al.* 2001). Regarding their functionality, it has been proven that *cyp51A* and *cyp51B* share the same substrate and display comparable functions. Neither of the two copies is individually essential in *A. fumigatus*, but the deletion of both genes is lethal for the fungus. However, deletion or silencing of *cyp51A* increases azole susceptibility without affecting the *cyp51B* expression whereas the *cyp51B* deletion does not affect azole susceptibility in this species (Hu *et al.* 2007; Martel *et al.* 2010; Mellado *et al.* 2005; Mousavi *et al.* 2015). These characteristics imply that *cyp51A* encodes major enzymatic activity and greater impact in the context of azole response than *cyp51B*. In addition, it seems that in the species containing three copies of the gene, *cyp51A* features might be displayed by *cyp51C* instead (Dudakova *et al.* 2017; Hagiwara *et al.* 2016b), although this is still unclear and currently under study (Paul *et al.* 2018).

cyp51 overexpression

Since azoles bind the Cyp51 enzyme, larger amounts of these proteins within the cell could bypass the inhibitory effect of the drugs. In this sense, *cyp51* gene expression could be a key aspect to understanding azole tolerance. In fact, introduction of extra copies of the *A. nidulans pdmA* gene (*cyp51A* ortholog) in *A. fumigatus* caused a decrease in the azole

susceptibility displayed by the latter (Osherov *et al.* 2001). However, it is still unclear if the same effect would be obtained for the *cyp51B* gene. In other *A. fumigatus* and *A. flavus* studies, no clear correlation between *cyp51* expression and azole resistance is found. As a matter of fact, the expression profiles of these genes barely vary among wt and non-wt strains, even after exposure to azoles (Buied *et al.* 2013; Liu *et al.* 2012; Paul *et al.* 2018).

Transcriptional upregulation of *cyp51* as response to azole toxicity and stress has been described to be mediated by transcription factors and regulation of transcriptional activators. Specifically, tandem repeats (TRs) of 34 bp (TR_{34}), 46 bp (TR_{46}) or 53 bp (TR_{53}) in the promoter region of *A. fumigatus cyp51A* enhances its expression (Hodiamont *et al.* 2009; Mellado *et al.* 2007; Snelders *et al.* 2011, 2015). These TRs are commonly found in combination with amino acid substitutions along the gene sequence (TR_{34}/L98H, TR_{46}/Y121F and TR_{46}/T289A) in non-wt strains (Mellado *et al.* 2007; Rodriguez-Tudela *et al.* 2008; van der Linden *et al.* 2013), except for the TR_{53}, which has not been linked to any other event (Garcia-Rubio *et al.* 2018). In addition, many genes coding for sterol-biosynthesis pathway harbor sterol regulatory binding elements in their promoter regions (Dhingra & Cramer 2017). In particular, recent studies have identified Transcription Factors (TFs) such as SrbA, HapE and AtrR, that bind to *A. fumigatus cyp51* promoters and modulate their expression. Point mutations on some of these regulators as well as their deletion have been described to significantly alter azole susceptibility by *cyp51* upregulation (Blosser & Cramer 2012; Camps *et al.* 2012a; Hagiwara *et al.* 2016a, 2017; Paul *et al.* 2019).

Cyp51 amino acid substitutions

Polymorphisms in both, Cyp51 azole-binding amino acids and other protein sequence regions that might affect enzymatic activity, can decrease the affinity of the enzyme towards azole drugs (Parker *et al.* 2014). Therefore, non-synonymous single point mutations in the *cyp51A* gene might alter the structure, stability and functionality of Cyp51, thus hindering substrate recognition, which leads to azole resistance (Liu *et al.* 2016; Warrilow *et al.* 2015). Several Cyp51A polymorphisms in *A. fumigatus* consisting of amino acid substitutions in positions G54, Y121, G138, P216, F219, M220, A284, Y431, G432, G434 and G448 have been reliably correlated with reduced azole susceptibility (Alanio *et al.* 2011; Albarrag *et al.* 2011; Bader *et al.* 2013; Bellete *et al.* 2010; Bueid *et al.* 2010; Camps *et al.* 2012b; Howard *et al.* 2009; Kidd *et al.* 2015; Krishnan-Natesan *et al.* 2012; Lescar *et al.* 2014; Mann *et al.* 2003; Mellado *et al.* 2004; Nascimento *et al.* 2003; Snelders *et al.* 2010; Wiederhold *et al.* 2016), contrasting with Cyp51B, in which no polymorphisms seem to be related to azole resistance (Dudakova *et al.* 2017; Hagiwara *et al.* 2016b). Regarding another species of the genus such *A. flavus*, mutations in the three Cyp51 enzymes (A, B and C) have been found in isolates with reduced azole susceptibility, however these have not been fully validated (Krishnan-Natesan *et al.* 2008; Paul *et al.* 2015; Sharma *et al.* 2018). In other species of *Aspergillus*, studies are scarce or not conclusive to date.

B. Azole resistance mediated by multidrug efflux pumps

Multidrug efflux pumps comprise transmembrane proteins that mediate active extrusion of antimicrobial molecules or toxic compounds and endogenous metabolites to the extracellular space (Rajendran *et al.* 2011).

Interestingly, a large number of different efflux pump protein-encoding genes are present in *Aspergillus* (Coleman & Mylonakis 2009), which are considered relevant determinants in drug resistance. More specifically, two types of efflux superfamilies modulate azole extrusion from fungal cells, *i.e.* the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters. While ABC transporters are composed by two transmembrane and two cytoplasmic nucleotide-binding domains and use the energy from ATP hydrolysis to extrude any molecules across the fungal membrane, the MFS transporters display from 12 to 14 transmembrane domains and use the proton-motive force to achieve drug efflux (Law *et al.* 2008; Perlin *et al.* 2015).

There are 45 ABC and 275 MFS transporters identified in the genome of *A. fumigatus* (Meneau *et al.* 2016), although only a few of them have been identified as drug transporters, which can be also named multidrug resistance (MDR) or pleiotropic drug resistance (PDR) proteins (Sipos & Kuchler 2006). In many cases the overexpression of these transporters result in inefficient effect of the drug because it does not reach the necessary intracellular concentration to exert its antifungal effect (Perlin *et al.* 2015).

C. Other azole resistance mechanisms

Very recently, it has been reported that the fungicidal effect exerted by azoles could be bypassed by cellular fungal stress responses or other molecular mechanisms that could be largely contributing to azole resistance. Specifically, one of these mechanisms involves the 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase enzyme of the ergosterol biosynthetic pathway, encoded by the *hmg1* gene in *A. fumigatus*

(see figure 13). This enzyme has a sterol-sensing domain involved in the negative regulation of its own activity (Rybak *et al.* 2019). Mutations located at the beginning of this domain (F262del, S305P, P309L and I412S) that might affect activity regulation have been described as candidate mediators of azole resistance, as they have been proven to increase azole MICs without altering *cyp51* expression (Rybak *et al.* 2019).

Another azole-derived effect within the fungal cell is the production of mitochondrial reactive oxygen species (ROS), which seems to contribute to fungal growth inhibition. The amino acid substitution E180D in the 29.9-kD subunit of the mitochondrial complex I has been found in azole-resistant *A. fumigatus* isolates, suggesting that resistance in this case could be due to activity loss of this complex. Furthermore, inhibition of this mitochondrial complex has proven to lead to azole resistance (Bromley *et al.* 2016). Mitochondria relevance in azole response has been recently reinforced, since it has been reported that VRC induces the sudden expulsive release of cytoplasm, arrest of mitochondrial dynamics with mitochondrial fragmentation and final lysis of the mitochondria, which eventually causes fungal death (Geißel *et al.* 2018).

Signalling pathways such the stress response or the cell wall integrity pathways have also been described to play a part in azole response and thus could contribute to azole resistance. Specifically, inhibition of the stress-related heat shock protein Hsp90 functions results in an increase of the azole susceptibility, proving the role of this enzyme in azole response, tolerance and resistance (Cowen 2009; Lamoth *et al.* 2014). In the case of the cell wall integrity pathway, a recent study has described the fungal chain of events derived from azole response. These seem to be related to increased β-1,3-glucan synthesis in patches all along the hyphae walls that

deform the cell membrane and trigger cell wall stress. Subsequently, the cell wall salvage system is activated, leading to fungal cell integrity failure and death (Geißel *et al.* 2018). Moreover, disruption of the *A. fumigatus* Mkk2 signalling kinase, which is a central modulator of cell wall biosynthesis results in an increase in azole susceptibility (Dirr *et al.* 2010). Also, loss of the endoplasmic reticulum-localized protein PerA (important for the fungal cell wall integrity) has been linked to triazoles increased susceptibility (Chung *et al.* 2014), thus providing evidence of the importance of the cell wall integrity pathway in the azole response.

Finally, another proposed and studied mechanism that might take part in azole resistance is biofilm formation. Biofilms can be defined as structured microbial cell populations that are attached to surfaces and embedded in a self-produced polysaccharide extracellular cell matrix (ECM). Fungal biofilms show reduced antifungal susceptibility when compared to planktonic cells, probably due to their structural complexity, the protection provided by the ECM and the upregulation of efflux pump genes (Delattin *et al.* 2014; Fanning & Mitchell 2012).

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STUDY AND CHARACTERIZATION OF AZOLE RESISTANCE IN ASPERGILLUS SECTION NIGRI
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INTEREST, HYPOTHESIS AND OBJECTIVES

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Some *Aspergillus* species belonging to section *Nigri* have been recently acquiring clinical importance, since they are continuously isolated from IA affected patients and constitute the second leading cause of IA after *A. fumigatus.* While azole response and resistance has been extensively studied in *A. fumigatus,* resistance mechanisms in non-*fumigatus* species remain poorly studied and practically unknown.

Characterization of azole susceptibility as well as the molecular mechanisms behind azole resistance in relevant species of section *Nigri* is of crucial importance to acquire better knowledge on azole resistance emergence, which might be potentially useful to palliate this problem in the clinical field.

On this basis, the central hypothesis of the studies presented in this dissertation is focused towards deciphering whether azole resistance mechanisms in species of *Aspergillus* section *Nigri* might differ from those of *A. fumigatus.*

Thus, the general objective of the project consists of characterizing azole resistance in species of *Aspergillus* section *Nigri* by *in vitro* analyses of the *cyp51* genes as well as exploring potentially undescribed mechanisms involved in such resistance.

The specific aims of this work are:

- To compile and update the current knowledge on azole resistance in non-*fumigatus* species.
- To *in silico* characterize the *cyp51* genes in relevant species of section *Nigri.*

- To establish the role of *cyp51A* and *cyp51B* in azole resistance by means of gene expression and amino acid substitutions analyses testing strains with different azole susceptibility.
- To generate knock-out *cyp51A* and *cyp51B* mutants from strains with different genetic backgrounds and assess their contribution to azole susceptibility.
- To analyze ergosterol content and its depletion upon azole exposure in strains with different azole susceptibility.
- To phenotypically characterize cell wall and fungal membrane in strains displaying diverse azole susceptibility.
- To examine potentially undescribed azole resistance mechanisms, such as azole degradation by laccase enzymes with gene expression and protein activity assays.
- To perform phagocytic killing assays to establish a possible correlation between drug susceptibility and macrophage clearance.

MATERIALS AND METHODS

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4.1. Culture media

All culture media were sterilized by autoclave (121 ºC for 15 min) unless otherwise indicated.

4.1.1. PDB (Potato Dextrose Broth)

Liquid medium with 200 g/L potato infusion, 20 g/L dextrose and distilled water.

4.1.2. YG (Yeast extract and glucose)

Liquid medium with 5g/L yeast extract, 20 g/L glucose and distilled water.

4.1.3. RPMI-1640 (Roswell Park Memorial Institute Medium)

Synthetic liquid medium (Gibco, Oxoid, Sigma-Aldrich) with L-glutamine lacking sodium bicarbonate. Buffered with 0.165 M MOPS [3-(N-morpholino) propanesulfonic acid], dissolved in distilled water, adjusted to pH 7 and filter sterilized.

4.1.4. DMEM (Dulbecco's Modified Eagle Medium)

Liquid medium (Biowest) with L-glutamine, 10 % (v/v) heat-inactivated fetal bovine serum and 1 % penicillin/streptomycin.

4.1.5. PDA (Potato Dextrose Agar)

Solid medium (Pronadisa) with 4 g/L potato starch, 20 g/L dextrose, 15 g/L agar and distilled water.

4.1.6. SM (Minimal Media)

Solid medium with 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.4 g/L KH_2PO_4, 0.2 g/L KCl, 1g/L NH_4NO_3, 0.01 g/L $ZnSO_4$, 0.01 g/L $MnCl_2$, 0.01 g/L $FeSO_4$, 10 g/L glucose, 15 g/L agar and distilled water.

4.1.7. AMM (*Aspergillus* Minimal Medium)

Solid medium with 5 % (v/v) 20x salt solution[1], 1 % (v/v) 100x nitrogen source[2], 0.1 % (v/v) trace elements[3], 10 g/L glucose, 15 g/L agar and distilled water.

4.1.8. SMM (Stabilized Minimal Medium)

Solid medium with 218.6 g/L sorbitol, 5 % (v/v) 20x salt solution, 1 % (v/v) 100x nitrogen source, 0.1 % (v/v) trace elements, 10 g/L glucose, 15 g/L agar and distilled water. Adjusted to pH 6.5.

4.2. Biological material and maintenance conditions

4.2.1. Fungal strains

Thirty-six *Aspergillus* section *Nigri* strains from clinical and environmental origins stored at the Facultat de Medicina i ciències de la salut de Reus (FMR) fungal collection were used in the *in vitro* susceptibility study (Table 4). These had been previously identified by ITS,

[1] **20x salt solution**: 10.4 g/L KCl, 10.4 g/L $MgSO_4 \cdot 7H_2O$ and 30.4 g/L of KH_2PO_4 and distilled water.
[2] **100x nitrogen source**: 85.2 g/L $NaNO_3$ and distilled water.
[3] **Trace elements**: 22 g/L $ZnSO_4 \cdot 7H_2O$, 11 g/L H_3BO_3, 5 g/L $MnCl_2 \cdot 4H_2O$, 5 g/L $FeSO_4 \cdot 7H_2O$, 1.6 g/L $CoCl_2 \cdot 5H_2O$, 1.6 g/L $CuSO_4 \cdot 5H_2O$, 1.1 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 50 g/L Na_4EDTA and distilled water. Adjusted to pH 6.5 -6.8.

benA and *caM* gene markers sequencing. Strain ATCC 204304 of *A. flavus* was used as the quality control when required.

All strains were retrieved from PDA slants cultures immersed in mineral oil, lyophilized cultures or 5 mm^3 cubes submerged in distilled sterile water. Before they were used, all strains were grown on PDA plates for 3-5 days at 35 ºC twice.

Table 4. *Aspergillus* section *Nigri* strains used in the *in vitro* susceptibility study.

Strain	Species	Strain	Species
FMR 6267	*A. niger*	**FMR 13541**	*A. awamori*
FMR 7131	*A. japonicus*	**FMR 14592**	*A. japonicus*
FMR 9393	*A. niger*	**FMR 14630**	*A. tubingensis*
FMR 11248	*A. niger*	**FMR 14635**	*A. tubingensis*
FMR 11250	*A. niger*	**FMR 14638**	*A. carbonarius*
FMR 11253	*A. niger*	**FMR 14640**	*A. niger*
FMR 11254	*A. niger*	**FMR 14712**	*A. tubingensis*
FMR 11894	*A. niger*	**FMR 14714**	*A. niger*
FMR 11896	*A. niger*	**FMR 15326**	*A. neoniger*
FMR 11897	*A. niger*	**FMR 15385**	*A. niger*
FMR 11898	*A. niger*	**FMR 15386**	*A. brasiliensis*
FMR 11900	*A. niger*	**FMR 15387**	*A. niger*
FMR 11901	*A. niger*	**FMR 15388**	*A. niger*
FMR 11906	*A. tubingensis*	**FMR 15389**	*A. tubingensis*
FMR 13537	*A. awamori*	**FMR 15396**	*A. niger*
FMR 13538	*A. awamori*	**FMR 15413**	*A. acidus*
FMR 13539	*A. awamori*	**FMR 15441**	*A. niger*
FMR 13540	*A. awamori*	**FMR 17207**	*A. tubingensis*

Fourteen of these strains, belonging to species *A. niger, A. tubingensis* and *A. brasiliensis* were selected for molecular and further studies (Table 5).

Table 5. Selected strains for further studies.

Strain	Species	Origin
FMR 11254	*A. niger*	Clinical, Spain
FMR 11894	*A. niger*	Clinical, United States
FMR 11898	*A. niger*	Clinical, United States
FMR 11900	*A. niger*	Clinical, United States
FMR 11906	*A. tubingensis*	Clinical, Brasil
FMR 14630	*A. tubingensis*	Environmental, Spain
FMR 14635	*A. tubingensis*	Environmental, Venezuela
FMR 14712	*A. tubingensis*	Environmental, México
FMR 15385	*A. niger*	Environmental, Brasil
FMR 15386	*A. brasiliensis*	Environmental, Spain
FMR 15388	*A. niger*	Environmental, Spain
FMR 15389	*A. tubingensis*	Environmental, Spain
FMR 15441	*A. niger*	Environmental, Spain
FMR 17207	*A. tubingensis*	Environmental, Vietnam

Conidial suspensions from these strains were conserved in 30 % glycerol (v/v) at -80ºC or 50 % glycerol (v/v) at -20ºC. These suspensions were used as work stocks for the obtention of fresh new cultures.

4.2.2. Macrophage cell line

Adherent macrophage-like cell line J774A.1 from BALB/c mice were used to assess fungal virulence.

Cells were incubated and maintained in tissue culture flasks at 37ºC under 5 % CO_2 in DMEM (Biowest) containing L-glutamine, 10 % (v/v) heat-inactivated fetal bovine serum and 1 % penicillin/streptomycin.

4.3. Fungal growth conditions

4.3.1. Conidia inocula preparation

For conidia inocula preparation, strains were grown on PDA for 3 to 5 days at 35 ºC and conidia were collected by flooding the PDA culture plates with 5 mL of 0.1 % Tween 20 and gentle agitation. Inocula were adjusted by hemocytometer count to different concentrations when indicated.

4.3.2. Conidia germination and growth

For genomic DNA (gDNA) extraction, conidial suspensions were incubated in PDB for 24 h at 37 ºC and 180 rpm. Subsequently, mycelia were collected by Monodur nylon filtration (pore diameter size:10-15 µm), washed with distilled sterile water and stored at -80 ºC until use.

For gene expression studies and sterol extraction, $1x10^7$ conidia/mL were germinated in 25 or 50 mL of YG medium respectively for 12 h at 37 ºC and 180 rpm. After germination, mycelia were aseptically harvested by Monodur nylon filtration and transferred to fresh YG medium supplemented with VRC (Pfizer) at a final concentration of 4 µg/mL when required. Cultures were further incubated at 37 ºC and 180 rpm, for 8 h and mycelia were collected by Monodur nylon filtration, washed with distilled water, frozen in liquid nitrogen and stored at -80 ºC until used. For sterol extraction, the final step after mycelia collection consisted of lyophilization using the AdVantage Freeze Dryer (The VirTis Company Inc) (Figure 16).

For extracellular protein studies, 250 mL of YG medium were inoculated with $1x10^7$ conidia/mL and samples were incubated at 37 ºC and 180 rpm for 12 h. After this incubation, mycelia were aseptically harvested by Monodur nylon filtration and transferred to fresh YG medium

supplemented with VRC (Pfizer) at 4 µg/mL of final concentration when required. Then, cultures where further incubated at 37 ºC and 180 rpm for 24 h, mycelia were collected by Monodur nylon filtration and culture media supernatant was sterile filtered and kept on ice (Figure 16).

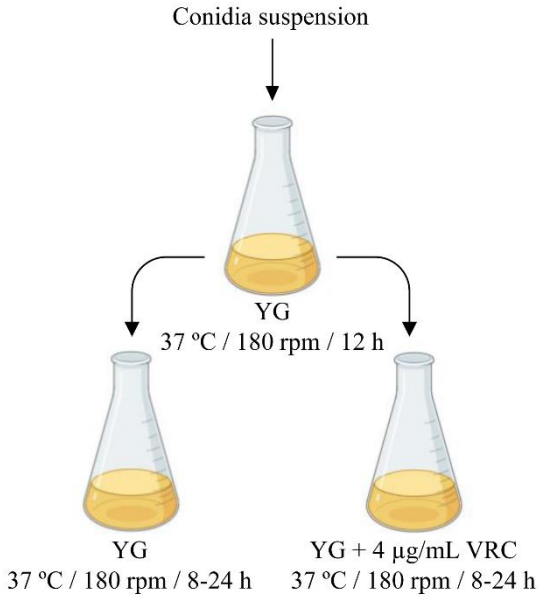

Figure 16. Growth conditions scheme for gene expression analyses, sterol extraction and extracellular protein obtention.

For conidia swelling determination, $1x10^7$ conidia/mL of every strain were inoculated into YG medium and incubated for 14 h at 37ºC and 180 rpm. Samples were aseptically taken at different times and placed on slides to assess conidia size at each time. Images were taken with a Zeiss Axio-Imager M1 light with a DeltaPix Infinity × digital camera and analysis of conidia swelling was carried by measuring conidia diameter with the ImageJ software v1.52a for Windows.

4.4. *In silico* studies

4.4.1. DNA and protein sequences obtention and analysis

Genomic sequences of well annotated strains *A. niger* CBS 513.88, *A. tubingensis* CBS 134.48 and *A. brasiliensis* CBS 101740 were used for all the *in silico* studies.

Sequences were retrieved from the Joint Genome Institute (JGI) and the Aspergillus Genome Database (AspGD). The BLASTn or BLASTp tools from the NCBI database were used for orthologs identification using *A. fumigatus* Af293 sequences as templates.

Different bioinformatic software were used to compare and analyze sequences: Visualization and sequence editing were carried in SeqBuilder (DNASTAR) and SnapGene viewer (GSL Biotech). Nucleotide and protein alignments were accomplished by using the ClustalW algorithm of the MegAlign software (DNASTAR). Sequence assembly and analysis was achieved with the SeqMan (DNASTAR) and MEGA version 7 software.

Amino acid sequence analysis, residues and groups of interest identification in the proteins of study was carried out by comparison with the *A. fumigatus* proteins structure deposited in the SWISS-MODEL database (IDs: 4uyl.2, 4uym.1 and 5fbr.1). Prediction of protein topology and domains was performed with the TMHMM v2.0 server and the InterPro platform.

Additionally, to evaluate Cyp51 enzymes conservation along the genus *Aspergillus,* the MEGA7 software was used to build a phylogenetic tree performing a non-parametric bootstrap analysis with 1000 repetitions.

4.4.2. Oligonucleotides design

Oligonucleotides for amplification, sequencing and CRISPR-Cas9 genetic transformation (Tables 6 - 10) were designed with the Oligo7 software (Molecular Biology Insights) and the OligoAnalyzer tool available at the Integrated DNA Technologies (IDT) website.

Sequence stability and other physical parameters such as melting temperature (Tm), G + C and A + T content were optimized. Hairpins and primer dimer formation were not accepted. Moreover, lack of secondary priming sites in the template and low specific binding at the 3' end to avoid mispriming were also assured.

Table 6. Oligonucleotides used for *cyp51* gene expression analyses.

Primer	Sequence (5' – 3')	Gene	Species
cyp51A-F1	GAACCCAGACGAGGAGAAG	*cyp51A*	All[1]
cyp51A-R1	TCGCAGCATGATCCAAGAAC	*cyp51A*	*A. niger / A. brasiliensis*
cyp51A-R2	CGCAACATAATCCAAGAGCTA	*cyp51A*	*A. tubingensis*
cyp51B-F1	GTTTCCATTCATAGGTAGCAC	*cyp51B*	All
cyp51B-R1	ACTTCTTCAGCACAGACATCA	*cyp51B*	All
act-F2	ACCCTCAGATACCCCATTGA	*actA*	All
act-R2	CTGGGTCATCTTCTCACGG	*actA*	All

1 All: *A. niger*, *A, tubingensis* and *A. brasiliensis.*

Table 7. Oligonucleotides for *cyp51A* amplification and sequencing.

Primer	Sequence (5' – 3')	Species
cyp51A-F1	GAACCCAGACGAGGAGAAG	All[1]
An-F2	CGATAGTCTTAAATGTCACGC	*A. niger*
An-R3	GCTCCCAACCCACTATAGC	*A. niger*
Anb-R2	TGAGGCGAGTAGAACATGGT	*A. niger/A.brasiliensis*
Ax-R3	ACCTCCTCGCCCTGCAAC	All
Asptu-F1	ATGGCATATCTTGCTGTTGCA	*A. tubingensis*

Asptu-R1	TTAGTTCAAGGACCCCTTGGA	*A. tubingensis*
Asptu-R2	CTGGTGAGGCGAGTAGAAC	*A. tubingensis*
Asptu-R3	CTTGGAGTTGTCTTGGATGC	*A. tubingensis*
Aspbt-F2	TGCTCGTTGCGATAGTCTTG	*A. tubingensis* / *A. brasiliensis*
Aspbr-F1	ATGGCATTCCTTGCTATCGC	*A. brasiliensis*
Aspbr-R1	TCAGTTCGATGACCCCTTGG	*A. brasiliensis*
Aspbr-R3	CGGAGAAGAGAGTCGAGTAA	*A. brasiliensis*

[1] All: *A. niger*, *A, tubingensis* and *A. brasiliensis.*

Table 8. Oligonucleotides used for *cyp51A* or *cyp51B* gene deletion. In capital letters, homologous region to the DNA template. In lowercase, homologous region to the Hyg^R repair template.

Primer	**Sequence (5' – 3')**	**Species**
Hyg Fw tail	CTTGCTCTTGACTTCCTCTTTACAACAA	*A. niger*
Ancyp51A	TCTTTCTCATCAagcttgcatgcctgcaggtc	
Hyg Rv tail	AGGTACTAGTTGGGAAAAGACTGGAGA	*A. niger*
Ancyp51A	CGAAAGGTAACCTGCcatcgatgatatcagatc	
Hyg Fw tail	CCCAAAGTGCACTTTCCTGCGCCGTTAC	*A. niger*
Ancyp51B	AAGCAATTTACAagcttgcatgcctgcaggtc	
Hyg Rv tail	ATTTCATCGTATTACATGAGAGGTTTAA	*A. niger*
Ancyp51B	GTACAATCACCGAAcatcgatgatatcagatc	
Hyg Fw tail	TCCCCATCAATTGGTCCTTTTTCCTGCC	*A. tubingensis*
Atcyp51A	TACGGTCGCTTCagcttgcatgcctgcaggtc	
Hyg Rv tail	CTAGTTGGGAAAGACTGGAGATGAAAG	*A. tubingensis*
Atcyp51A	ATAGCTTGCAGAGAAcatcgatgatatcagatc	
Hyg Fw tail	CTTCCTTGTGAACACAACCAACTCACTC	*A. tubingensis*
Atcyp51B	CAATTGTCTGTTagcttgcatgcctgcaggtc	
Hyg Rv tail	GGTTTGAGAACAATCACAAAAGGCGGT	*A. tubingensis*
Atcyp51B	TTCTATGCAGTAACAcatcgatgatatcagatc	

Table 9. crRNA sequences used for genetic transformation using the CRISPR-Cas9 technique.

crRNA	Sequence (5' – 3')	Species
Ancyp51A 5' gRNA	CAACAATCTTTCTCATCAAC	*A. niger*
Ancyp51A 3' gRNA	GAATTAGGCCACCTTCTTGC	*A. niger*
Ancyp51B 5' gRNA	CCGTTACAAGCAATTTACAC	*A. niger*
Ancyp51b 3' gRNA	TACTGTATAGAAACCGCGTT	*A. niger*
Atcyp51A 5' gRNA	TCCTGCCTACGGTCGCTTCC	*A. tubingensis*
Atcyp51A 3' gRNA	AAAGATAGCTTGCAGAGAAG	*A. tubingensis*
Atcyp51B 5' gRNA	CTCACTCCAATTGTCTGTTC	*A. tubingensis*
Atcyp51B 3' gRNA	CGGTTTCTATGCAGTAAGAG	*A. tubingensis*

Table 10.Oligonucleotides used as PCR primers for *cyp51A* and *cyp51B* knock-out screening.

Primer	Sequence (5' – 3')	Species
Ancyp51A Fw scr	GAACGATGCAGCTCAGTGCCA	*A. niger*
Ancyp51A Rev scr	ACACGAGGGATCTCACGTGC	*A. niger*
Ancyp51B Fw scr	CCTGCTACTAGGAGAGCCGC	*A. niger*
Ancyp51B Rev scr	GCCTGTATGGGACAGTCGCT	*A. niger*
Atcyp51A Fw scr	CCGATGTAGCTCAGTGCCAGG	*A. tubingensis*
Atcyp51A Rev scr	ACTGGGCAGCGGTCATATCTC	*A. tubingensis*
Atcyp51B Fw scr	GTGCACTTTCCTGCGCTGTTG	*A. tubingensis*
Atcyp51B Rev scr	CCGGCCATTAAAGCCCCAAG	*A. tubingensis*
Hyg Fw intern	CGATTCCTTGCGGTCCGAAT	All[1]
Hyg Rev intern	ATCGGCACTTTGCATCGGC	All

1 All: *A. niger*, *A, tubingensis* and *A. brasiliensis.*

Table 11. Oligonucleotides for *A. niger* multicopper oxidase family (laccase and laccase-like genes) gene expression analyses.

Primer	Sequence (5' – 3')	Gene
AnmcoA-F1	CCGACTGGCGATTCTTGAC	*mcoA*
AnmcoA-R1	ACATAGAAGGTGGCATAGCG	*mcoA*
AnmcoB-F1	CCTGAATGTCGAAAATGCCAA	*mcoB*
AnmcoB-R1	ACGGAGGACCAGATGAAGG	*mcoB*
AnmcoC-F1	GCGTGAACCAAGGCGACC	*mcoC*

AnmcoC-R1	TCTTGCTAAACGGTCTCTGC	*mcoC*
AnmcoD-F1	GCACAAACTCCATTACCCAAT	*mcoD*
AnmcoD-R1	GTTTCGTTGTTCTCCTTAACC	*mcoD*
AnmcoE-F1	AAAAGCTCTCCATAAACCACAA	*mcoD*
AnmcoE-R1	TACGGGTGCTTGGTAGGTC	*mcoE*
AnmcoF-F1	GAGACGGATGCCTTGAACAA	*mcoE*
AnmcoF-R1	AGGGGAGATGGATGATGCG	*mcoF*
AnmcoG-F1	TTGCTACTCCTTGGGCTTCT	*mcoG*
AnmcoG-R1	CCATTGTTTTCCATGCGATTC	*mcoG*
AnmcoH-F1	ACCGTGACCTATGACTGGAA	*mcoH*
AnmcoH-R1	GGTGAAGTTGTACGTGATGG	*mcoH*
AnmcoI-F1	GACCCCACTACCACGCAAA	*mcoI*
AnmcoI-R2	GAGGGGATTAGACGGATTAG	*mcoI*
AnmcoJ-F1	CAGTATCCGCTTCCACCAAA	*mcoJ*
AnmcoJ-R2	GAATTACGCCTGTGGGTGC	*mcoJ*
AnmcoK-F1	CACTTCCACGGCATGTACC	*mcoK*
AnmcoK-R2	TGGGATTATACAGCGACATGA	*mcoK*
AnbrnA-F1	ATGGAAAGCCCGCCTACAG	*brnA*
AnbrnA-R2	TTATCAGGATTCATTGCACGG	*brnA*
AnmcoM-F1	TGAACGATACTCTGTGGGTG	*mcoM*
AnmcoM-R2	TGCTGTTGTACTTGTCGGTG	*mcoM*
AnmcoN-F1	TCCTCATCAACGGGCAATTC	*mcoN*
AnmcoN-R2	CGCTGTAATGGGAATGGTAC	*mcoN*
AnmcoO-F1	CAACCCTCTCCTTCTCGATT	*mcoO*
AnmcoO-R2	GATGTCGATGTAAGGGGTAG	*mcoO*
AnmcoP-F1	AATCAGATCATCAACGGGACT	*mcoP*
AnmcoP-R1	AGAGCACAGGGGTCGATAG	*mcoP*

4.5. Nucleic acids manipulation

4.5.1. Genomic DNA extraction and purification

Genomic DNA (gDNA) extraction was carried out as previously described (Torres *et al.* 1993) with some modifications. Mycelia were ground in liquid nitrogen with mortar and pestle, and 100 mg of pulverized

mycelia were transferred to a tube with 1 mL of extraction buffer CTAB[4] (cetyl trimethylammonium bromide). After vigorous homogenization, 4 µL of β-mercaptoethanol (Sigma) were added, the tube was filled with a solution of chloroform:isoamilalcohol (24:1 v/v) and incubated for 30 min at 65 ºC. Samples were further incubated at room temperature for 15 additional minutes and centrifuged for 5 min at 7000 rpm. Supernatants were recovered and transferred to new tubes, 1 mL of cold 100 % ethanol was added, and tubes were incubated at -20ºC for 30 minutes. To precipitate DNA, tubes were centrifuged at 13000 rpm and 4 ºC for 30 min, and two washes with 1mL of cold 75 % ethanol were carried at 13000 rpm and 4 ºC for 5 min centrifugations. Finally, the resulting DNA pellets were resuspended with 50-100 µL of sterile deionized water and RNAse treatment was followed by incubating the samples with 4 µL of RNAse (Thermo Fisher) at 37 ºC for 30 min.

For DNA purification, samples were subjected to an additional precipitation consisting of the addition of 0.1 volumes of sodium acetate 3 M (pH 5.4) and incubation with 2.5 volumes of cold 100 % ethanol for 30 min at -20 ºC. Then, samples were centrifuged at 14000 rpm and 4 ºC for 30 min and two washes with cold 70 % ethanol were performed at 14000 rpm and 4ºC for 5 min. The resulting pellets were resuspended in 50 – 100 µL of sterile deionized water.

In some cases, an alternative protocol was followed. In these occasions, mycelia were lyophilized in 2 mL screw-cap tubes containing glass beads. Samples were bead-beated in three rounds of 1 min each cooling on ice

[4] **CTAB buffer**: 12.1 g/L Tris-Base, 7.44 g/L EDTA, 81.8 g/L NaCl and 20 g/L hexadecyltrimethylammonium bromide dissolved at 60 ºC and adjusted to pH 8.0 with NaOH. Stored at 37 ºC to avoid precipitation.

between cycles. Afterwards, 600 µL of extraction buffer TENS[5] was added to each sample, tubes were vigorously vortex mixed and incubated at 65 ºC for 1 h. Next, 600 µL of phenol:chloroform:isoamylalcohol (25:24:1) were added and samples were vortex mixed and centrifuged at 13000 rpm for 10 min. Supernatants were removed and transferred to a new 1.5 mL tube. An equal volume of chloroform:isoamylalcohol (24:1), samples were mixed by inversion and centrifuged at 13000 rpm for 10 min. Supernatants were removed and dispensed into new 1.5 mL tubes, and 1.6 µL of RNAse A were added. After incubation at 37 ºC for 1 h, DNA was precipitated by the addition of 0.1 volumes of sodium oxaloacetate 3 M (pH 5.3) and 2 volumes of cold 100 % ethanol. Samples were incubated at -20 ºC for 30 min and centrifuged at 14000 rpm and 4 ºC for 30 min and two washes with cold 70 % ethanol were performed at 14000 rpm and 4ºC for 5 min. The resulting pellets were resuspended in 50 – 100 µL of sterile deionized water.

Finally, in all cases, DNA samples quantity and quality were checked by running aliquots in agarose gels (Pronadisa) stained with RedSafe (iNtRON Biotechnology) and spectrophotometric analysis in a NanoDrop2000 spectrophotometer (Thermo Fisher).

4.5.2. Total RNA extraction and purification

Total RNA was extracted from mycelia produced in the conditions specified in section 3. Frozen mycelia were ground in liquid nitrogen with mortar and pestle and 100 mg of pulverized mycelia were transferred to a tube with 1 mL of TRIzol® reagent (Thermo Fisher). After vigorous

[5] **TENS buffer**: 3.02 g/L Tris-HCl, 9.30 g/L EDTA 1.46 g/L NaCl and 1 % (v/v) SDS.

homogenization, samples were centrifuged at 12000 rpm and 4 ºC for 10 min. Supernatants were transferred to new tubes and incubated on ice for 10 min to allow complete dissociation of nucleoprotein complexes. Next, 200 µL of chloroform were added, samples were vigorously homogenized and further incubated on ice for an additional 5 min. Tubes were centrifuged for 15 min at 12000 rpm and 4 ºC, which resulted in a three-phase solution. The upper phase was collected and transferred to a new tube and 500 µL of isopropanol were added. After mixing by inversion and incubating for10 min on ice, centrifugation at 12000 rpm and 4 ºC for 10 min was performed and supernatants were removed. RNA pellets were precipitated and washed with 1 mL of cold 75 % ethanol and 7500 rpm for 5 min at 4 ºC centrifugation. Finally, the resulting RNA pellets were resuspended in 100 µL of sterile DNAse-free and RNAse-free water and tubes were incubated at 55 ºC for 10 min.

For RNA purification, the NucleoSpin® RNA kit (Macherey-Nagel) were used following the manufacturer's instructions. Briefly, RNA samples, were mixed with 600 µL buffer RA1:96 % ethanol (1:1 v/v) and transferred to NucleoSpin® RNA columns, which were centrifuged at 11000 rpm for 30 sec for the RNA. The flow through-was discarded, 350 µL of Membrane Desalting Buffer (MDB) were added into the column and samples were centrifuged at 11000 rpm for 30 sec. Following the centrifugation, on-column DNAse treatment was carried out by adding the digestion mixture prepared with 10 µL rDNAse and 90 µL of Reaction Buffer for rDNAse and incubating for 15 min at room temperature. Subsequently, 200 µL of RAW2 buffer were added to wash and dry the silica membrane, tubes were centrifuged at 11000 rpm for 30 sec and flow-through was discarded. Next, two steps of RA3 buffer addition and

centrifugation were carried by adding 600 µL and 250 µL of RA3 buffer, respectively and centrifuging at 11000 rpm for 30 sec and 2 min in every case. Finally, flow-through was discarded, columns were placed into nuclease-free collection tubes and RNA was eluted in 60 µL of RNAse-free and DNAse-free water by centrifuging 1 min at 11000 rpm.

Alternatively, RNA samples were subjected to a DNAse treatment with DNAse I (Thermo Fisher) for 30 min at 37 ºC following the manufacturer's instructions. Samples were then precipitated by adding to the tubes 2.5 M LiCl, followed by a 30 min incubation at -20 ºC and centrifugation at 16000 rpm and 4 ºC for 20 min. Afterwards, RNA pellets were washed with cold 75 % ethanol and resuspended in RNAse-free and DNAse-free water.

Finally, in all cases, RNA samples quantity and quality were checked by running aliquots in agarose gels (Pronadisa) stained with RedSafe (iNtRON Biotechnology) and spectrophotometric analysis in NanoDrop2000 spectrophotometer (Thermo Fisher).

4.6. Nucleic acids electrophoresis

4.6.1. Agarose gels

DNA and RNA molecules were separated and identified by electrophoresis using agarose (Pronadisa) gels prepared in TAE[6] buffer and stained with Ethidium Bromide (Bio-Rad) or RedSafe (iNtRON

[6] **TAE buffer**: Prepared as a stock of 50x TAE buffer and diluted with distilled water as required. For the 50x solution: 242.2 g/L Tris Base, 60.5 mL/L acetic acid, and 18.61 g/L EDTA.

Biotechnology). Agarose concentration of the gels ranged between 0.7 and 1.5 % (w/v) depending on the molecule fragments to be separated.

Samples were loaded with an agarose-gel loading dye[7], electrophoresis was performed at a constant voltage of 1-4 V/cm and fragment sizes were estimated with the GeneRuler™ Plus DNA ladder molecular marker (Thermo Fisher). Gel imaging was carried out with the ChemiDoc ™ system coupled with the ImageLab™ software (Bio-Rad).

4.6.2. DNA purification from agarose gels

Two different commercial kits were used to recover and purify DNA fragments from agarose gels: the GeneJET Gel Extraction Kit (Thermo Scientific) or the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare).

In both cases, the gel slice containing the DNA fragment was excised, placed into a pre-weighted tube and weighted. Next, 1:1 volume of binding/capture buffer was added to the tube and incubated at 60 ºC until the agarose slice was completely dissolved. The mixture was then placed into a purification column, incubated for 1 min at room temperature and centrifuged. The flow-through was discarded and wash buffer was added to the column and centrifuged. Finally, the flow-through was discarded and elution was carried by adding 30 – 60 µL of sterile deionized water and centrifuging to collect DNA in a new tube. Purified DNA was stored at -20 ºC until use.

[7] **Agarose-gel loading dye**: 0.25 % (w/v) Bromophenol Blue, 0.25 % (w/v) xylene cyanol and 40 % (w/v) sacarose in 5x TAE.

4.7. Polymerase Chain Reaction (PCR)

Depending on experiment purposes, DNA amplifications were carried out in thermocyclers (BioRad, Applied Biosystems) with different polymerases: EmeraldAMP® GT PCR (Takara), DreamTaq Green (Thermo Scientific), NEBNext® High-Fidelity (New England BioLabs) or BioTaq™ (Bioline) following the manufacturer's instructions. Reaction setups (in 50 µL of final volume) and cycling conditions for every polymerase are specified in Table 12.

Table 12. DNA amplification conditions for PCR.

Master mix	Reaction Setup	Cycling conditions
EmeraldAMP® GT PCR	25 µL Master Mix 0.2 µM Primer 1 0.2 µM Primer 2 ≤ 500 ng DNA	98 ºC (1 min) 98 ºC (10 s) T_m (30 s) 72 ºC (1 min/kb) 72 ºC (7 min) 4 ºC (∞)
DreamTaq Green	25 µL Master Mix 0.5 µM Primer 1 0.5 µM Primer 2 10 pg - 1µg DNA	95 ºC (1 min) 95 ºC (30 s) T_m (30 s) 72 ºC (1 min/kb) 72 ºC (5 - 15 min) 4 ºC (∞)
NEBNext® High-Fidelity	25 µL Master Mix 0.5 µM Primer 1 0.5 µM Primer 2 1 ng – 1µg gDNA or 1 pg – 1 ng pDNA	98 ºC (30 s) 98 ºC (5 - 10 s) T_m (10 - 30 s) 72 ºC (20 – 30 s/kb) 72 ºC (2 min) 4 ºC (∞)
BioTaq™	5 units BioTaq (1 µL) 0.2 µM Primer 1 0.2 µM Primer 2 1x NH_4 reaction buffer 3 mM $MgCl_2$ solution 1 mM dNTPs 100 ng - 1 µg DNA	95 ºC (5 min) 95 ºC (30 s) T_m (30 s) 72 ºC (30 s/kb) 72 ºC (10 min) 4 ºC (∞)

4.8. DNA sequencing

Amplification and sequencing of *cyp51A* from gDNA was performed with the Oligo7- designed primers specified (see section 4.4.2) and the PCR cycling conditions stated in Table 12 (section 4.7). PCR products were visualized after electrophoresis in agarose gels (see section 4.6) and precipitated to discard any possible contaminants that could have affected the sequencing reaction (as explained in section 4.5.1).

Sequencing was carried out at Macrogen Spain (Macrogen, Inc) and resulting sequences assembly and analyses were performed as stated in section 4.4.1.

4.9. Gene expression analysis

4.9.1. RNA reverse transcription (RT-PCR)

Purified RNA was reverse transcribed into cDNA using the iScript™ cDNA synthesis kit (BioRad) following the manufacturer's instructions.

Reactions comprised 1 µg of purified RNA, 0.1 µM of retrotranscriptase (RT) enzyme, 0.1 µM of buffer and RNAse-free water in a final volume of 20 µL. Retrotranscription conditions consisted of incubation at 25 ºC for 5 min and 46 ºC for 20 min with a final incubation at 95 ºC for 1 min to inactivate the reverse transcriptase enzyme. After cDNA synthesis, standard PCRs of the housekeeping *actA* (actin) gene were performed using 1 µL of cDNA as template in order to check its integrity.

4.9.2. Quantitative Real Time PCR (qPCR)

Gene expression analyses were carried out by quantitative real-time PCR using the synthetized cDNA diluted 1:1 in deionized steril water as template and fragments were amplified with the appropriated primer pair (see section 4.4.2).

Reactions were prepared in a final volume of 15 µL using the FastStart Universal SYBR Green Master Mix (Roche Diagnostics) or the TB Green ™ Premix Ex Taq ™ (Tli RNase H Plus) (Takara Bio) in 96-well plates (Applied Biosystems) and analyzed in a StepOne™ Plus Real-Time PCR system (Applied Biosystems). For every sample, three technical and biological replicates were performed.

Specific conditions for every case are specified in Table 13.

Table 13. qPCR amplification conditions.

Master mix	Reaction setup	Cycling conditions	
FastStart Universal SYBR Green Master Mix	7.5 µL Master Mix 6.9 µL cDNA 300 nM Primer 1 300 nM Primer 2	94 ºC (5 min) 94 ºC (30 s) T_m (30 s) 72 ºC (30 s) 80 ºC (20 s)	x 45
TB Green ™ Premix Ex Taq ™	7.5 µL Master Mix 6.6 µL cDNA 0.3 µL ROX 300 nM Primer 1 300 nM Primer 2	95 ºC (30 s) 95 ºC (5 s) T_m (30 s) 72 ºC (30 s)	x 45

In all cases, a melting curve was obtained after PCR completion to check amplification specificity under the following conditions: 95 ºC (15 s), 60 ºC (15 s) and 95 ºC (15 s).

Serial dilutions of cDNA were used as template in qPCR amplification reactions to assess the efficiency (E) of every amplification. The E value was obtained from the slope of the linear regression between the $\log_{10}$ of the template concentration and the Ct value according to the equation:

$$E=10^{\left(-\frac{1}{slope}\right)}.$$

Expression levels of the target genes were assessed by normalizing their cycle thresholds (Ct) with those of the housekeeping gene *actA*. Expression levels calculations were carried with the E corrected ΔCt method (Pfaffl 2001) in which the relative expression ratio is calculated from the E value for every gene of the study:

$$\text{Expression ratio} = \frac{E_{target}^{\ Ct\ target}}{E_{reference}^{\ Ct\ reference}}$$

In which:

- E_{target} : Efficiency for the target gene amplification
- $E_{reference}$: Efficiency for the reference gene amplification
- $Ct_{\ target}$: Target gene threshold cycle
- $Ct_{\ reference}$: Reference gene threshold cycle

4.10. Genetic transformation

Single gene deletions of *cyp51A* and *cyp51B* were generated by the CRISPR-Cas9 technique in different *A. niger* and *A. tubingensis* strains (FMRs 15441, 14712, 14635 and 15388). To that purpose, the *in vitro* knock-out strategy (Figure 17) was based on the use of a repair template

(HygR) flanked by microhomology regions adjacent to *cyp51A* or *cyp51B* assembled with dual Cas9 ribonucleoproteins (RNPs), as previously described (Al Abdallah *et al.* 2017) .

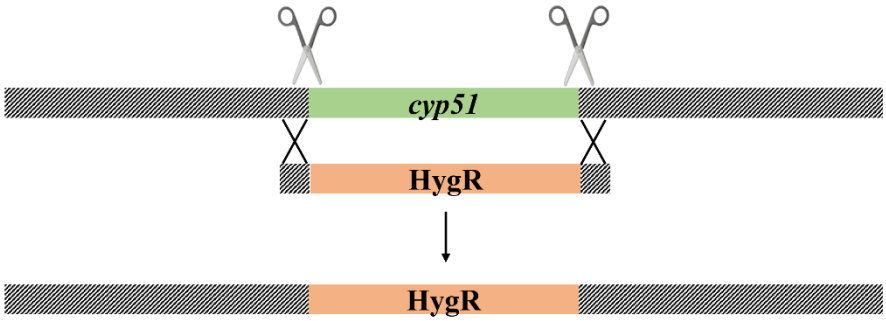

Figure 17. CRISPR-Cas9 gene deletion strategy.

In this system, the Cas9 enzyme (DNA nuclease) forms an RNP complex with a guide RNA (gRNA), which consists of a CRISPR RNA (crRNA) and a transactivating CRISPR RNA (tracrRNA) duplex. The crRNA contains a 20-base sequence named "protospacer," which facilitates DNA cleavage by binding to complementary regions in the target sequence. DNA cleavage is only achieved if the protospacer is followed by a Protospacer-Adjacent Motif (PAM) in the target sequence, which consists of an NGG motif. In addition, the crRNA contains a 22-base stretch for binding the tracrRNA, which regulates crRNA maturation and the DNA cleavage by crRNA-bound Cas9.

4.10.1. Design and assembly CRISPR-Cas9 gRNAs

To delete the entire coding sequence of *cyp51A* or *cyp51B*, two crRNAs to direct Cas9 cleavage within the 5' untranslated region (UTR) and the 3' UTR of *cyp51A* and *cyp51B*, respectively were designed for each species and target gene (Table 9).

To do so, two PAMs consisting of NGG motifs were identified and located upstream and downstream of the *cyp51* start and stop codons respectively. crRNA sequences consisted of the sequences comprising the 20 nucleotides upstream each PAM site. To check the specificity of each sequence, a BLAST was performed to ensure that protospacers did not display more than 15 bp identities to off-target (yet PAM-adjacent) regions of the genome (Cho *et al.* 2014).

Guide RNA (gRNA) was built by mixing in a PCR tube 5 µL of the crRNA, 5 µL of the tracrRNA and 5 µL of duplexing buffer (IDT). Tubes were incubated at 95 ºC for 5 min, allowed to cool down at room temperature, and kept on ice if going to be used or stored at -20 ºC until use.

4.10.2. Amplification of the HygR repair template

A repair template consisting of hygromycin B phosphotransferase expression cassette (HygR), was used to replace the cleaved *cyp51A* or *cyp51B* genes, which was also used as the selectable marker for the CRISPR-Cas9-mediated gene deletion.

The HygR template was amplified from plasmid pUCGH (Langfelder *et al.* 2001) using the primers stated in Table 8. The obtained PCR fragments were purified from agarose gels (as explained in section 4.6.2) and used as the repair templates with microhomology regions targeting the *cyp51A* or *cyp51B* genes.

4.10.3. Protoplast obtention

Transformation was performed in fungal cells with debilitated cell walls known as protoplasts. Protoplasts were obtained following a

previously described protocol (Arentshorst *et al.* 2012) with some modifications. Specifically, $1.2x10^6$ conidia/mL were inoculated into 100 mL of YG medium and incubated for 14 h at 28 ºC and 180 rpm. Then, hyphae were harvested by pouring through sterile myracloth and washed with sterile deionized water poured over the hyphal mat. Subsequently, hyphal mats were transferred to 50 mL Falcon tubes and 20 mL of protoplasting solution[8] in OM[9] was added to each tube. Enzymatic treatment was performed by incubation at 32 ºC and 75 rpm for 2 h. During the incubation, protoplast formation was monitored once or twice by removing aliquots to visualize at the microscope and clumps were broken up by gently pipetting up and down. When cultures contained adequate number of quality protoplasts, 10 mL of trapping buffer[10] were overlayed and tubes were centrifuged for 15 min at 3500 rpm and 4 ºC with no centrifuge break. A thin white layer of protoplasts formed at the interface of the two layers, which was transferred to a new Falcon tube. Next, 3 volumes of STC[11] were added to the isolated protoplasts and tubes were centrifuged for 10 min at 3500 rpm and 4 ºC. Resulting supernatant was poured off and protoplasts were resuspended in STC until a barely opaque suspension was obtained (approximately $5x10^7$ conidia/mL). Protoplasts were kept on ice at all times.

[8] **Protoplasting solution**: sterile filtered 5 % (w/v) commercial fungal cell wall lytic enzymes Extralyse (Laffort) in OM solution.
[9] **OM (Osmotic Media)**: sterile filtered 1.2 M $MgSO_4 \cdot 7H_2O$, 10 mM sodium phosphate buffer, adjusted to pH 6.5.
[10] **Trapping buffer**: sterile filtered 0.6 M sorbitol and 0.1 M Tris-HCl, adjusted to pH 7.
[11] **STC**: sterile filtered 1.2 M sorbitol, 10 mM $CaCl_2$ and 10 mM Tris-HCl, adjusted to pH 7.5

4.10.4. RNP complex formation

RNP complex was formed by mixing 1.5 µL of 5'gRNA, 1.5 µL of 3'gRNA, 1.5 µL of Cas9 (diluted 1:10 in working buffer) and 22 µL of working buffer in a 1.5 mL tube. Cas9 and working buffer were commercially obtained from IDT.

Finally, tubes were incubated at room temperature for 5 min to allow the complex to form.

4.10.5. Protoplast transformation and transformants selection

In the same tube where the RNP complex was formed (section 4.10.4.), 200 µL of the obtained protoplasts (section 4.10.3.), 2 µg of the repair template PCR product (section 4.10.2) and 25 µL of PEG-$CaCl_2$[12] were added and incubated on ice for 50 min. Next, the mixture was transferred to a culture tube and 1.25 mL of PEG-$CaCl_2$ were added. Samples were incubated at room temperature for 20 min and after the incubation volume was brought up to 3 mL with STC. Finally, 500 µL of the transformation mixture was plated onto 6 SMM plates, which were incubated overnight at room temperature to allow protoplasts' regeneration and expression of the resistance gene.

Subsequently, 10 mL of SMM top agar[13] containing hygromycin at a final concentration of 150 µg/mL was overlayed and allowed to solidify. Plates were then incubated at 30 ºC until colonies were visible.

[12] **PEG-$CaCl_2$**: sterile filtered 6g PEG 3350 in 4.5 mL Tris-HCl and 0.5 mL of 1 M $CaCl_2 \cdot 2H_2O$.
[13] **SMM top agar**: SMM medium with 0.7 g/L agar.

Growing colonies were picked and transferred to new SMM plates supplemented with 150 µg/mL of hygromycin for the obtention of single pure colonies. Plates were again incubated at 30 ºC until colonies were grown.

4.10.6. Verification of mutant strains

Once single colonies were obtained from the selective media plates, genomic DNA was isolated as explained in section 4.5.1. and amplified by different PCRs using the primers in Table 10 to verify the correct substitution of the target gene by homologous recombination with the resistance repair template (HygR).

4.11. Antifungal susceptibility testing

In vitro susceptibility testing was carried out according to the CLSI protocol M38 3rd Edition for broth microdilution (CLSI 2017).

Stock solutions of ITC (Janssen Pharmaceutica), PSC (Schering-Plough Research Institute) and VRC (Pfizer) were prepared in dimethyl sulfoxide (DMSO) (Table 14). Antifungal stocks were prepared to a concentration 100 times higher (100x) than the highest assayed concentration and after, 2-fold dilutions performed in DMSO. Finally, all drugs were diluted 1:50 in RPMI 1640 medium and 100 µL of each drug concentration to be assayed were dispensed onto rows in 96-well microdilution trays with rounded bottom. The last two wells of every row consisted of drug-free RPMI 1640 destined to be growth control and sterility control (Figure 18). Trays were covered with an adherent cover

and stored at -20 ºC until use for a maximum of 6 months to ensure the drugs potency.

Table 14. Antifungal drugs for *in vitro* susceptibility studies.

Antifungal	Manufacturer	Solvent	Purity
ITC	Janssen Pharmaceutica	DMSO	100 %
PSC	Schering-Plough Research Institute	DMSO	98 %
VRC	Pfizer	DMSO	100 %

Inocula were prepared as previously stated (see section 4.3.1) and adjusted by hemocytometer count to $5x10^6$ conidia/mL. These suspensions were then diluted 1:50 in RPMI 1640 medium and 100 µL of the final inocula were dispensed into each well, with the exception of the sterility control well, in which 100 µL RPMI 1640 were dispensed (Figure 18). The *A. flavus* strain ATCC 204304 was used as the quality control strain. Conidia concentration and viability were verified by culturing an aliquot of the inoculum onto PDA plates.

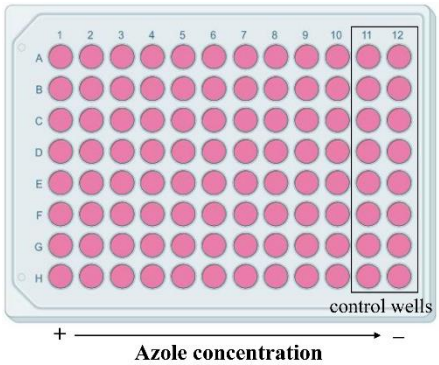

Figure 18. Microplate setup for azole susceptibility testing.

Microplates were incubated for 48 h at 35 ºC without light nor agitation and MICs were determined by direct visualization with an inverted mirror. MICs for the quality control strain were assessed in the first place to check whether susceptibility values were within the ranges stablished by the CLSI protocol and thus ensure microplates' quality. MICs corresponded to the lowest drug concentration that completely inhibited fungal growth. Strains were classified as wt and non-wt according to the currently established

ECVs for ITC (4 µg/mL), PSC (2 µg/mL), and VRC (2 µg/mL) and section *Nigri*, since no clinical breakpoints are available yet for *Aspergillus* spp. (Espinel-Ingroff & Turnidge 2016). Strains displaying ITC, PSC, or VRC MICs above the ECVs were categorized as non-wt, while those displaying MICs below the ECVs were categorized as wt.

4.12. Sterol extraction and quantification

Total sterol extraction was performed as previously described (Breivik & Owades 1957) with modifications. Cultures were prepared and carried out as explained in section 4.3.2. in both, presence and absence of VRC, and the collected mycelia was lyophilized with the AdVantage Freeze Dryer (The VirTis Company Inc). Finally, mycelia were ground with a grinder and total dry weight was determined.

Then, 60 mg (dry weight) of each sample were placed into borosilicate glass screw-cap tubes, 6 mL of 25 % alcoholic potassium hydroxide (KOH/EtOH) solution [14] were added to each tube and vortex mixed for 1 min. Samples were incubated at 85 ºC for 1 h in a water bath, and after allowing them to cool at room temperature, sterols were extracted by the addition of 2 mL of sterile distilled water and 6 mL of n-heptane. Samples were vortex mixed again for 3 min and the upper-layer, consisting of the n-heptane layer, was transferred to a new borosilicate glass screw-cap tube and stored at -20 ºC for a maximum of 20 h.

Sterol quantification was spectrophotometrically determined as described elsewhere (Arthington-Skaggs *et al.* 1999) in the range of 240 to

[14] **KOH/EtOH**: 25 g of KOH and 35 mL of sterile distilled water brought to 100 mL with 100 % ethanol.

300 nm with a NanoDrop2000 spectrophotometer (Thermo Fisher). Presence of ergosterol and the intermediate sterol 24(28) dehydroergosterol (DHE) in the extracted samples resulted in four-peaked curves.

As both, ergosterol and 24(28)DHE absorb at 281.5 nm, but only 24(28)DHE shows intense absorption at 230 nm, the amount of ergosterol can be determined by subtracting the 24(28)DHE content from the total amount of absorption. Thus, ergosterol content was calculated as a percentage of the dry weight using the following equations:

$$\%\ \text{ergosterol} + \%\ 24(28)\text{DHE} = \frac{\frac{A_{281.5}}{290}}{\text{dry weight}}$$

$$\%\ 24(28)\text{DHE} = \frac{\frac{A_{230}}{518}}{\text{dry weight}}$$

$$\%\ \text{ergosterol} = [\%\ \text{ergosterol} + \%\ 24(28)\text{DHE}] - \%24(28)\text{DHE}$$

In which:

- $A_{281.5}$: Maximum absorbance of ergosterol + 24(28)DHE
- A_{230} : Maximum absorbance of 24(28)DHE
- 290 and 518: molar extinction coefficients (in percentage per cm) for crystalline ergosterol and 24(28)DHE, respectively.

4.13. Cell wall and membrane stress studies

4.13.1. Cell wall and membrane characterization

For membrane and cell wall stress assays, 10^6 conidia/mL suspensions (starting inoculum) were serially diluted to obtain 10^5 and 10^4 conidia/mL.

Next, each inoculum concentration was spotted onto SM agar supplemented with the indicated agents when required.

To qualitatively assess cell wall composition and integrity of section *Nigri* strains, SM was supplemented with the cell wall disturbing agents Calcofluor White (CFW) at a final concentration of 200 μg/mL, Congo Red (CR) at 400 μg/mL or Caffeine at 20 mM. Membrane disturbing agents Sodium dodecyl sulfate (SDS) 0.01 % and ethylenediaminetetraacetic acid (EDTA) 50 mM were used as SM supplements in order to evaluate membrane fluidity and permeability. All plates were incubated at 28 ºC and growth was assessed for every strain and condition.

In addition, membrane and cell wall assays were quantitatively performed following a recently published method (Cánovas *et al.* 2017). Briefly, SM and SM supplemented with the indicated agents as indicated above, were placed in 96-wells plates. Afterwards, 30 μL of a 10^6 conidia/mL suspension of every strain were inoculated, and plates were incubated at 28 ºC to assess fungal growth. Growth quantification was carried out by 595 nm absorbance reading at different time intervals in a Tecan Sunrise™ microplate reader.

4.13.2. Conidia resistance to disruption

Resistance of conidial walls to mechanical disruption was evaluated as elsewhere described (Valsecchi *et al.* 2017). Specifically, 500 μL of 10^7 conidia/mL suspensions of every strain were placed in 2 mL tubes with glass beads of 425 -600 μm (Sigma). Samples were subjected to disruption in a MP Biomedicals™ FastPrep ™ cell breaker (MP Biomedicals) for 1 min and resulting suspensions were plated on PDA, which were incubated at 30 ºC until visible colonies could be counted. In parallel, non-disrupted

suspensions equally prepared and plated were used as controls to allow percentage of conidia survival calculation.

4.14. Extracellular protein manipulation

4.14.1 Protein concentration

Supernatants obtained from cultures in VRC induction were obtained as explained in section 4.3.2 and transferred to dialysis tubing cellulose membranes with pore diameter of 14 kDa (Sigma) to retain proteins of greater molecular weight, such as laccases. Samples were dialyzed as depicted in Figure 19 at 4 ºC with 5 L of cold distilled water with gentle agitation for 72 h (with water changes every 8 h or 24 h). Subsequently, membranes were kept in external contact with polyethylene glycol (PEG) 35000 (Sigma) (Di Pietro & Roncero 1996) for 6 - 8 h. Then, they were carefully washed with cold distilled water and dialyzed for an additional 1,5 - 2 h to remove all possible PEG leftovers.

Figure 19. Protein dialysis scheme.

To avoid possible proteolysis during the extraction and all the steps of protein purification, protease inhibitors were added to avoid internal degradation of the protein extracts. Specifically, 1 mM of phenylmethylsulfonyl fluoride (PMSF)[15] (Sigma) and 1 % (v/v) of protease inhibitor cocktail 5 (PanReac Applichem, ITW reagents). Extracts were stored in 2 mL tubes at -20 ºC until used.

[15] **PMSF**: 100 mM of PMSF dissolved in ethanol.

4.14.2. Protein quantification

Proteins in the extract were quantified by the Bradford method using Bovine Serum Albumin (BSA) as standard with the Bradford Protein Assay Kit (Takara bio) following the manufacturer's instructions. In this method, a BSA solution was diluted to different concentrations (from 0 to 1000 µg/mL) and 4 µL of dilution were added to 200 µL of pre-warmed Bradford Dye Reagent. After an incubation for 5 min at room temperature, absorbance was measured with NanoDrop2000 spectrophotometer (Thermo Fisher) at 595 nm and a standard curve with the absorbance values and the BSA concentrations was generated.

Protein concentration determination in the dialyzed extracts was achieved following the same technical approach and extrapolating concentration values from the standard curve previously generated.

4.14.3. Laccase activity assays

Laccase activity in every extract was assessed by an enzymatic assay previously described (Palmieri *et al.* 1997; Weenink *et al.* 2006) with modifications. For every sample, the assay mixture contained 5 µg of total protein and 2 mM of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma) in 0.1 M sodium acetate buffer at pH 3 (final volume = 400 µL). ABTS oxidation by laccases was monitored by incubating all samples at 30 ºC and measuring the absorbance at 414 nm in a NanoDrop2000 spectrophotometer (Thermo Fisher) at different time points.

Additionally, laccase activity was assessed by spotting $5x10^4$ conidia in the centre of PDA plates supplemented with 20 g/L of bromophenol blue

(which is a laccase substrate) and different concentrations of VRC (0, 0.25, 1 and 2 μg/mL). Plates were incubated for 5 days at 30 ºC and scanned in order to measure colony diameter and clear halos around laccase producing colonies. Normalization of colony diameter and clear halo was performed.

4.15. Macrophage infection

The virulence of three section *Nigri* strains (FMRs 11900, 14712 and 11898) with different VRC susceptibilities was evaluated with the macrophage-like cell line J774A.1. This cell line was maintained in tissue culture flasks as explained in section 4.2.2.

For the virulence assessment, previous protocols (Slesiona *et al.* 2012; Wasylnka & Moore 2003) were followed with some modifications. Macrophages were scraped from the tissue culture flasks, seeded into 6-well culture plates with complete DMEM medium and incubated at 37 ºC and 5 % CO_2 for 24 h approximately until they reached a confluence of 100 % ($1,2x10^6$ cells/well). Before the infection, $6x10^5$ conidia/mL suspensions were prepared in non-supplemented DMEM and incubated for 2h at 37 ºC and 180 rpm to allow conidial swelling. In parallel, suspensions of $6x10^5$ conidia/mL were inoculated into YG and incubated for 1,5 h at 37ºC and 180 rpm, after which VRC was added at a concentration of half the MIC for every strain and further incubated at 37ºC and 180 rpm for 1 h. Finally, VRC-exposed conidia were centrifuged, washed with sterile distilled water and resuspended in non-supplemented DMEM.

For the macrophage infection, growth medium from the cells was discarded, and macrophages were infected with 2 mL of the conidia suspensions at a MOI (Multiplicity Of Infection) of 1. Infection was carried

out at 37 ºC and 5 % CO_2 for 4 h. Subsequently, DMEM media from the culture plates was removed, wells were carefully washed with PBS twice to remove non-adherent macrophages and cells were lysed with 0.1 % triton in cold sterile deionized water. Lysates were diluted and plated on PDA plates for viable fungal cell count. All experiments were performed in triplicate.

In addition, to assess possible differences in conidia internalization among strains and/or conditions samples were taken from the cell culture plates after the infections and placed onto slides, which were analyzed by microscope visualization to count macrophages with 0 to 6 internalized conidia.

4.16. Statistical analyses

The GraphPad Prism 6.0 software for windows was used to perform all statistical analyses. P values ≤ 0.05 were considered statistically significant.

Relative gene expression values, ergosterol and 24(28)DHE contents as well as conidial resistance to physical disruption results were analyzed with the Mann-Whitney U T-test. In this nonparametric test, data from each group is arranged and compared by assigned ranges, without comparing medians or means.

Differences in fungal viability and conidia internalization after macrophage infection were analyzed with a two-way ANOVA considering strain-related differences and VRC exposure, since this test is used to determine how a specific response is affected by two different factors.

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Alba Pérez Cantero

RESULTS

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5.1. Azole resistance mechanisms in *Aspergillus*: update and recent advances

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Review

Azole resistance mechanisms in *Aspergillus*: update and recent advances

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ABSTRACT

Aspergillus fumigatus is the main causal agent of invasive aspergillosis (IA), however other species of the genus can also cause IA, such as *Aspergillus flavus, Aspergillus terreus, Aspergillus niger* and related cryptic species. This infectious disease mainly affects immunosuppressed patients and is linked to elevated mortality rates. As voriconazole is the treatment of choice for this condition, the relevant increase in the number of azole-resistant isolates in recent years has gathered alarming attention, as it also translates into an increase in clinical failures. In this review, we summarise and discuss the azole resistance molecular data described to date in the most clinically prevalent sections of *Aspergillus*, including mechanisms involving the target proteins Cyp51 and ATP-binding cassette (ABC) or major facilitator superfamily (MFS) efflux pumps. Other resistance mechanisms proposed but not yet fully characterised are also discussed.

1. Introduction

Aspergillus is a broad fungal genus comprising more than 300 different species distributed ubiquitously worldwide. Several species of the genus are used biotechnologically in the medical and industrial fields owing to their ability to produce important metabolites. However, *Aspergillus* is also responsible for important economic losses as it can negatively affect crops. The genus is currently organised in different subgenera and sections [1,2], with the sections *Fumigati, Flavi, Terrei* and *Nigri* being the most clinically relevant due to their great impact as human opportunistic pathogens [1].

Aspergillus infections show a wide range of clinical manifestations, including infections that drive hypersensitivity reactions such allergic *Aspergillus* sinusitis [3] and allergic bronchopulmonary aspergillosis [4], infections via the skin that result in cutaneous aspergillosis [5] and aspergilloma and chronic conditions such as chronic pulmonary aspergillosis [6]. The most problematic pathology in terms of patient outcome and disease management is invasive aspergillosis (IA), a life-threatening condition that, although it has been reported to affect immunocompetent patients on rare occasions [7,8], mainly affects immunocompromised patients such those suffering from cancer, solid and haematological malignancies as well as those undergoing chemotherapy, corticosteroid treatment or transplantation [9,10]. The presentation of the disease generally differs between neutropenic and non-neutropenic patients. IA in non-neutropenic patients is characterised by few symptoms such as fever, cough and chest pain, whilst pneumonia and higher fatality rates are observed in neutropenic patients suffering from IA [11].

Scarce data are available on the epidemiology of IA. Although *Aspergillus fumigatus* continues to be considered the most frequent causal agent of the condition [12], rates of infection due to other emerging species are uncertain and probably underestimated as a result of inaccurate molecular identification. Nonetheless, it has been reported that cryptic species belonging to the sections *Flavi, Nigri* and *Terrei* are nowadays also frequently isolated from IA patients [12–15]. In general, approximately 200 000 cases of IA are estimated to occur every year worldwide, which may account for only approximately one-half of actual cases as a direct consequence of misdiagnosis. In fact, lack of accuracy in diagnosis or treatment strategy results in IA mortality rates varying from 50% to 100 % [16].

As currently stated in the aspergillosis management guidelines, the recommended treatment for IA is voriconazole (VRC); alternative treatments consist of liposomal amphotericin B and isavuconazole. Other treatments used as salvage therapy are amphotericin B lipid complex, caspofungin, micafungin, posaconazole (PSC) and itraconazole (ITC). In addition, PSC and VRC constitute the recommended prophylactic measures for aspergillosis prevention [10].

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Specifically, azoles have been described to target the cytochrome P450 sterol 14α-demethylase enzyme (Cyp51) by non-competitive binding, causing inhibition of the demethylation of ergosterol precursors and thus blocking ergosterol biosynthesis [17]. However, a few studies suggest that azoles act as competitive inhibitors of Cyp51 instead, as their interactions consist of reversible and competitive binding to iron of the heme group and residues in its close proximity [18,19]. In any case, the result is the accumulation of 14-methylated sterols that cause alterations in membrane fluidity and its final disruption, which in turn reduces the activity of membrane-bound enzymes and leads to inhibition of cell growth and proliferation. Hence, it is believed that azoles may disrupt the role of ergosterol in stimulating growth and proliferation [17,18,20,21].

Nevertheless, the incidence of azole-resistant *Aspergillus* isolates has increased alarmingly in recent years, which is thought to result from the use of azoles both in clinical and agricultural settings, directly contributing to therapeutic failures [22–26].

The aim of this review is to compile azole resistance data described to date in the most prevalent sections of *Aspergillus*.

2. Azole resistance

Azole resistance is the ability of fungal strains to overcome doses of azole drugs that exert antifungal activity in susceptible isolates. The threshold values that distinguish resistant from susceptible strains are established through determination of the Minimum Inhibitory Concentration (MIC) of a drug against a broad range of strains. Specifically, MIC distributions together with pharmacokinetic/pharmacodynamic profiles allow the establishment of Clinical BreakPoints (CBPs), which are used as predictors to anticipate treatment effectiveness in patients.

In the case of *Aspergillus* spp., different reference methods have been established to characterise isolates on the basis of their antifungal susceptibility, such those developed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI) [27–30].

Whereas some CBPs have been established for a few antifungals against *Aspergillus* spp. by the EUCAST method [31], there are no available CBPs set by the CLSI method, although it does provide Epidemiological Cutoff Values (ECVs) for *Aspergillus* instead [32,33]. ECVs categorise isolates into wild-type (WT) or non-wild-type (non-WT), with the latter being indicative of decreased susceptibility to a particular antifungal agent. This reduction in susceptibility can be linked to potential acquired resistance mechanisms, although, unlike CBPs, it cannot be considered a predictor of a patient's response to therapy [34].

To decipher the mechanisms responsible for antifungal resistance, it is essential to consider the effect of the drug within the cell. Specifically, azoles exert an inherent antifungal potency by binding the Cyp51 protein. Nevertheless, the precise physiological effects derived from Cyp51 inhibition on fungal cell biology remain unclear, and the specifics of the fungicidal effect of azoles in *Aspergillus* are currently under study [35].

Traditionally, researchers have considered two fundamental aspects regarding the nature of azole resistance in *Aspergillus*: the relevance of polymorphisms in Cyp51 proteins, which are related to a decrease in azole affinity; and the transcriptional response of the fungus, which appears to be crucial for fungal adaptation to azole stress [36]. On this basis, the most studied molecular mechanisms contributing to the appearance of resistant phenotypes in *Aspergillus* can be classified into: (i) alterations in the Cyp51 protein that reduce the affinity between the azole drug and its target; (ii) overexpression of the target enzyme, which increases the azole concentration necessary to inhibit fungal growth; and (iii) upregulation of efflux pump systems to decrease the intracellular drug concentration. However, other mechanisms, including biofilm formation, cellular stress response, drug enzymatic degradation, and activation of alternative pathways to bypass the effects of the drug, have also been proposed to contribute to antimicrobial resistance [25,37] (Fig. 1).

2.1. cyp51 genes

Among the studied resistance mechanisms in *Aspergillus*, the Cyp51 enzyme encoded by the *cyp51* gene (*ERG11* in yeasts) is the major candidate responsible for azole resistance at the molecular level. Remarkably, many filamentous ascomycetes have undergone gene duplication as occurred in *Aspergillus*, which contains varying numbers of *cyp51* paralogues among species located on different chromosomes throughout the genome. Whilst some species such *A. fumigatus, Aspergillus nidulans* and *Aspergillus niger* present two paralogues (*cyp51A* and *cyp51B*), others species such as *Aspergillus flavus* and *Aspergillus oryzae* carry three paralogues (*cyp51A, cyp51B* and *cyp51C*) [20,25]. In addition, *Aspergillus terreus* and section *Nigri* species *Aspergillus carbonarius* also appear to display three *cyp51* paralogues, which has not been reported so far to our knowledge (accession nos. of these proteins are XP_001218650 and OOF93749, respectively) (Fig. 2).

Interestingly, Cyp51 enzymes share a common ancient origin and, assuming that the different *cyp51* forms diverged upon evolution, we find similar sequence identities among them in *Aspergillus*. However, they are evolutionarily differentiated into two lineages of paralogous proteins (Cyp51A and Cyp51B). The third paralogue (Cyp51C) appears to have originated from duplication of both genes (*cyp51A* or *cyp51B*) depending on the species (Fig. 2). In this sense, some authors have proposed that *cyp51* duplications derive from evolutionary mechanisms that govern azole toxicity adaptation [38,39]. As gene duplications are a great source of genetic adaptive potential, if selection favoured cells with additional copies of *cyp51* to have better growth under selective conditions, we should expect those strains with two or more copies of the gene to have greater tolerance to azoles than those with only one copy. Nevertheless, this hypothesis will need to be corroborated by future studies.

Moreover, functional analyses have demonstrated that both enzymes share the same substrate and display comparable functions. This has been observed by heterologous expression of *A. fumigatus cyp51A* and *cyp51B* in a *Saccharomyces cerevisiae cyp51*-defective mutant, which resulted in effective complementation in terms of ergosterol content and azole tolerance [40]. In the specific case of *cyp51A*, its heterologous expression in a *cyp51*defective strain of *S. cerevisiae* caused a decrease in azole susceptibility, although not for all of the azoles tested [40]. These findings have been reinforced by further small interfering RNA (siRNA) silencing studies in which *cyp51A* silencing has been seen to increase azole susceptibility in a non-WT *A. fumigatus* strain [41]. By contrast, in the case of *cyp51B*, neither its deletion nor its heterologous expression has any effect on azole susceptibility [40,42] (Table 1).

Although it has been demonstrated that neither of the two *cyp51* forms is individually essential in *A. fumigatus*, the lack of both genes has a lethal result in this organism [42]. Curiously, deletion of *cyp51A* increases azole susceptibility without altering *cyp51B* expression levels [43], meaning that *cyp51* redundancy does not lead to genetic compensation by transcriptional adaptation.

As reviewed elsewhere, for those species carrying an additional *cyp51C* copy of the gene, *cyp51A* characteristics could be displayed by *cyp51C* instead [25,39]. However, a recent study performed on section *Flavi* appears to indicate that 14α-demethylase activity is largely contributed by *cyp51A* and *cyp51B* in this section, since basal expression of *cyp51C* was extremely low or undetectable in

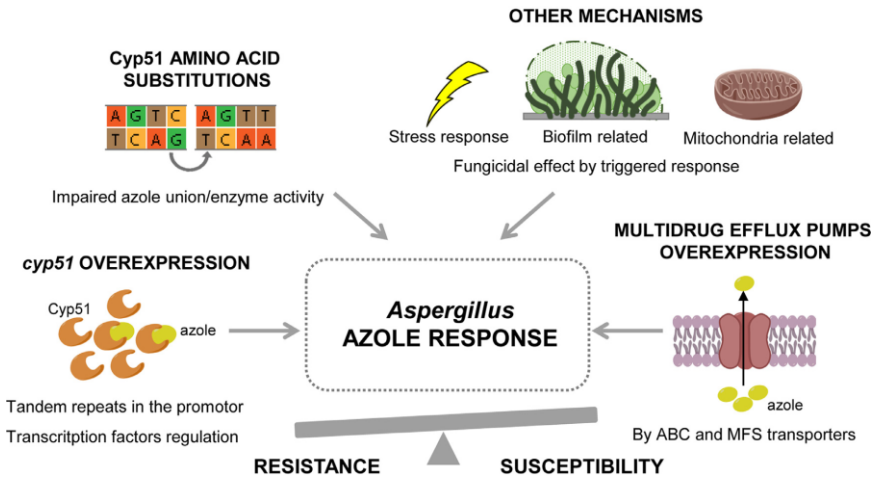

Fig. 1. Summary of azole resistance mechanisms described in *Aspergillus* spp. ABC, ATP-binding cassette; MFS, major facilitator superfamily.

Table 1
Reported genes in *Aspergillus fumigatus* involved in azole resistance.

Gene	Accession no.	Mutant phenotype(s)	Expression studies
Ergosterol synthesis			
cyp51A	Afu4g06890	Increased azole susceptibility without *cyp51B* expression alteration [43]	Induced by azoles [18] Overexpression reduces azole susceptibility [76] Silencing by siRNA increases azole susceptibility [41]
cyp51B	Afu7g03740	No effect on azole susceptibility [42]	Not induced by azoles [18]
ABC transporters			
cdr1B (*abcB, abcC, atrG*)	Afu1g14330	Large increase in azole susceptibility [67,100,101]	Upregulated by VRC [102]
mdr1 (abcA')	Afu5g06070	No effect on in vitro azole susceptibility [67] *mdr1* overproduction increases in vivo azole tolerance [100]	Highly induced by VRC and ITC and occasionally overexpressed in non-WT strains [100,102,103]
mdr2	Afu4g10000	No effect on azole susceptibility [67]	Overexpressed in non-WT strains [103]
mdr3	Afu3g03500	No effect on azole susceptibility [67]	Overexpressed in non-WT strains [51,103]
mdr4	Afu1g12690	No effect on azole susceptibility [67]	Occasionally upregulated by ITC [51,103]
abcD	Afu6g03470	–	Upregulated by VRC [102]
abcE	Afu7g00480	–	Upregulated by VRC [102]
atrI	Afu3g07300	–	Higher expression in non-WT strains [98]
atrF	Afu6g04360	No effect on azole susceptibility [67]	Overexpressed and upregulated by ITC in non-WT strains [98,105]
MFS transporters			
mdrA	Afu1g13800	Increased susceptibility to ITC and VRC [98]	–
mfs56	Afu1g05010	No effect on azole susceptibility [67]	Overexpressed in some non-WT strains but no general correlation with MICs [67]
mfsA	Afu8g05710	–	Upregulated by VRC [102]
mfsB	Afu1g15490	–	Upregulated by VRC [102]
mfsC	Afu1g03200	–	Upregulated by VRC [102]

siRNA, small interfering RNA; ABC, ATP-binding cassette; VRC, voriconazole; ITC, itraconazole; WT, wild-type; MFS, major facilitator superfamily; MIC, minimum inhibitory concentration.

contrast to the greater gene expression of *cyp51A* and *cyp51B* for the strains analysed [44]. Accordingly, further studies are needed to characterise the true involvement of each Cyp51 paralogue in the azole response in these species.

Taken together, the features displayed by these genes imply that *cyp51A* (or *cyp51C* in some species) encodes major enzymatic activity and at the same time has a greater influence in terms of azole response. As for *cyp51B*, it constitutes a functionally redundant enzyme that could have potential alternative functions yet to be defined [45].

2.1.1. Cyp51 protein sequence variations

At the structural level, Cyp51 enzymes are widely conserved proteins owing to their essential role in ergosterol biosynthesis and narrow substrate specificity. Therefore, alterations in *cyp51* gene sequences may occur in regions that do not compromise their functional activity [46]. However, polymorphisms in azole-binding amino acids that do not compromise Cyp51 activity could decrease the affinity of the enzyme towards these drugs [17]. Consequently, single point mutations in the *cyp51A* gene causing amino acid substitutions within the Cyp51A protein have emerged as the

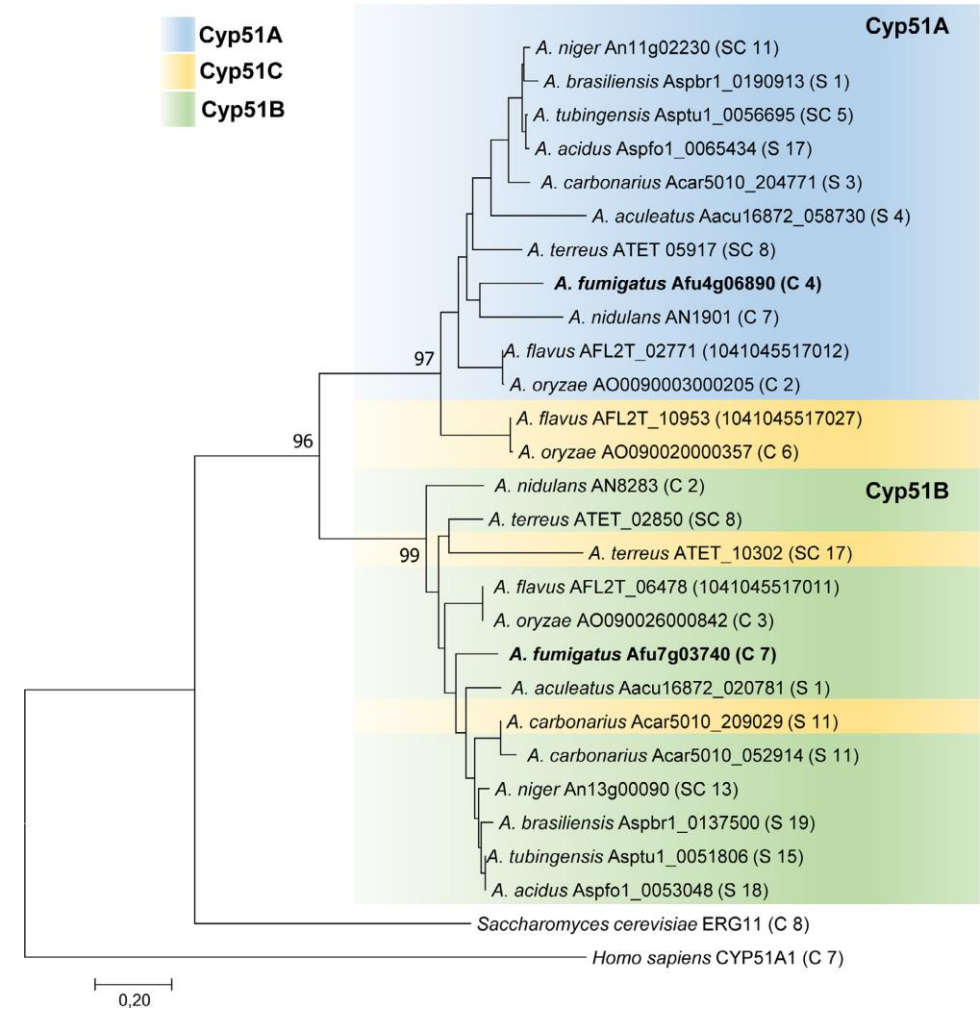

Fig. 2. Cyp51 phylogenetic tree of *Aspergillus* spp. Sequences were retrieved from the Joint Genome Institute (JGI) database and the *Aspergillus* Genome Database (AspGD). The phylogenetic tree was constructed using MEGA7 software. Genome localisation of the *cyp51* genes is indicated as SC (supercontig), S (scaffold) and C (chromosome).

major resistance-mediating mechanism in *Aspergillus*. The presence of such mutations might alter the structure, stability and functionality of Cyp51, thus hindering substrate recognition and eventually leading to different azole resistance patterns [45,47]. Screening of specific variants of the *cyp51* gene associated with increased resistance to azoles has led to extensive genotyping studies in which some *Aspergillus* spp. (mostly *A. fumigatus*) have been characterised in the search for potential mutations that could explain resistant phenotypes. It is worth mentioning, however, that some non-synonymous mutations within the *cyp51A* gene initially associated with resistance have later been reported in WT strains as well [48,49], suggesting that some association studies can lead to errors due to the small number of strains analysed.

So far, Cyp51A polymorphisms in *A. fumigatus* consisting of amino acid substitutions in positions G54, Y121, G138, P216, F219, M220, A284, Y431, G432, G434 and G448 have been reliably correlated with reduced azole susceptibility [50–64] (Table 2; Fig. 3). In contrast, no polymorphisms in Cyp51B appears to contribute to azole resistance in this species [25,39].

Whilst some point mutations in *A. fumigatus* Cyp51A have been validated as an important resistance mechanism, their role in azole susceptibility in other species of the genus remains unclear and poorly studied. In fact, reports on non-WT *Aspergillus* spp. isolates lacking *cyp51* mutations are abundant [53,65–67]. In this regard, a mutation (S240A) in the *A. flavus* Cyp51C protein initially reported to reduce VRC susceptibility [68] was later found both in WT and non-WT strains, thus leading to the hypothesis that it represented a geographical variation instead [69]. Furthermore, several mutations in the three Cyp51 enzymes (A, B and C) have been reported in *A. flavus* isolates with reduced VRC

Table 2
Reported amino acid changes in the Cyp51 protein with effects on azole susceptibility in *Aspergillus* spp.

Species	Substitution	Reduced susceptibility
A. fumigatus	G54R, -W, -E, -K[a]	ITC and/or PSC [50,51]
	Y121F[a]	VRC [52]
	G138C, -S[a]	ITC, PSC, VRC [53,59]
	P216L[a]	ITC, PSC [55,57,62]
	F219I, -C, -S[a]	ITC, PSC [53,61,62]
	M220V, -K, -T, -I[a]	ITC and/or PSC and/or VRC [53,54,58]
	A284T[a]	ITC, PSC, VRC [57]
	Y431C, -S[a]	PSC, VRC and/or ITC [55,59,63]
	G432S[a]	ITC [64]
	G434C[a]	ITC, PSC, VRC [55,59]
	G448S[a]	ITC, VRC [56,60]
	TR34/L98H[a]	ITC, PSC, VRC [80,84]
	TR46/Y121F/T289A[a]	VRC [85]
	TR53[a]	ITC, VRC [83]
A. flavus	Y132N/T469S[a]	VRC [65]
	K197N[a]	VRC [65]
	K197N/D282E/M288L[a]	VRC [65]
	R450S[a]	VRC [70]
	Q354K[b]	VRC [70]
	S196F/A324P/N423D/V465M[c]	VRC [70]
	Y319H[c]	VRC [69]
A. niger	V104I[a]	ITC [73]
	H382R[a]	ITC [73]
	I377V/S507I/L511M[a]	ITC, VRC [73]
A. tubingensis	L21F[a]	ITC and/or PSC [72,73]
	A9V/L21F/A140V/P413S/D505E[a]	ITC [73]
	T321A[a]	VRC [73]
	A185G/T321A/N327S/V422I/L492M/I503F/Q504P[a]	ITC, VRC [73]
A. terreus	M217T, -V, -I[a]	PSC and/or ITC [74,75]

ITC, itraconazole; PSC, posaconazole; VRC, voriconazole.
[a] Substitution in Cyp51A.
[b] Substitution in Cyp51B.
[c] Substitution in Cyp51C.

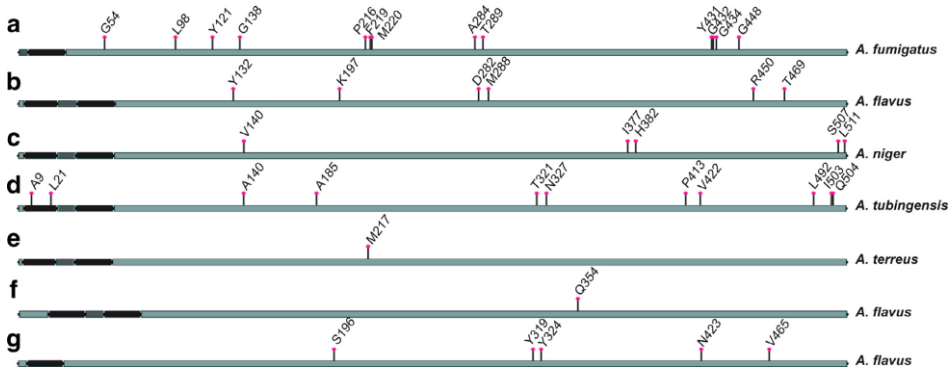

Fig. 3. Cyp51 amino acids involved in azole resistance: (a–e) amino acids in Cyp51A of *A. fumigatus* (a), *A. flavus* (b), *A. niger* (c), *A. tubingensis* (d) and *A. terreus* (e); and (f,g) amino acids in Cyp51B (f) and Cyp51C (g) from *A. flavus*. Conserved domains are highlighted: transmembrane domains associated with the endoplasmic reticulum (black) and cytoplasmic regions (grey).

susceptibility, although more studies are required to validate them. This is the case of amino acid changes R450S and K197N or the combinations K197N/D282E/M288L and Y132N/T469S, which have been found in the Cyp51A enzyme, whilst in the case of the Cyp51B protein only the variation Q354K has been reported. Finally, Y319H and the combination S196F/A324P/N423D/V465M have been identified in Cyp51C [65,69,70].

Doubts regarding the involvement of Cyp51 mutations in azole resistance arise in the case of section *Nigri*, as elevated azole MICs cannot be explained by polymorphisms in numerous cases of this section [71]. Reported Cyp51A mutations in *A. niger* and *Aspergillus tubingensis* appear to mostly account for reduced ITC and/or VRC susceptibility. In particular, the reported amino acid substitutions in *A. niger* consist of V104I and H382R and the combination I377V/S507I/L511M. In the case of *A. tubingensis*, L21F and T321A or the combinations A9V/L21F/A140V/P413S/D505E or A185G/T321A/327S/V422I/L492M/I503F/Q504P have been reported [72,73].

Studies on the azole resistance of section *Terrei* are scarce and, to date, only amino acid changes in Cyp51A position M217 have been described to have effects on ITC and PSC susceptibility [74,75].

The reported amino acid changes identified to date with effects on azole susceptibility are summarised in Table 2 and are presented in Fig. 3.

2.1.2. cyp51 overexpression

Another major mechanism hypothesised to be responsible for the acquisition of azole resistance in *Aspergillus* consists of *cyp51* overexpression. It is reasonable to believe that if azoles bind the Cyp51 enzyme, a greater abundance of these proteins within the cell could rescue the fungus from the inhibitory effect of the drugs. Therefore, differences in the expression profile of both genes could be a key aspect to understanding azole tolerance.

Despite the great similarity displayed by Cyp51 proteins, both isoenzymes diverge in their transcriptional regulation in *Aspergillus*. In general terms, whilst *cyp51A* expression appears to be inducible by azoles, the *cyp51B* gene maintains a constitutive pattern of expression in *A. fumigatus* [18]. Furthermore, the importance of *cyp51A* expression in azole resistance was proven by heterologous expression assays in which a decrease in *A. fumigatus* ITC susceptibility was conferred by the introduction of extra copies of the *A. nidulans pdmA* (*cyp51A*) gene [76]. However, it remains to be determined whether the same effect occurs for the *cyp51B* gene.

On this basis, one would expect a clear correlation between *cyp51A* expression and resistance. However, numerous studies present patterns of expression that do not fit this hypothesis. For instance, in a study where *A. fumigatus* clinical isolates did not show either *cyp51A* point mutations or *cyp51A* overexpression, *cyp51B* was overexpressed in one of the non-WT strains tested [77], suggesting that *cyp51B* is not constitutively expressed in all strains and that it could, unusually, be involved in azole response as well.

Regarding other species of the genus, *cyp51A* and *cyp51B* expression levels in *A. flavus* do not seem to be related to VRC resistance. As a matter of fact, the expression profiles of these genes barely vary among WT and non-WT strains, even after exposure to azoles [44,68]. Moreover, *cyp51C* displays a very low expression level compared with its paralogues and its expression is not induced by VRC [44]. This leads us to believe that sterol 14α-demethylase activity in this species is mainly due to the expression of *cyp51A* and *cyp51B* genes, even though *cyp51C* had been initially accepted to account for this [25,39].

Similarly, it has been shown that transcript levels of *cyp51A* and *cyp51B* cannot explain azole resistance in species from section *Nigri*. Specifically, *cyp51* basal expression does not appear to be related to azole susceptibility [73,78] and, despite the fact that *cyp51A* is upregulated after azole exposure, *cyp51B* displays a low constitutive expression in contrast [78]. This seems to indicate that Cyp51A is the major player in azole response in this section, although expression profiles do not show a correlation with azole susceptibility.

In addition, there are specific cases in which overexpression of *cyp51* occurs along with other factors also associated with resistance, making it difficult to establish reliable associations. In fact, overexpression of the *cyp51A* gene was detected in four of five non-WT isolates of *A. flavus* [79] that also displayed increased *mdr1* (multidrug efflux pump) transcript levels, making difficult to distinguish the degree of involvement in azole resistance of *cyp51A* or *mdr1* by themselves. Similar results have also been reported in a recent study on *A. flavus*, despite in this case overexpression of *cyp51A, cyp51B* and *cyp51C* along with *mdr1, mdr2, atrF* and *mfs1* (multidrug efflux pumps) was found in a single non-WT isolate [70].

Transcriptional upregulation of *cyp51* is the result of an efficient response to azole toxicity mediated by transcription factors and regulation of transcriptional activators. In certain cases where there are tandem repeats (TRs) of 34 bp (TR_{34}), 46 bp (TR_{46}) or 53 bp (TR_{53}) in the promoter region of *A. fumigatus cyp51A*, expression of this gene is enhanced [80–83]. TRs have been observed in strains that display reduced susceptibility to ITC and VRC [83]. However, they are frequently found in combination with amino acid substitutions (TR_{34}/L98H, TR_{46}/Y121F and TR_{46}/T289A) in the *cyp51A* of non-WT strains [80,84,85], with the exception of TR_{53} that has not been linked to any other *cyp51A* modification [86]. It is hard to distinguish whether TRs or point mutations are responsible for the resistance phenotype in the case of TR_{34} and TR_{46}, thus mutant strains harbouring TR_{34} or TR_{46} alone or in combination with different amino acid substitutions have been generated and characterised. The results showed that strains with TRs display increased expression of *cyp51A*, suggesting that these sequences could indeed act as transcriptional enhancers. However, the contribution of these TRs to azole resistance appears to be insignificant [81,82].

As sterol biosynthesis is highly regulated, many genes coding for enzymes in this pathway harbour sterol regulatory binding elements in their promoter region [87]. Relatively little is known about transcription factors (TFs) that regulate *Aspergillus cyp51* expression. Recent studies have identified TFs that bind to *A. fumigatus cyp51* promoters and regulate their expression, such as SrbA, HapE and AtrR. In particular, SrbA is a transcriptional regulator belonging to the sterol regulatory element binding protein (SREBP) family. This regulator has been described to participate in fungal growth during hypoxic conditions, sterol biosynthesis, cell polarity, hyphal morphogenesis, *A. fumigatus* virulence and *cyp51* transcriptional regulation, as its deletion increases azole susceptibility [88,89]. HapE is a CCAAT-binding TF complex subunit that negatively regulates *cyp51* transcription. A single mutation in amino acid position 88 of this negative regulator as well as its deletion cause overexpression of *cyp51A*, resulting in a decrease in azole susceptibility [90].

In addition, recent investigations on the role of the zinc finger AtrR have revealed that this TF regulates *cyp51* expression, among many other genes, and its deletion in *A. fumigatus* not only reduces *cyp51A* and *cyp51B* transcription but also virulence, indicating the essential role of this TF in fungal pathogenesis and resistance [91,92].

Furthermore, cytochrome b_5 CybE has also been found to regulate *cyp51A* transcription levels in *A. fumigatus* [93]. In this particular case, deletion of *cybE* resulted in the compensatory upregulation of *cyp51A;* however, its deletion also caused an increase in VRC susceptibility and accumulation of the ergosterol precursor eburicol [93].

2.2. Multidrug efflux pumps

Multidrug efflux pumps consist of transmembrane proteins that mediate active extrusion of antimicrobial molecules or toxic compounds and endogenous metabolites to the extracellular space [94]. Thus, efflux activity constitutes a determinant factor to be considered in drug resistance and fungal survival. Considering the great number of efflux pump protein-encoding genes present in the *Aspergillus* genome, functional redundancy could be expected in terms of antifungal transport [95].

Currently, two types of efflux superfamilies are known to modulate azole extrusion from fungal cells: the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters. Both the structure and the mechanisms of action differ between the two types of proteins. ABC transporters are constituted by two transmembrane and two cytoplasmic nucleotide-binding domains and use the energy derived from ATP hydrolysis to extrude the substrate across the membrane, whilst MFS transporters contain 12–14 transmembrane domains and in almost all cases use the proton-motive force to achieve drug efflux [96,97]. There are

45 ABC and 275 MFS transporters identified in the *A. fumigatus* genome [98], although only a few of them have been identified as drug transporters, which are also referred as multidrug resistance (MDR) or pleiotropic drug resistance (PDR) proteins [99]. To elucidate the contribution of efflux pumps to azole resistance, intensive research by means of expression analysis and site-directed mutagenesis has been performed in *A. fumigatus* and, to a lesser extent, in other *Aspergillus* spp. In most cases, overexpression of these transporters can cause the intracellular drug concentration to not reach the level necessary to be effective against the fungal cell [96]. However, their expression does not show a clear correlation with azole MICs (Table 1).

The ABC transporters investigated so far in terms of azole resistance include *cdr1B, mdr1, mdr2, mdr3, mdr4, abcD, abcE, atrI, atrB, atrC* and *atrF*. In the case of *A. fumigatus, cdr1B* (*abcB*) has been reported to contribute to azole resistance since its disruption largely increases azole susceptibility [67,100,101], demonstrating its importance in azole resistance. None the less, despite its clear upregulation upon VRC exposure [102], its relevance in the underlying resistance mechanism remains uncertain. This transporter has also been found to be important in *A. flavus* VRC resistance as it was found to be more expressed than other efflux pumps investigated (*mdr2, mdr4, mfs1* and *atrF*) in basal conditions and upregulated upon azole exposure [44]. However, this observation is not completely reliable considering the limited number of strains analysed and the fact that only a few non-WT strains exhibited overexpression of these transporters.

In addition, deletion of the ABC transporters *mdr1* (*abcA*), *mdr2, mdr3* and *mdr4* in *A. fumigatus* does not have any clear effect on azole susceptibility, although *mdr1* expression is highly induced by ITC and VRC. Moreover, non-WT isolates have been reported to show upregulation of theses efflux pumps under azole exposure in *A. fumigatus* and other *Aspergillus* spp., although when overexpressed azole susceptibility was not always reduced [51,67,70,79,100,102–104]. Similarly, the less studied transporters *abcD, abcE, atr1* and *atrF* also showed patterns of upregulation under azole induction in *A. fumigatus* and *A. flavus*, with their expression being commonly higher in non-WT strains [98,102–105].

There are fewer published studies regarding the MFS transporters and those analysed so far only include *mdrA, mfs56, mfs1, mfsA, mfsB* and *mfsC*. Among them, only *mdrA* and *mfs56* have been studied through deletion experiments in *A. fumigatus*, resulting in an increase of ITC and VRC susceptibility in the Δ*mdrA* mutant [98], whilst disruption of *mfs56* did not affect azole susceptibility [67], thus indicating its minor importance in azole resistance events. Regarding gene expression responses during azole exposure, only *mfsA, mfsB* and *mfsC* have been confirmed to be upregulated in *A. fumigatus*. [102]. In the case of *A. flavus*, transporter *mfs1* expression has been studied under VRC exposure, showing no correlation with azole susceptibility [44].

An important limitation of these studies, however, lies in the few strains tested in every case (in some cases, analyses are limited to only one strain), thus not allowing generalisation and extrapolation of the results. Further studies are needed to better understand the real involvement of efflux pumps in azole resistance in *Aspergillus*.

2.3. Other resistance mechanisms

Although it is widely accepted that azole-related ergosterol depletion is the main cause of fungal viability and growth inhibition, little is known about the exact mechanism triggered by azoles to exert their fungicidal effect on *Aspergillus*. In this context, it has been reported that resistance events, although proven to be mediated by Cyp51, could result from the cellular stress responses displayed by the fungal cell or other molecular mechanisms. The cell wall integrity pathway as well as other intracellular pathways are some examples of mechanisms that have recently been identified to play a role in *Aspergillus* azole response and resistance, as will be further explained.

2.3.1. Amino acid substitutions in HMG-CoA

Ergosterol biosynthesis takes place in a complex pathway that appears to be specific to fungal taxa and includes about 20 different enzymes [106]. Catalysis of the first step in this metabolic pathway is carried out by the 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase protein [87], encoded by the *hmg1* gene in *A. fumigatus*. This enzyme contains a sterol-sensing domain that is involved in negative regulation of its own reductase activity through direct interaction with sterols [107]. Recently, mutations located at the beginning of this sensing domain have been described as candidate mediators of azole resistance. As a matter of fact, in a recent study 52% of clinical *A. fumigatus* non-WT isolates tested were found to contain mutations in this protein. Specifically, mutations F262del, S305P, P309L and I412S notably increased VRC, PSC, ITC and isavuconazole MICs, leading to an accumulation of ergosterol precursors without altering *cyp51* gene expression [107]. Moreover, introduction of three of these mutations (F262del, S305P and I412S) in a WT genetic background resulted in a reduction in triazole susceptibility, and correction of these substitutions restored susceptibility [107]. Mutations in the *hmg1* gene have also been examined in another study, however the substitution S269F (potentially involved in azole resistance) did not have any effects on azole susceptibility when introduced into a WT isolate, thus rejecting any role in azole resistance [108].

2.3.2. Stress response

One of the effects of azole exposure is the production of mitochondrial reactive oxygen species (ROS), which appears to contribute to inhibition of fungal growth. As azoles promote the accumulation of ROS, the involvement of the mitochondrial complex constitutes a field of interest in terms of azole resistance. Inhibition of mitochondrial complex I has been shown to abolish the release of deleterious ROS in an azole-exposed *A. fumigatus* [109]. In addition, amino acid substitution E180D in the 29.9-kD subunit of mitochondrial complex I has been found in azole-resistant *A. fumigatus* isolates, suggesting that resistance could arise from loss of complex I activity. Moreover, inhibition of this mitochondrial complex led to azole resistance in *A. fumigatus* [110].

The importance of mitochondria in the azole response has also been described in other studies. As a matter of fact, it has recently been reported that VRC induces a sequence of events that include a sudden expulsive release of cytoplasm, arrest of mitochondrial dynamics, mitochondrial fragmentation and finally lysis of the mitochondria, eventually leading to fungal death in *A. fumigatus* [35]. This clearly suggests an essential involvement of mitochondria in the azole response, although it is not yet fully characterised.

The connection between oxidative stress adaptation and azole resistance is reinforced by previous studies on Yap1, a transcription factor that regulates defence mechanisms against ROS and azole antifungal drugs in *A. fumigatus* [111,112]. When truncated, this version of the *yap1* gene has been observed to confer attenuated susceptibility to VRC [111]. Yap1 has also been considered to participate in VRC resistance in *A. flavus*, since a point mutation causing the amino acid substitution L558W in this factor led to ATP transporter AtrF upregulation, which in turn is correlated with low VRC susceptibility [113].

Recently, many signalling pathways have also been shown to mediate responses to azole toxicity and to play key roles in azole tolerance. For instance, it has been shown that compromising the function of heat shock protein 90 (Hsp90), one of the most studied stress-related proteins, enhances the activity of many antifungal

drugs. This protein is a molecular chaperone that interacts with a diverse number of proteins by which gene expression is controlled during stress conditions. Among others, Hsp90 activates phosphatase calcineurin by interacting with its catalytic subunit to regulate the stress response, including azole-induced stress in *A. fumigatus*. Inhibition of Hsp90 (with geldanamycin) or of calcineurin (with FK506) increases azole susceptibility, proving the involvement of these proteins in azole resistance [114,115].

Likewise, the damage resistance protein (Dap) family, comprising DapA, DapB and DapC, responds to azole stress and controls ergosterol biosynthesis by SrbA regulation. Specifically, deletion of *dapA* increases azole susceptibility, therefore demonstrating its potential involvement in azole resistance [116,117].

The calcium signalling pathway also constitutes another example of this kind. Whilst the role of Ca^{2+}-mediated signal transduction in growth, development, proliferation, secretion, transportation and stress response is well recognised, adaptation to drug toxicity could also involve this signalling pathway in fungi [118]. Following ITC exposure, induction of calcium signalling is achieved, demonstrating the role of the pathway in the azole-induced stress response. Moreover, deletion of *cnaA* or *crzA* genes of this pathway increases azole susceptibility [119].

An additional example in this field is the case of the stress-related cell wall integrity pathway. Interestingly, a recent study has provided details on the cellular chain of events promoted by azoles that lead to *A. fumigatus* death. Curiously, they appear to be related to increased β-1,3-glucan synthesis in patches all along the hyphae walls that deform the cell membrane and trigger cell wall stress. Subsequently, the cell wall salvage system is activated, leading to fungal cell integrity failure and death [35].

Furthermore, disruption of the *A. fumigatus* Mkk2 signalling kinase, which is a central modulator of cell wall biosynthesis and organisation, results in an increase in susceptibility to PSC and VRC as well as a reduction in virulence and adherence of this species [120]. Also in the context of *A. fumigatus* cell wall integrity, loss of the endoplasmic reticulum-localised protein PerA is associated with impaired cell wall integrity and increased susceptibility to triazoles [121], providing evidence of the importance of the cell wall integrity pathway in the azole response.

2.3.3. Biofilm formation

Biofilms are structured microbial cell populations attached to surfaces and embedded in a self-produced polysaccharide extracellular cell matrix (ECM). Fungal biofilms display reduced susceptibility to the host immune system as well as to antifungal drugs compared with planktonic cells, which is thought to arise from their biofilm structural complexity, protection provided by the ECM and upregulation of efflux pump genes [122,123].

In this context, there are some proposals on the role of biofilms in *A. fumigatus* azole resistance. It seems that the cell density reached in a mature biofilm might hinder drug penetration, similar to the effect hypothesised for the role of the ECM in azole resistance as its hydrophobic nature cohesively binds hyphae [124]. In addition, upregulation of the ABC transporter Mdr4 has also been observed in *A. fumigatus* biofilm, which could contribute to azole resistance [94].

2.3.4. Cholesterol import

Another mechanism suggested to be involved in *Aspergillus* azole resistance consists of exogenous cholesterol import [125]. It has been demonstrated that import of exogenous cholesterol in *A. fumigatus* is a highly regulated process linked to enhanced fungal growth. Interestingly, this import is enhanced by the presence of azoles and attenuates their effects, probably due to the fact that although imported cholesterol appears to be stored in lipid particles, some of it is also incorporated into the fungal membrane. This suggests a possible compensation of fungal ergosterol depletion, which in turns appears to be related to negative consequences in terms of azoles efficacy [126].

3. Conclusion

Although in the last years genetic and molecular studies have shed light on different mechanisms contributing to azole resistance, it is clear that azole resistance is a complex and multifactorial event that requires several elements. To date, resistance mechanisms are not fully characterised in *A. fumigatus*, which is the most prevalent species causing IA, and there is much to explore in the emergent species *A. flavus, A. terreus, A. niger* and cryptic species. In this sense, more studies including a larger number of isolates are necessary to determine the real importance of all of these factors as well as to clarify controversy around the most studied mechanisms, such as *cyp51* overexpression and the contribution of multidrug efflux pumps. Another limitation of major concern regarding Cyp51 amino acid substitutions is that although there are studies demonstrating azole binding impairment by analysis of structural protein changes, in many others the location of the mutations (i.e. near the heme group or the azole-binding residues) is not investigated. Therefore, such studies could lead to unreliable associations between amino acid substitutions and azole resistance.

Addressing these limitations will be of major significance to truly understand the molecular mechanisms of azole resistance in *Aspergillus*. In addition, multiple lines of evidence converge into the hypothesis that the intracellular stress response constitutes another key factor in azole tolerance, corroborating the complexity of drug resistance. It will be essential to go in depth in the study of these pathways as well as other potential mechanisms such as drug degradation or activation of alternative pathways to bypass the drug effects. To do so, as proposed elsewhere [127], further analysis based on forward genetics, by generation of drug-resistant mutants and genetic screening of WT and non-WT strains will reveal the gene networks mediating resistance to azoles in *Aspergillus*.

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STUDY AND CHARACTERIZATION OF AZOLE RESISTANCE IN ASPERGILLUS SECTION NIGRI
Alba Pérez Cantero

5.2. New insights into the *cyp51* contribution to azole resistance in *Aspergillus* section *Nigri*

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MECHANISMS OF RESISTANCE

New Insights into the Cyp51 Contribution to Azole Resistance in *Aspergillus* Section *Nigri*

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ABSTRACT Invasive aspergillosis (IA) is a severe condition mainly caused by *Aspergillus fumigatus*, although other species of the genus, such as section *Nigri* members, can also be involved. Voriconazole (VRC) is the recommended treatment for IA; however, the prevalence of azole-resistant *Aspergillus* isolates has alarmingly increased in recent years, and the underlying resistance mechanisms in non-*fumigatus* species remain unclear. We have determined the *in vitro* susceptibility of 36 strains from section *Nigri* to VRC, posaconazole (POS), and itraconazole (ITC), and we have explored the role of Cyp51A and Cyp51B, both targets of azoles, in azole resistance. The three drugs were highly active; POS displayed the best *in vitro* activity, while ITC and VRC showed MICs above the established epidemiological cutoff values in 9 and 16% of the strains, respectively. Furthermore, expression studies of *cyp51A* and *cyp51B* in control condition and after VRC exposure were performed in 14 strains with different VRC susceptibility. We found higher transcription of *cyp51A*, which was upregulated upon VRC exposure, but no correlation between MICs and *cyp51* transcription levels was observed. In addition, *cyp51A* sequence analyses revealed nonsynonymous mutations present in both, wild-type and non-wild-type strains of *A. niger* and *A. tubingensis*. Nevertheless, a few mutations were exclusively present in non-wild-type *A. tubingensis* strains. Altogether, our results suggest that azole resistance in section *Nigri* is not clearly explained by Cyp51A protein alteration or by *cyp51* gene upregulation, which indicates that other mechanisms might be involved.

KEYWORDS *Aspergillus* section *Nigri*, Cyp51, amino acid substitutions, azole resistance, gene expression

Aspergillus is a widely distributed genus able to cause human opportunistic infections (1), with invasive aspergillosis (IA) being the most problematic condition in terms of mortality, which ranges from 50 to 100% (2). Immunocompromised patients, such those suffering from neutropenia and cancer and patients undergoing organ or hematopoietic stem cell transplantation, are the most susceptible (3, 4). Although *A. fumigatus* is the most frequent causal agent of IA (5), species from other sections of the genus, such as *Flavi*, *Terrei*, and *Nigri*, can also be involved (6). In particular, members of the section *Nigri*, including *A. niger* and the cryptic species *A. tubingensis*, *A. brasiliensis*, *A. awamori*, *A. japonicus*, and *A. carbonarius*, among others (1), have been reported as the second leading cause of IA, along with sections *Flavi* and *Terrei* (6–11).

In the current guidelines for diagnosis and management of IA, voriconazole (VRC) constitutes the first-line therapy (12). However, in recent years there has been an alarming increase in *Aspergillus* isolates showing resistance to azoles, which is correlated with poor therapeutic outcome, entirely reducing treatment options (5, 13–15). In the particular case of *Aspergillus*, which generally displays elevated azole susceptibility (16, 17), cases of azole resistance have been constantly increasing since the first reports in the 1990s and have been associated with both azole-treated and azole-naive patients

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(13). This acquired resistance can be explained by the fungistatic effect of azoles but also by azole exposure in the clinical and environmental settings (5, 13, 18).

The azole mode of action consists of noncompetitive binding to the Cyp51 enzyme, a 14α-demethylase from the ergosterol biosynthetic pathway, which leads to ergosterol synthesis inhibition and, consequently, cell membrane disruption (19). In this sense, several azole resistance mechanisms in *A. fumigatus* have been postulated. The presence of amino acid substitutions in the Cyp51 target proteins and overexpression of the *cyp51* genes or efflux pumps, such as the ATP binding cassette transporter Cdr1B, constitute the most important mechanisms described thus far (7, 20).

In this context, the study of *cyp51* genes has emerged as a main topic in azole susceptibility profiling in fungal pathogens. Some filamentous fungi, including *Aspergillus*, carry multiple *cyp51* genes in their genomes. This genetic redundancy is widely believed to facilitate adaptation to fungicides exposure, leading to an increase in azole resistance (21). Specifically, *A. fumigatus*, *A. terreus*, and *A. niger* contain two *cyp51* paralogs (*cyp51A* and *cyp51B*), while *A. flavus* contains three *cyp51* genes (*cyp51A*, *cyp51B*, and *cyp51C*) (7, 22). Moreover, previous studies conducted in *A. fumigatus* have indicated functional diversification and different contribution to azole resistance of *cyp51* paralogs (22).

Since azole resistance mechanisms in non-*fumigatus* species, such as the members of section *Nigri*, remain practically unknown, characterization of *cyp51* genes might lead to increase our knowledge on azole resistance emergence.

In this study, we performed gene expression studies of *cyp51A* and *cyp51B* in a set of clinical and environmental strains belonging to *Aspergillus* section *Nigri*, as well as sequence analyses of the *cyp51A* gene, in order to potentially correlate gene expression and/or nonsynonymous mutations with VRC resistance in members of this section.

RESULTS

Antifungal susceptibility. The MICs of itraconazole (ITC), posaconazole (POS), and VRC were determined in 36 strains belonging to section *Nigri*. The results are shown in Fig. 1 and also Table S1 in the supplemental material. All the assayed drugs were active against the tested strains, with the lowest modal MIC being that of POS (0.06 μg/ml), followed by ITC (1 μg/ml) and VRC (2 μg/ml). In all strains the POS MICs were below the epidemiological cutoff value (ECV) established for this drug against section *Nigri*, whereas the ITC and VRC MIC values were lower than the ECVs established in 9 and 16% of the strains, respectively. The ITC MIC was equal to the ECV in only one strain of *A. tubingensis*, while in the case of VRC, this was observed in eleven strains (species *A. niger*, *A. tubingensis*, *A. neoniger*, and *A. acidus*).

Cross-resistance between ITC and VRC was observed in four strains (9% of the total) belonging to *A. niger*, *A. tubingensis*, and *A. brasiliensis* species.

***In silico* identification and sequence analysis of *cyp51* genes in section *Nigri* species.** We conducted an *in silico* analysis in the *A. niger*, *A. tubingensis*, and *A. brasiliensis* genomes to identify *cyp51* genes and their deduced amino acid sequences. The results of BLASTP using the amino acid sequence of *A. fumigatus* Cyp51A (Afu4g06890) as a query revealed high homology with *A. niger* An11g02230, *A. tubingensis* Asptu1_0056695, and *A. brasiliensis* Aspbr1_0190913, showing a 79.7 to 80.2% identity range. Likewise, BLASTP search using *A. fumigatus* Cyp51B (Afu7g03740) as a query revealed high homology with *A. niger* An13g0009, *A. tubingensis* Asptu1_0051806, and *A. brasiliensis* Aspbr1_0137500, showing a 84.2 to 85.1% identity range.

In addition, sequence alignment of Cyp51A and Cyp51B revealed moderate pairwise similarity among these paralogous proteins in every species, displaying identity values of 62.5% for *A. fumigatus*, and from 63 to 63.8% for the non-*fumigatus* species studied. The detailed view reveals conserved residues involving the putative azole binding site among both proteins in all species.

Unlike *A. fumigatus*, which contains a single transmembrane domain, *A. niger*, *A. tubingensis*, and *A. brasiliensis* exhibit two predicted transmembrane domains in both Cyp51A and Cyp51B, comprising amino acids 5 to 24 and 37 to 59 and amino acids 20

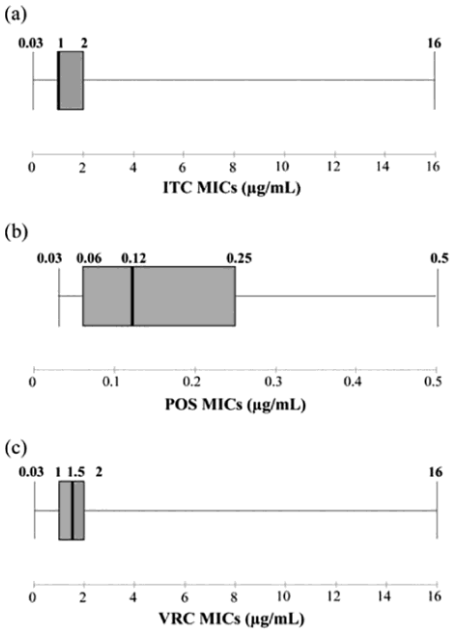

FIG 1 Box plot representation of *Aspergillus* section *Nigri* susceptibility pattern to the triazoles ITC (a), POS (b), and VRC (c). The box edges represent the first and third quartiles, respectively. Thick lines denote median values, while minimum and maximum values are depicted by horizontal lines.

to 42 and 54 to 73, respectively. Protein models are represented in Fig. 2. The sequence alignment, azole-binding residues, transmembrane domains, and the heme prosthetic group are represented in Fig. S1 (Cyp51A) and Fig. S2 (Cyp51B). The Cyp51A and Cyp51B sequence alignment of every species is represented in Fig. S3.

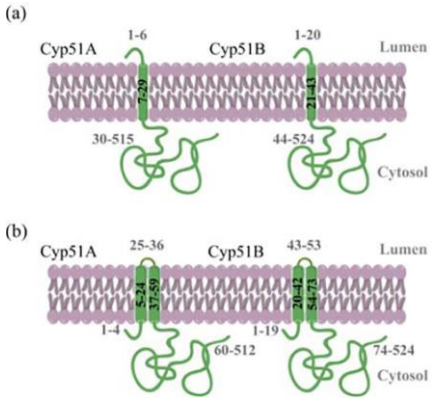

FIG 2 Protein topology of *A. fumigatus* (a) and *A. niger*, *A. tubingensis*, and *A. brasiliensis* (b) Cyp51A and Cyp51B associated with the endoplasmic reticulum membrane. The amino acid positions comprised by each protein structure are also indicated.

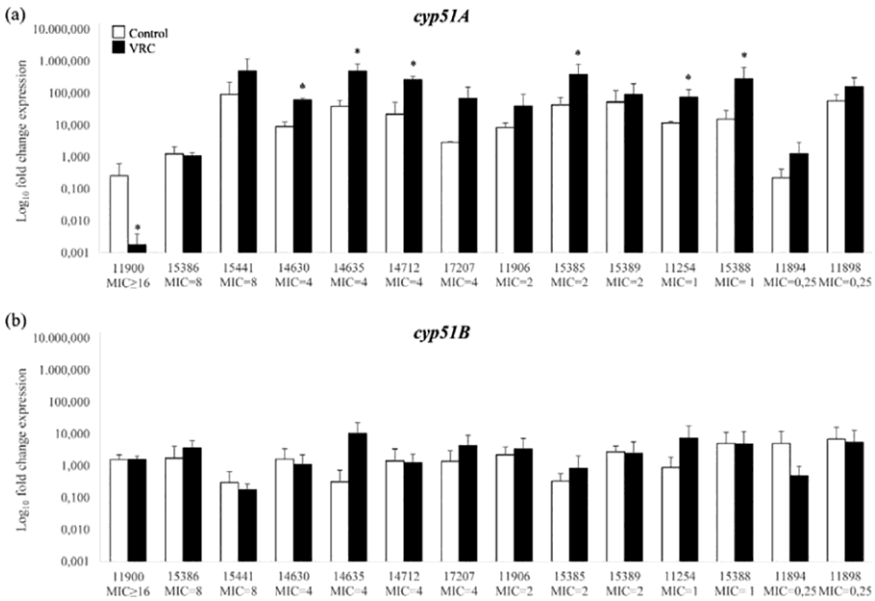

FIG 3 Expression analysis results of *cyp51A* (a) and *cyp51B* (b) genes in control condition and after exposure to 4 μg/ml VRC for 8 h. *actA* was used as the housekeeping gene to normalize transcription levels. MIC values expressed in μg/ml are shown below isolates labels. Statistical significance ($P \leq 0.05$) is marked by an asterisk (*).

Expression analysis of *cyp51A* and *cyp51B* in azole-inducing conditions. To analyze the expression patterns of *cyp51A* and *cyp51B* in control condition and during exposure to VRC, transcription levels of both genes were determined in 14 strains of section *Nigri* by quantitative reverse transcription-PCR. The results show higher expression of the *cyp51A* gene compared to that of the *cyp51B* in both conditions and all strains. We observed remarkable fluctuations of *cyp51A* transcription among strains, ranging from 2×10^{-1} to 5×10^{2} times fold with respect to the housekeeping gene (Fig. 3). Exposure to VRC resulted in statistically significant *cyp51A* induction ($P \leq 0.008$) in six strains (43%) categorized as wild type (wt) and non-wild type (non-wt). In addition, another 43% of the tested strains showed a non-statistically significant increased transcription of this gene upon drug exposure. Exceptionally, *cyp51A* was similarly expressed in the control condition and under VRC exposure in the *A. brasiliensis* strain (Fig. 3a). Unexpectedly, the strain displaying the highest VRC MIC showed low levels of *cyp51A* transcription, which were significantly reduced in VRC presence. Therefore, we could not establish any pattern of expression of *cyp51A* along the increasing MIC scale.

Regarding *cyp51B*, expression levels ranged from 3×10^{-1} to 7 times fold respect to the housekeeping gene, and VRC exposure did not result in significant overexpression (Fig. 3b). These data suggest that *cyp51B* gene expression is not inducible by VRC, in contrast to the *cyp51A* gene.

***cyp51A* sequencing and amino acid substitutions identification.** In order to identify nonsynonymous mutations in *cyp51A* that could be correlated with VRC resistance within the section *Nigri*, Cyp51A protein sequence was analyzed in the 14 selected strains. All *A. tubingensis* strains but one and all *A. niger* strains displayed amino acid substitutions in the Cyp51A protein compared to the reference sequences.

In the particular case of *A. tubingensis*, the amino acid change H467Q was exclusively detected in environmental non-wt strains in combination with substitutions K64E or

TABLE 1 Nonsynonymous nucleotide mutations in gDNA and their corresponding amino acid substitutions in the deduced Cyp51A protein sequence[a]

Species	FMR strain	Nucleotide change	Amino acid substitution[b]	VRC MIC (μg/ml)[c]	Origin
A. tubingensis	14630	C475T	A140V	**4**	Environmental
		C1293T	P413S		
	14635			**4**	Environmental
	14712	G1185A	**V377I**	**4**	Environmental
		C1457G	**H467Q**		
	17207	A190G	**K64E**	**4**	Environmental
		C1457G	**H467Q**		
	11906	C475T	A140V	2	Clinical
		C1293T	P413S		
	15389	G1017A	A321T	2	Environmental
A. niger	11900	A169G	T57A	**16**	Clinical
		A740G	Q228R		
	15441	A740G	Q228R	**8**	Environmental
	15385	A169G	T57A	2	Environmental
		A740G	Q228R		
	11254	A169G	T57A	1	Clinical
		A740G	Q228R		
	15388	A169G	T57A	1	Environmental
		A740G	Q228R		
		T1095A	S346R		
	11894	A169G	T57A	0.25	Clinical
		A571G	I172V		
		(CGC)610(AGT)	A185S		
		A740G	Q228R		
		C782T	T242M		
		A1189G	I378V		
		A1318C	M421L		
		(CTG)1321(ATC)	V422I		
	11898	A169G	T57A	0.25	Clinical
		A740G	Q228R		
A. brasiliensis	15386			**8**	Environmental

[a]Nomenclature refers to the nucleotide/amino acid present in the reference sequence, followed by its position and the nucleotide/amino acid substitution in the analyzed strain.
[b]Amino acid substitutions indicated in boldface have a potential role in VRC resistance.
[c]MIC values indicated in boldface are greater than the established ECV for VRC (2 μg/ml).

V377I. In addition, other mutations were found in both wt and non-wt strains of this species, such A140V and P413S, whereas the amino acid change A321T was only found in an environmental strain that displayed elevated susceptibility to VRC (Table 1). Regarding *A. niger*, none of the substitutions found in the Cyp51A protein could be linked to resistant phenotypes. Q228R was a common change in all strains, which was frequently found in combination with T57A, located in the second transmembrane domain of the *A. niger* Cyp51A. In addition to these, an environmental wt strain showed the S346R change. Multiple substitutions were observed in the case of the clinical wt strain FMR 11894 beyond Q228R and T57A: I172V, A185S, T242M, I378V, M420L, and V422I. The *A. brasiliensis* non-wt strain did not display any amino acid substitution in the Cyp51A protein.

The detected nucleotide and amino acid modifications compared to the deposited sequences in the Aspergillus Genome Database are summarized in Table 1. In addition, Cyp51A sequence alignments with domain identifications and the amino acid changes found in our strains are marked in Fig. S1.

DISCUSSION

Although resistance mechanisms in *A. fumigatus* have been extensively studied, there are very few studies carried out on members of *Aspergillus* section *Nigri*. Thus, we sought to test the *in vitro* susceptibility of clinical and environmental isolates of relevant species of *Aspergillus* section *Nigri*, as well as to characterize possible resistance mechanisms present in our set of strains.

The three azoles tested here (VRC, PSC, and ITC) showed good activity, with POS being the most active one, a finding in accordance with previous studies (23–25). The low modal POS MIC and its narrow MIC range are also indicative of its elevated *in vitro* activity against these fungi. Both ITC and VRC exhibited good *in vitro* activity, even though low susceptibility to VRC was more common than to ITC, which is also in line with previous results (26). In addition, ITC non-wt isolates were found more frequently among *A. tubingensis* isolates than in the other species of this section that we tested, as already described (23, 26, 27). In accordance with previous works (23, 27), cross-resistance was not common in our isolates.

Since Cyp51 proteins constitute the targets for azoles, a high level of their transcripts could confer azole resistance by maintaining the cellular ergosterol levels. In fact, it has been documented that the introduction of extra copies of the *A. nidulans cyp51A* gene (*pdmA*) in *A. fumigatus* caused a decrease in ITC susceptibility in the latter (28), suggesting that *cyp51A* overexpression might confer azole resistance. However, this mechanism does not seem to be the key factor conditioning azole resistance, since no overexpression of *cyp51A* was detected in a set of non-wt *A. fumigatus* strains in a subsequent study (29). Great variability in expression patterns was also described in studies focusing on other species of the genus, thus providing controversial results. Specifically, in *A. flavus*, *cyp51* transcript levels were not correlated to VRC-resistant phenotypes in a study (30), whereas 80% of non-wt strains displayed *cyp51A* overexpression in another one (31), although in the latter work resistance was linked to multidrug efflux pump overexpression as well.

Our results suggest, for the first time in section *Nigri* to our knowledge, that *cyp51A* actively participates in the transcriptional response to azole stress in these fungi, which is clearly upregulated after exposure to VRC. Nevertheless, transcript levels of *cyp51A* showed no correlation to the VRC MICs in this section, meaning that azole resistance is not explained by *cyp51A* overexpression. This has also been discussed in a recent study in which the basal expression of *cyp51A* in a few species of section *Nigri* did not correlate with the susceptibility patterns they displayed (26).

In contrast, *cyp51B* showed notably lower and stable expression in all the conditions we tested, suggesting a minor role in azole response. Accordingly, *cyp51A* has also been described to be the key player in *A. fumigatus* azole response, since its disruption results in reduced resistance to azoles, while azole susceptibility patterns are not altered in *cyp51B* knockout mutants (22, 32). However, this perception is not shared by everyone, since it has been suggested that *cyp51B* also contributes to azole resistance in *A. fumigatus* due to its induction upon ITC exposure, although only in one strain after ITC exposure (29). This was also observed in our study since a slight but not significant upregulation of *cyp51B* was detected upon VRC exposure. Considering that these are isolated facts, the involvement of *cyp51B* in azole resistance seems negligible.

Interestingly, an opposite pattern was detected in a transcriptome analysis performed on a strain of *A. fumigatus* with elevated azole susceptibility in which both *cyp51* genes decreased their expression after VRC exposure (33). Surprisingly, we have observed this inhibition in the strain showing the highest VRC MIC (FMR 11900), which displayed a drastic reduction of *cyp51A* transcription under VRC. These controversial data reinforce the hypothesis that other mechanisms and not *cyp51* expression could be contributing to the appearance of resistant phenotypes in *Aspergillus*.

In another line, differences in the Cyp51A protein sequence and structure are also important parameters to be considered, as changes in the conformation of the protein can vary its stability and flexibility, reducing azole binding affinity (34, 35). It should be noted that the detailed study of the Cyp51 proteins revealed some structural and sequence differences among the studied species of section *Nigri* and *A. fumigatus*, such as the presence of two transmembrane domains in section *Nigri* in contrast to only one of *A. fumigatus*. Although the transmembrane domain present in *A. fumigatus* has been described already, there are slight differences between the previously reported amino acids that comprise the transmembrane region in this species and our predicted data (36).

In *A. fumigatus*, many cases of azole resistance have been linked to *cyp51A* single point mutations that lead to amino acid alterations in the Cyp51A protein, as well as tandem repeats in the promoter region of the *cyp51A* gene that enhance its expression (37–41). Interestingly, no amino acid substitutions in the Cyp51B protein have been correlated to azole resistance so far, which is also indicative of its smaller role in azole response.

However, there has been some controversy, since some of the Cyp51A mutations previously described have also been found in highly azole-susceptible *A. fumigatus* and other *Aspergillus* spp. (23, 27, 42, 43). In fact, some of these amino acid differences believed to confer azole resistance have been related to different regional origins rather than resistance events (42).

Regarding our results in the species of section *Nigri*, sequence analysis of Cyp51A revealed no general correlation between amino acid changes and azole resistance, since most of the mutations were present in both wt and non-wt strains. This is the case for A140V and P413S substitutions in *A. tubingensis* or T57A and Q228R in *A. niger*, which were previously reported by others as well (23, 26). Moreover, amino acid A321 in *A. tubingensis* Cyp51A has been described as a residue that could confer azole resistance (26). Our results though, seem to contradict this, since five of our strains with different VRC MICs presented Ala in this position and a non-wt strain presented Thr in the same position. This straightforwardly suggests that amino acid changes in this position are not related to azole resistance. In addition, we found that two non-wt strains belonging to *A. tubingensis* (FMR 14635) and *A. brasiliensis* (FMR 15386) did not present any amino acid substitution, meaning that elevated VRC MICs found for these strains could be due to other mechanisms. Nevertheless, other non-wt strains (FMR 14712 and FMR 17207) presented in an exclusive manner a few Cyp51A substitutions. To our knowledge, the mutations H467Q, V377I, or K64E found in these isolates have not been reported to date; however, it remains unclear whether they are the cause of azole resistance in these strains, and further studies are needed to clarify their exact role. In particular, the substitutions K64E, V377I, and H467Q do not correspond to any characterized azole binding site residues, and therefore azole union should not be altered. Nonetheless, it remains unknown whether these mutations have any effect on the conformation of the protein or its binding pocket, preventing optimal binding.

Although *cyp51* expression levels and protein affinity to VRC may be crucial to dealing with azole toxicity inside the cell, our data show that neither *cyp51A* transcription nor amino acid changes are straightforwardly linked to resistant phenotypes in *Aspergillus* section *Nigri*. This contrasts with what occurs in *A. fumigatus* (26, 27). Together, these findings suggest that resistance is a complex phenomenon in which other molecular mechanisms are involved, especially in non-*fumigatus* species. In this sense, more studies with larger sets of strains are needed, and potential new resistance mechanisms should be further explored.

MATERIALS AND METHODS

Strains, media, and growth conditions. A total of 36 environmental and clinical strains belonging to *Aspergillus* section *Nigri* (*A. niger*, *A. tubingensis*, *A. brasiliensis*, *A. awamori*, *A. japonicus*, *A. carbonarius*, *A. neoniger*, and *A. acidus*), previously identified by ITS, *benA*, and *cam* gene marker sequencing, were included in the *in vitro* susceptibility study (see Table S1 in the supplemental material).

Fourteen of these strains, belonging to *A. niger*, *A. tubingensis*, and *A. brasiliensis*, were selected for further studies on the basis of their different *in vitro* VRC susceptibility to characterize potential resistance mechanisms (Table S1). Five of the strains were clinical and had been previously isolated from sputum or skin lesions in Texas, Brazil, and Spain. The rest had been isolated from environmental sites in different countries (Spain, Venezuela, Mexico, Vietnam, and Brazil).

Strains were stored as mineral oil cultures or lyophilized cultures and before used they were grown on potato dextrose agar (PDA) media (Conda-Pronadisa) for 3 to 5 days at 35°C twice. For conidiation, strains were grown for 3 to 5 days on PDA at 35°C, and conidia were collected by flooding the PDA culture plates with 5 ml of 0.1% Tween 20 solution and gentle agitation. For genomic DNA (gDNA) extraction, cultures were incubated in potato dextrose broth (PDB) as previously described with some modifications (44). Briefly, conidial suspensions were incubated in PDB for 24 h at 37°C and 180 rpm, and mycelia were collected by monodur filtration, washed with distilled sterile water, and stored at −80°C until use. For gene expression analyses, 1×10^7 conidia/ml were germinated in YG medium (0.5% yeast extract, 2% glucose) for 12 h at 37°C and 180 rpm. Mycelia were harvested by filtration and aseptically

TABLE 2 Primers used in this study designed with Oligo7 software[a]

Primer	Sequence (5′–3′)	Species	Use
cyp51A-F1	GAACCCAGACGAGGAGAAG	All[b]	RT-qPCR/Seq
cyp51A-R1	TCGCAGCATGATCCAAGAAC	*A. niger*-*A. brasiliensis*	RT-qPCR
cyp51A-R2	CGCAACATAATCCAAGAGCTA	*A. tubingensis*	RT-qPCR
cyp51B-F1	GTTTCCATTCATAGGTAGCAC	All	RT-qPCR
cyp51B-R1	ACTTCTTCAGCACAGACATCA	All	RT-qPCR
act-F2	ACCCTCAGATACCCCATTGA	All	RT-qPCR
act-R2	CTGGGTCATCTTCTCACGG	All	RT-qPCR
An-F2	CGATAGTCTTAAATGTCACGC	*A. niger*	Seq
An-R3	GCTCCCAACCCACTATAGC	*A. niger*	Seq
Anb-R2	TGAGGCGAGTAGAACATGGT	*A. niger*-*A. brasiliensis*	Seq
Ax-R3	ACCTCCTCGCCCTGCAAC	All	Seq
Asptu-F1	ATGGCATATCTTGCTGTTGCA	*A. tubingensis*	Seq
Asptu-R1	TTAGTTCAAGGACCCCTTGGA	*A. tubingensis*	Seq
Asptu-R2	CTGGTGAGGCGAGTAGAAC	*A. tubingensis*	Seq
Asptu-R3	CTTGGAGTTGTCTTGGATGC	*A. tubingensis*	Seq
Aspbt-F2	TGCTCGTTGCGATAGTCTTG	*A. tubingensis*-*A. brasiliensis*	Seq
Aspbr-F1	ATGGCATTCCTTGCTATCGC	*A. brasiliensis*	Seq
Aspbr-R1	TCAGTTCGATGACCCCTTGG	*A. brasiliensis*	Seq
Aspbr-R3	CGGAGAAGAGAGTCGAGTAA	*A. brasiliensis*	Seq

[a]Abbreviations: F, forward; R, reverse; RT-qPCR, quantitative real-time PCR; Seq, sequencing.
[b]All = *A. niger*, *A. tubingensis*, and *A. brasiliensis*.

transferred to fresh YG medium supplemented with VRC (Pfizer, Inc.) at a final concentration of 4 μg/ml when required. Cultures were incubated at 37°C and 180 rpm for 8 h and mycelia were collected by monodur filtration, washed with distilled water, and stored at −80°C until use.

Antifungal susceptibility testing. *In vitro* susceptibility testing was carried out according to Clinical and Laboratory Standards Institute (CLSI) protocol M38 for broth microdilution (45). Briefly, stock solutions of ITC (Janssen Pharmaceutica), POS (Schering-Plough Research Institute), and VRC (Pfizer, Inc.) were prepared in sterile water or dimethyl sulfoxide. All drugs were diluted in RPMI 1640 medium and dispensed in 96-well microdilution trays, which were inoculated with a conidial suspension of every strain previously adjusted by using a hemocytometer count. MICs of triazoles were determined after 48 h of incubation at 35°C without light nor agitation by direct visualization with an inverted mirror. MICs corresponded to the lowest drug concentration that completely inhibited fungal growth. *A. flavus* strain ATCC 204304 was used as the quality control strain.

Strains were classified as wt and non-wt according to the currently established ECVs for ITC (4 μg/ml), POS (2 μg/ml), and VRC (2 μg/ml) and section *Nigri*, since no clinical breakpoints are available yet for *Aspergillus* (46). The strains displaying ITC, POS, or VRC MICs above the ECVs were categorized as non-wt, while those displaying MICs below the ECVs were categorized as wt.

DNA and RNA isolation. For DNA and RNA extraction, mycelia were ground in liquid nitrogen with mortar and pestle. Total gDNA was extracted from ground mycelia according to a previously reported protocol (47). Total RNA was extracted from ground mycelia with TRIzol reagent (Thermo Fisher) as previously described (48). RNA was purified with a NucleoSpin RNA kit (Macherey-Nagel, Germany), including on-column DNase digestion. The resulting DNA and RNA pellets were resuspended in DNase- and RNase-free purified water, and the quantity and quality were checked by running aliquots in RedSafe-stained agarose gels and spectrophotometric analysis in NanoDrop 2000 spectrophotometer (Thermo Fisher).

Gene and protein identification and analysis in section *Nigri*. *cyp51A*, *cyp51B*, and actin (*actA*) orthologs were identified in *A. niger*, *A. tubingensis*, and *A. brasiliensis* by BLASTP searches (Basic Local Alignment Search Tool) in the Aspergillus Genome Database, using *A. fumigatus* Af293 protein sequences as templates. Alignments of the deduced amino acid sequence were performed with ClustalW algorithm of MegaLign software (DNASTAR). Sequence analysis and identification of the putative azole-binding residues and heme prosthetic group in *A. niger*, *A. tubingensis*, and *A. brasiliensis* was performed by comparison with *A. fumigatus* protein structure deposited in the SWISS-MODEL database (IDs: 4uyl.2, 4uym.1, and 5frb.1) and data published by others (49–51). Prediction of transmembrane helices and topology of proteins was performed with the TMHMM v2.0 server and the InterPro platform.

Retrotranscription and quantitative real-time PCR. For gene expression studies, total RNA was reverse transcribed into first-strand complementary DNA (cDNA) using the iScript cDNA synthesis kit (Bio-Rad) and following the manufacturer's indications. Reverse transcription-PCRs were performed in a StepOne Plus real-time PCR system (Applied Biosystems) using 7.5 μl of FastStart Universal SYBR green Master mix (Roche Diagnostics), 6.9 μl of cDNA template, and 300 nM of each gene-specific primer (Table 2) in a final reaction volume of 15 μl. All primer pairs amplified products of 162 to 207 bp. The thermal cycling conditions were as follows: 94°C (5 min); 45 cycles of 94°C (30 s), 60°C (30 s), and 72°C (30 s); and finally 80°C (20 s). A melting curve was obtained immediately after PCR completion to check amplification specificity under the following conditions: 95°C (15 s), 60°C (15 s), and 95°C (15 s). Expression levels were calculated by normalizing *cyp51A* and *cyp51B* cycle thresholds (C_T) with those of the housekeeping gene *actA* (52) using the efficiency (E) corrected ΔC_T method (53). With this method, the relative expression

ratio is calculated from the quantitative real-time PCR (RT-qPCR) E value assessed for the three genes in the study (Fig. S4), which were within an acceptable E value range (54), and the C_T in each case.

***cyp51A* sequencing and protein analysis.** Amplification of *cyp51A* was carried out by standard PCR using the following thermal cycling protocol: 98°C (1 min); followed by 35 cycles of 98°C (10 s), 62°C (30 s), and 72°C (1.5 min); and finally 72°C (10 min).

Amplicons were sequenced with the primers listed in Table 2 at Macrogen Europe (Macrogen, Inc., Madrid, Spain), and sequences were analyzed using SeqMan (DNASTAR) and MEGA version 7 software. Alignments of the deduced amino acid sequences were performed using the ClustalW algorithm of MegaAlign software (DNASTAR).

Statistical analysis. A Mann-Whitney test was used to compare relative gene expression values. All statistical analyses were performed with GraphPad Prism 6.0 for Windows. *P* values of ≤0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00543-19.

SUPPLEMENTAL FILE 1, PDF file, 2.1 MB.

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5.3. Analysis of the *cyp51* genes contribution to azole resistance in *Aspergillus* section *Nigri* with the CRISPR-Cas9 technique

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STUDY AND CHARACTERIZATION OF AZOLE RESISTANCE IN ASPERGILLUS SECTION NIGRI
Alba Pérez Cantero

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Abstract	Cyp51 contribution to azole resistance has been broadly studied and characterized in Aspergillus fumigatus, whereas it remains poorly investigated in other clinically relevant species of the genus, such as those of section Nigri. In this work, we aimed to analyze the impact of cyp51 genes (cyp51A and cyp51B) on the voriconazole (VRC) response and resistance of A. niger and A. tubingensis. We generated CRISPR-Cas9 cyp51A and cyp51B knock-out mutants from strains with different genetic backgrounds and diverse patterns of VRC susceptibility. Single gene deletions of cyp51 genes resulted in 2 to 16-fold decrease of the VRC Minimal Inhibitory Concentration (MIC) values, which were below the VRC Epidemiological Cutoff Value (ECV) stablished by the Clinical and Laboratory Standards Institute (CLSI) irrespective of their parental strains susceptibilities. Gene expression studies in the tested species confirmed that cyp51A participates more actively than cyp51B in the transcriptional response of azole stress. However, ergosterol quantification revealed that both enzymes comparably impact the total ergosterol content within the cell, as basal and VRC-induced changes to ergosterol content was similar in all cases. These data might contribute to expand our knowledge on Aspergillus azole resistance, especially in non-fumigatus species.
Editor	Prof. Andreas H Groll

ABSTRACT

Cyp51 contribution to azole resistance has been broadly studied and characterized in *Aspergillus fumigatus*, whereas it remains poorly investigated in other clinically relevant species of the genus, such as those of section *Nigri*. In this work, we aimed to analyze the impact of *cyp51* genes (*cyp51A* and *cyp51B*) on the voriconazole (VRC) response and resistance of *A. niger* and *A. tubingensis*. We generated CRISPR-Cas9 *cyp51A* and *cyp51B* knock-out mutants from strains with different genetic backgrounds and diverse patterns of VRC susceptibility. Single gene deletions of *cyp51* genes resulted in 2 to 16-fold decrease of the VRC Minimal Inhibitory Concentration (MIC) values, which were below the VRC Epidemiological Cutoff Value (ECV) stablished by the Clinical and Laboratory Standards Institute (CLSI) irrespective of their parental strains susceptibilities. Gene expression studies in the tested species confirmed that *cyp51A* participates more actively than *cyp51B* in the transcriptional response of azole stress. However, ergosterol quantification revealed that both enzymes impact similarly the total ergosterol content within the cell, as basal and VRC-induced changes to ergosterol content was similar in all cases. These data might contribute to expand our knowledge on *Aspergillus* azole resistance, especially in non-*fumigatus* species.

INTRODUCTION

Sterol 14 α-demethylase (Cyp51) enzymes are included in the cytochrome P450 protein superfamily, which is present in all biological kingdoms. In fungi, these enzymes display essential functions within the ergosterol biosynthetic pathway, since they catalyze the oxidative removal of 14 α-methyl groups from sterol precursors to be transformed into ergosterol. In

turn, ergosterol constitutes an essential component for the permeability and fluidity of fungal membranes and it is involved in several fungal regulatory and developmental processes (1, 2). Inhibition of Cyp51 enzymes, such as the effect exerted by azole antifungals, blocks ergosterol synthesis by impairing the demethylation of its precursors. This results in an accumulation of methylated sterols within the cell that affects the fluidity of the fungal membrane and leads to its disruption and the inhibition of cell proliferation (3–6).

In *Aspergillus,* Cyp51 enzymes are encoded by the *cyp51* genes and, interestingly, different species of the genus display varying number of *cyp51* paralogues in their genomes. For example, *Aspergillus fumigatus* and *Aspergillus niger* contain two *cyp51* copies (*cyp51A* and *cyp51B*) while *Aspergillus flavus* displays three different paralogues (*cyp51A*, *cyp51B* and *cyp51C*) (6, 7). In *A. fumigatus,* this genetic redundancy is translated into two Cyp51 proteins that carry equivalent enzymatic functions and share the same substrates. However, while Cyp51A seems to encode the predominant enzymatic activity and displays higher influence in azole stress response, Cyp51B has been considered a redundant gene that might carry out alternative functions (8, 9). As a matter of fact, azole resistance in this species has been broadly linked to overexpression or non-synonymous mutations of the *cyp51A* gene (10, 11), which contrasts to the unclear role of *cyp51B* in azole resistance (12, 13).

Despite the extensive data obtained from azole resistance analyses in *A. fumigatus,* the potential contribution of every Cyp51 paralog to azole response and/or resistance is limited and remains obscure in non-*fumigatus* species. This is the case of clinically important species of *Aspergillus*

section *Nigri* (*i.e. A. niger* and *A. tubingensis*), which are commonly found as causal agents of invasive aspergillosis (IA) (7, 14).

In a previous work (15) we explored the role of *Aspergillus* section *Nigri* Cyp51 enzymes in azole resistance, which could not be clearly explained by Cyp51A protein alteration or by *cyp51* gene upregulation, contrarily to what has been described in *A. fumigatus* (10, 11). Therefore, in the present study, we aimed to elucidate the role of *A. niger* and *A. tubingensis cyp51* paralogues in azole response and resistance. With this purpose, we have generated CRISPR-Cas9 knock-out (KO) mutants from strains with different genetic backgrounds and diverse azole susceptibility and we have assessed gene expression and ergosterol biosynthesis under standard conditions and azole stress.

RESULTS

Mutant generation with CRISPR-Cas9. A total of ten *cyp51* KO (Δ*cyp51A* or Δ*cyp51B*) strains from five parental isolates with diverse VRC susceptibility (FMRs 15441, 14712, 14635, 11906 and 15388) (15) were generated using a CRISPR-Cas9-driven gene targeting technique. After selection with hygromycin (Hyg), mutant confirmation was carried out with three different gDNA PCRs (Figure 1) combining four primers for every species and gene as follows: Fw scr – Rev scr, Fw scr – Rev intern and Fw intern – Rev scr. For the *A. niger* Δ*cyp51A* confirmation PCRs, the resulting bands sizes were: 4614 bp, 1814 bp and 2800 bp, whereas in the Δ*cyp51B* case, band sizes were 4710 bp, 1844 bp and 2866 bp, respectively. In the case of *A. tubingensis* Δ*cyp51A* PCRs, bands showed sizes of 4557 bp, 1844 bp and 2713 bp, while Δ*cyp51B* PCR fragments showed 4502 bp, 1777 bp and 2725 bp, respectively.

Antifungal susceptibility. The VRC MICs determined in the 10 KO strains (Δ*cyp51A* or Δ*cyp51B*) generated from section *Nigri* are shown in Table 1.

In general, the two single gene deletions impacted azole susceptibility by notably decreasing VRC MICs. Moreover, both single gene deletions resulted in MICs below the VRC ECV established by the CLSI (2 µg/mL) (16) regardless the MICs in the parental strains. Deletion of the *cyp51A* gene showed a 16-fold decrease in the VRC MIC value for the strain initially displaying the highest VRC MIC (FMR 15441), while a 2-fold decrease in the VRC MIC was observed for the FMR 15388 strain, which originally showed the lowest MIC of the set. The other *cyp51A* KO mutants also exhibited an increase in VRC susceptibility, being 4 times (FMRs 14712 and 11906) and 8 times (FMR 14635) more susceptible than their parental strains.

Regarding the effects of the *cyp51B* deletion on VRC susceptibility, MICs were reduced in all strains but FMR 15388, in which VRC susceptibility was not altered in comparison to its parental strain. Again, the strain initially displaying the highest VRC MIC (FMR 15441) suffered the most marked affectation, with an 8-fold decrease in the VRC MIC. With respect to the other *cyp51B* KOs, VRC MICs were 2-fold (FMRs 14712 and 11906) and 4-fold (FMR 14635) lower in comparison to the MIC values of the parental strains.

Gene expression analysis of *cyp51A* and *cyp51B* in azole-inducing conditions. Transcription levels of *cyp51A* and *cyp51B* were determined in the 10 KO mutant strains by quantitative reverse transcription-PCR.

Results from *cyp51A* gene expression (in Δ*cyp51B* strains), showed fluctuations in the *cyp51A* transcription among strains, ranging from 2- to

9-fold with respect to the housekeeping gene (Fig 2A). Exposure of these strains to VRC resulted in noticeable increase of *cyp51A* expression for all the cases, with statistically significant induction ($P = 0.0002$) of this gene in four strains (*i.e.* FMRs 15441 ΔB , 14712 ΔB, 11906 ΔB and 15388 ΔB). Regarding results on *cyp51B* expression (in Δ*cyp51A* strains), transcription levels ranged from $9x10^{-1}$ to 3- fold respect to the housekeeping gene (Fig 2B). In contrast to *cyp51A* observations, transcription of *cyp51B* upon VRC exposure was slightly affected, with a statistically significant increase in strains FMR 15441 ΔA ($P = 0.0002$) and FMR 14635 ΔA ($P = 0.0379$).

Ergosterol content. Percentage of ergosterol and the intermediate sterol 24(28) dehydroergosterol (DHE) dry weight were determined for all Δ*cyp51A* and Δ*cyp51B* generated mutants in standard conditions and upon VRC exposure stress. In general, basal ergosterol content in Δ*cyp51A* mutants showed minimal differences, since it ranged from 0.031 to 0.035 % (dry weight). In the case of Δ*cyp51B*, the ergosterol level rank was slightly broader, ranging from 0.026 to 0.040 % (dry weight). Comparison of Δ*cyp51A* and Δ*cyp51B* ergosterol basal quantification, revealed no general differences with only one (FMR 11906 ΔA and 11906 ΔB) significant exception ($P = 0.0286$). In addition, ergosterol was significantly depleted in all cases after VRC exposure, ranging from 0.009 to 0.016 % dry weight within all strains ($P \leq 0.0286$).

Regarding 24(28)DHE quantification, basal levels were very similar among all Δ*cyp51A* (0.022 to 0.026 % dry weight) and all Δ*cyp51B* (0.019 to 0.022 %) mutants. Interestingly, exposure to VRC resulted in very small variations when compared to the control condition, with the exception of the reduction observed for strains FMRs 15541 ΔA and 11906 ΔA or the increase displayed by FMR 14712 ΔB.

DISCUSSION

In the current guidelines for aspergillosis management, azoles, and more specifically VRC, are established as the first-line therapeutic and prophylactic treatments for most infections caused by *Aspergillus* (17). Nevertheless, azole resistance is continuously rising due to the wide use of azole fungicides in the environment (for plant and animal protection as well as food production) and in long-term human therapies (18, 19). This phenomenon has shown an important impact in the clinical management of *Aspergillus* infections, especially impairing IA patient outcomes. As a matter of fact, mortality rates linked to therapeutic failure in IA caused by resistant *A. fumigatus* isolates have been estimated to be around 90 %, while estimation of death rates in IA caused by strains with wild type susceptibilities is significantly lower (30-50 %) (20).

Although *A. fumigatus* is the most commonly isolated species from IA patients, other species, such section *Nigri* members, can also cause disseminated and invasive infections (7, 14). Interestingly, *A. niger* and related species have been described to show lower azole susceptibility than *A. fumigatus* (21), which, together with azole resistance events, might potentially impact clinical management and patient outcomes.

In a previous study we analyzed azole resistance in a set of section *Nigri* strains with different azole susceptibility patterns. We focused our investigation on *cyp51* expression and *cyp51A* non-synonymous mutations, but these could not be clearly associated to azole resistance within the section (15).

On this basis, we have focused the current work in the generation, for the first time to our knowledge, of single defective *cyp51A* and *cyp51B* mutants in section *Nigri* strains with different genetic backgrounds and diverse

VRC susceptibility to assess the real contribution of each *cyp51* paralog into azole response and resistance. With that purpose, we successfully used the CRISPR-Cas9 based methodology recently developed for *A. fumigatus* (22) with very few modifications.

Our results with the *cyp51* KO surprisingly show that VRC susceptibility is similarly contributed by the two Cyp51 paralogs (Cyp51A and Cyp51B) in the section, since the two single deletions reduced VRC MICs below the ECV established by the CLSI (16) irrespective of the parental strains susceptibility. Interestingly, VRC susceptibility in Δ*cyp51A* strains was slightly higher than in the Δ*cyp51B* strains. These results are in line to those obtained from *A. fumigatus* in a very recent study, where deletion of either *cyp51* paralog resulted in increased susceptibility, with a greater effect on VRC MICs after deletion of *cyp51A* (23). Curiously, previous *A. fumigatus* analyses showed that *cyp51A* deletion or silencing by siRNA increased azole susceptibility (9, 24) while deletion of *cyp51B* had no effects (25, 26). Deep analyses to elucidate the role of every paralog will be needed to resolve the conflicting results on this species.In addition, our data is in agreement with that obtained from *Aspergillus flavus*, since azole response seems to rely on *cyp51A* and *cyp51B* in that species (27), as it has been described to occur in *Fusarium oxysporum* (28). In the latter, *cyp51A* deletion clearly reduced azole MICs while *cyp51B* deletion increased azole susceptibility to a lesser extent. However, it is worth noting that these two species contain a third *cyp51* paralog (*cyp51C*) and its contribution to azole response in both cases seem negligible (27). In other filamentous fungi, such as *Rhizopus oryzae* (also containing the *cyp51B* paralog) azole resistance was similarly attributed to *cyp51A* (29).

Altogether, these data evidence that every *cyp51* paralog has different levels of involvement in azole response within the *Aspergillus* genus and other filamentous fungi. In this sense, assumption of analogous *cyp51* contribution towards azole response within the same genus or group of fungi is not acceptable.

Furthermore, regarding our expression results, no mechanism of transcriptional compensation appeared to occur when deleting either of the *cyp51* genes in the tested species of section *Nigri.* This is similar to what occurs in *A. fumigatus,* since transcriptional response of *cyp51B* after *cyp51A* deletion in this species remains unaltered (9). However, we generally found higher expression of *cyp51A* (in Δ*cyp51B* strains) than *cyp51B* expression (in Δ*cyp51A* strains) in the KO mutants. Moreover, minor expression differences were observed among *cyp51* basal expression of all strains tested here, like it had been described in wild type strains (15, 30), which reinforces the idea of no *cyp51* transcriptional compensation in section *Nigri*, even after deleting one of the two paralogs.

In accordance to what we had observed in our previous studies with wild type strains (15), VRC exposure in the KO mutants resulted in a clear upregulation of *cyp51A* expression while that of *cyp51B* was mildly affected by VRC. Greater impact on azole stress response of *cyp51A* than *cyp51B* has been suggested to occur in *A. fumigatus* by others (4), which could be supported to occur in section *Nigri* by our expression results. However, the basal ergosterol content within the cells did not differ between our Δ*cyp51A* and Δ*cyp51B* mutants. This suggests a similar contribution of both enzymes to the ergosterol biosynthetic pathway in the section. Nevertheless, and as we expected, ergosterol levels were notably lower in both, Δ*cyp51A* and Δ*cyp51B* strains than in their parental strains

(data and results not shown; manuscript in preparation). Reasonably, upon VRC exposure ergosterol was significantly and similarly depleted in all cases. We also investigated the intermediate sterol 24(28)DHE content in the KO mutants, since this sterol can replace ergosterol due to their structural and chemical resemblance (31, 32). As we found similar amount of this sterol in all strains in addition to its mild alteration upon VRC exposure, it seems that no evident compensatory effect of this sterol exists in section *Nigri*, even after deleting one of the two *cyp51* paralogs.

In summary, we have generated for the first time defective *cyp51* mutants in section *Nigri* strains with different genetic and VRC susceptibility backgrounds. We believe this approach has allowed us to investigate azole response within the section in a realistic and reliable manner, and thus our study might be useful to clearly decipher the *cyp51* role in azole stress, especially in *Aspergillus* section *Nigri.*

All in all, our study contributes new evidence to the understanding of azole resistance in non-*fumigatus* species of *Aspergillus*. In this context, we could not clearly correlate our *cyp51* expression, inducibility by VRC nor ergosterol content observations to initial VRC MICs displayed by our isolates, which reinforces the multifactorial nature and the molecular complexity of azole resistance in section *Nigri.* Therefore, deep analysis of the ergosterol biosynthetic pathway and the characterization of other potential resistance mechanisms in *Aspergillus* are essential to really establish its molecular basis.

MATERIALS AND METHODS

Strains, media and growth conditions. Five strains belonging to species *A. niger* and *A. tubingensis* of *Aspergillus* section *Nigri* (FMR 15441, FMR

14712, FMR 14635, FMR 11906 and FMR 15388) previously characterized and with different VRC susceptibilities (15) were used in this study. They were retrieved from the Facultat de Medicina de Reus (FMR) fungal collection and grown on potato dextrose agar (PDA) media (Conda-Pronadisa) for 3 to 5 days at 35º C twice before use.

For gene expression analyses, $1x10^7$ conidia/mL suspensions were germinated in YG medium (0.5 % yeast extract, 2 % glucose) at 37 ºC and 180 rpm for 12 h. After this, mycelia were filtered and aseptically transferred to new YG medium supplemented with VRC (Pfizer, Inc) at 4 µg/mL when required. Cultures were further incubated at 37 ºC and 180 rpm for 8 h and mycelia were harvested by filtration, washed with sterile distilled water, frozen in liquid nitrogen and stored at -80 ºC until use.

For sterol extraction, the same growth conditions were followed, with a final step consisting of lyophilization using the AdVantage Freeze Dryer (The VirTis Company Inc) and mechanical grinding.

Targeted gene deletion by CRISPR-Cas9. Generation of *cyp51A* or *cyp51B* single-gene KOs was carried following a previously described CRISPR-Cas9 protocol (22). Gene deletion was achieved by replacing the complete *cyp51A* or *cyp51B* ORFs with a repair template (Hyg^R) amplified from the pUCGH plasmid with primers introducing flanking microhomology regions of 40 bp adjacent to the upstream and downstream regions of either gene in every species (Table 2). Protoplast generation was performed as reported elsewhere (33) and fungal transformations were carried by using dual Cas9 ribonucleoprotein (RNP) complexes as follows: two crRNA sequences were designed to direct the Cas9 cleavage within the 5' UTR and the 3' UTR of each gene (Table 3), and were used, together with the tracrRNA, for the generation of the gRNA complexes. These

complexes were then combined with commercial Cas9 enzyme and Cas9 working buffer (20 mM HEPES, 150mM KCl, pH 7.5) to build the RNP complexes, which were used for protoplast transformation. The obtained transformants were phenotypically and molecularly analyzed to verify correct integration and recombination by means of hygromycin B (Hyg) resistance evaluation and by gDNA PCRs with the appropriate primer pair (*i.e.*: Fw scr – Rev scr, Fw scr – Rev intern and Fw intern – Rev scr) for every species and gene (Table 4 and Figure 1).

Antifungal susceptibility testing. For the *in vitro* susceptibility testing, the M38 protocol for broth microdilution developed by the Clinical and Laboratory Standards Institute (CLSI) (34) was followed. Briefly, stock solutions of VRC (Pfizer, Inc) were diluted in RPMI 1640 medium and dispensed in 96-well microdilution trays. Then, conidial suspensions of every strain were adjusted by hemocytometer count and inoculated onto every well. Trays were then incubated at 35 ºC for 48 h without light nor agitation. VRC MICs, corresponding to the lowest VRC concentration that completely inhibited fungal growth, were determined by direct visualization with an inverted mirror. *A. flavus* ATCC 204304 was used as the quality control strain.

RNA extraction and gene expression analysis. Mycelia were ground in liquid nitrogen with mortar and pestle and total RNA was extracted with the TRIzol reagent (Thermo Fisher) as described by others (35). The NucleoSpin RNA kit with an on-column DNAse digestion (Macherey-Nagel) was used to purify the RNA. Total RNA quality and quantity were determined by spectrophotometric analysis in NanoDrop2000 spectrophotometer (Thermo Fisher) and running aliquots in RedSafe-stained agarose gels.

For gene expression analysis, RNA was reverse transcribed into first-strand complementary DNA (cDNA) using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's instructions. Real time PCRs were carried in a StepOne Plus real-time PCR system (Applied biosystems) using 7.5 µL of TB Green Premix Ex Taq (Tli RNase H Plus) (Takara Bio), 6.6 µL of cDNA template, 0.3 µL of ROX and 300 nM of each gene-specific primer (15) in a final reaction volume of 15 µL. Thermal cycling conditions were as follows: 95 ºC (30 s), and 45 cycles of 95 ºC (5 s), 60 ºC (30 s) and 72 ºC (s). For all reactions, a melting curve was obtained after PCR completion to check amplification specificity under the following conditions: 95 ºC (15 s), 60 ºC (15 s) and 95 ºC (15 s).

Expression levels were calculated after normalization of *cyp51A* and *cyp51B* cycle thresholds (Ct) with those of the housekeeping gene *actA* (36).

Sterol extraction and quantification. Total sterol extraction was carried out as previously published (37) with some modifications. In brief, 60 mg (dry weight) of every sample were placed into borosilicate glass screw-cap tubes with 6 mL of 25 % alcoholic potassium hydroxide solution and vortex mixed for 1 min. After incubating the tubes at 85 ºC for 1 h in a water bath, 2 mL of sterile distilled water and 6 mL of n-heptane were added, tubes were vortex mixed and the upper-layer (n-heptane) was transferred to a new tube and stored at -20 ºC for a maximum of 20 h.

Sterol quantification was carried out as described elsewhere (31) by spectrophotometric scan between 240 and 300 nm with a NanoDrop2000 spectrophotometer (Thermo Fisher). Presence of ergosterol and the intermediate sterol 24(28)DHE in the extracted samples resulted in four-peaked curves. Both, ergosterol and 24(28)DHE absorb at 281.5 nm, but

24(28)DHE shows intense absorption at 230 nn, so ergosterol quantification can be determined by subtracting the 24(28)DHE content from the total sterol amount following the equations previously stablished by Arthington-Skaggs *et al.* (37).

Statistical analysis. The nonparametric Mann-Whitney test was used to compare relative gene expression values and ergosterol contents. All statistical analyses were performed with GraphPad Prism 6.0 for Windows. *P* values ≤ 0.05 were considered statistically significant.

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DECLARATION OF INTEREST

None to declare.

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TABLES

Table 1. Antifungal susceptibility testing results. Parental strains MICs were determined in a previous work (15). In bold, VRC MICs above the ECVs stablished by the CLSI.

Species	Parental strain	VRC MIC (µg/mL)	Δ*cyp51A* VRC MIC (µg/mL)	Δ*cyp51B* VRC MIC (µg/mL)
A. niger	FMR 15441	**8**	0.5	1
A. tubingensis	FMR 14635	**4**	0.5	1
A. tubingensis	FMR 14712	**4**	1	2
A. tubingensis	FMR 11906	2	0.5	1
A. niger	FMR 15388	1	0.5	1

Table 2. Oligonucleotides used for *cyp51* gene deletion. In capital letters, homologous region to the DNA template. In lowercase, homologous region to the Hyg^R repair template.

Primer	Sequence (5' – 3')	Species
Hyg Fw tail Ancyp51A	CTTGCTCTTGACTTCCTCTTTACAACAA TCTTTCTCATCAagcttgcatgcctgcaggtc	*A. niger*
Hyg Rv tail Ancyp51A	AGGTACTAGTTGGGAAAAGACTGGAGA CGAAAGGTAACCTGCcatcgatgatatcagatc	*A. niger*
Hyg Fw tail Ancyp51B	CCCAAAGTGCACTTTCCTGCGCCGTTAC AAGCAATTTACAagcttgcatgcctgcaggtc	*A. niger*
Hyg Rv tail Ancyp51B	ATTTCATCGTATTACATGAGAGGTTTAA GTACAATCACCGAAcatcgatgatatcagatc	*A. niger*
Hyg Fw tail Atcyp51A	TCCCCATCAATTGGTCCTTTTTCCTGCC TACGGTCGCTTCagcttgcatgcctgcaggtc	*A. tubingensis*

Hyg Rv tail Atcyp51A	CTAGTTGGGAAAGACTGGAGATGAAAG ATAGCTTGCAGAGAAcatcgatgatatcagatc	*A. tubingensis*
Hyg Fw tail Atcyp51B	CTTCCTTGTGAACACAACCAACTCACTC CAATTGTCTGTTagcttgcatgcctgcaggtc	*A. tubingensis*
Hyg Rv tail Atcyp51B	GGTTTGAGAACAATCACAAAAGGCGGT TTCTATGCAGTAACAcatcgatgatatcagatc	*A. tubingensis*

Table 3. crRNA sequences used for genetic transformation.

crRNA	**Sequence (5' – 3')**	**Species**
Ancyp51A 5' gRNA	CAACAATCTTTCTCATCAAC	*A. niger*
Ancyp51A 3' gRNA	GAATTAGGCCACCTTCTTGC	*A. niger*
Ancyp51B 5' gRNA	CCGTTACAAGCAATTTACAC	*A. niger*
Ancyp51b 3' gRNA	TACTGTATAGAAACCGCGTT	*A. niger*
Atcyp51A 5' gRNA	TCCTGCCTACGGTCGCTTCC	*A. tubingensis*
Atcyp51A 3' gRNA	AAAGATAGCTTGCAGAGAAG	*A. tubingensis*
Atcyp51B 5' gRNA	CTCACTCCAATTGTCTGTTC	*A. tubingensis*
Atcyp51B 3' gRNA	CGGTTTCTATGCAGTAAGAG	*A. tubingensis*

Table 4. Oligonucleotides used as PCR primers for *cyp51A* and *cyp51B* knock-out screening.

Primer	**Sequence (5' – 3')**	**Species**
Ancyp51A Fw scr	GAACGATGCAGCTCAGTGCCA	*A. niger*
Ancyp51A Rev scr	ACACGAGGGATCTCACGTGC	*A. niger*
Ancyp51B Fw scr	CCTGCTACTAGGAGAGCCGC	*A. niger*
Ancyp51B Rev scr	GCCTGTATGGGACAGTCGCT	*A. niger*
Atcyp51A Fw scr	CCGATGTAGCTCAGTGCCAGG	*A. tubingensis*
Atcyp51A Rev scr	ACTGGGCAGCGGTCATATCTC	*A. tubingensis*
Atcyp51B Fw scr	GTGCACTTTCCTGCGCTGTTG	*A. tubingensis*
Atcyp51B Rev scr	CCGGCCATTAAAGCCCCAAG	*A. tubingensis*

Hyg Fw intern	CGATTCCTTGCGGTCCGAAT	All
Hyg Rev intern	ATCGGCACTTTGCATCGGC	All

FIGURES

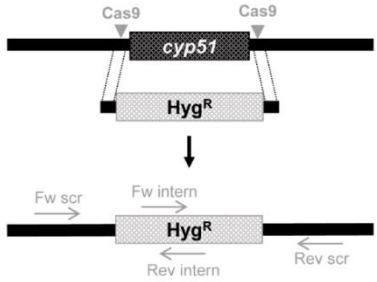

Figure 1. *cyp51* deletion strategy using a CRISPR-Cas9 gene targeting technique and a repair template with microhomology regions to the target genes. Mutant confirmation was carried by three different gDNA PCRs using four primers.

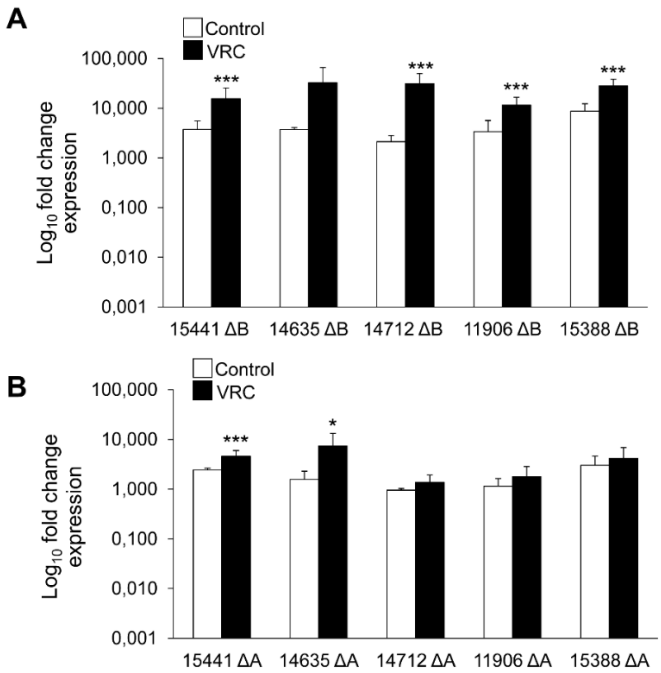

Figure 2. Gene expression analysis results of the (A) *cyp51A* gene in Δ*cyp51B* strains and the (B) *cyp51B* gene in Δ*cyp51A* strains. In white bars, results of the

control condition; in black bars, results after exposure to 4 µg/mL VRC for 8 h. The *actA* gene was used in all cases as the housekeeping gene to normalize transcription levels. Statistical significance is marked by asterisks (* $P \leq 0.05$; *** $P \leq 0.001$).

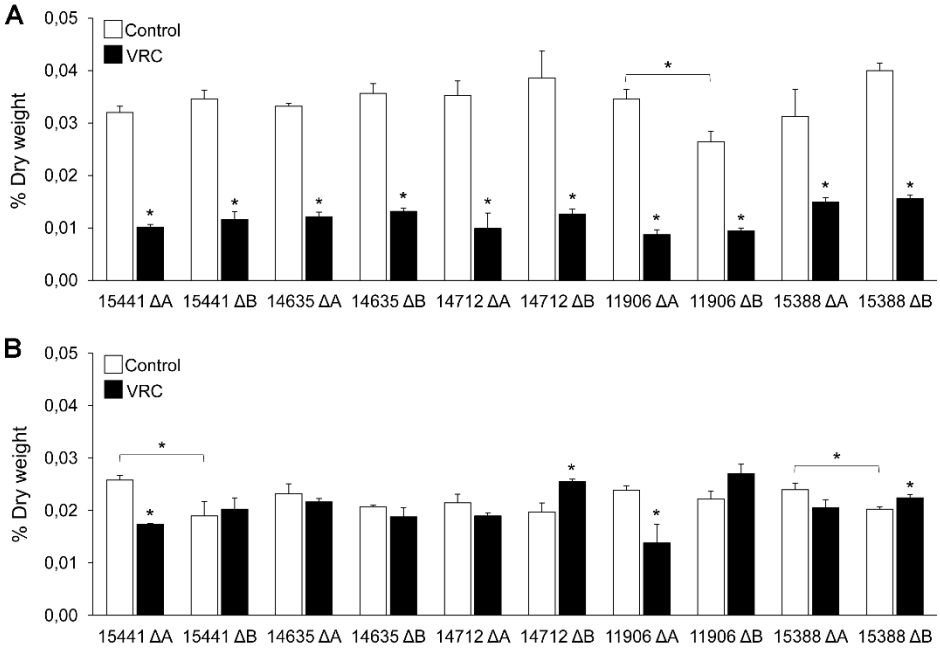

Figure 3. Quantification of (A) ergosterol and (B) the intermediate sterol 24(28)DHE, expressed as percentage of the mycelia dry weight. In white bars, results of the control condition; in black bars, results after exposure to 4 µg/mL VRC for 8 h. Statistical significance ($P \leq 0.05$) is marked by an asterisk (*).

5.4. Ergosterol quantification, membrane and cell wall characterization of *Aspergillus* section *Nigri* strains with different voriconazole susceptibility

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STUDY AND CHARACTERIZATION OF AZOLE RESISTANCE IN ASPERGILLUS SECTION NIGRI
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ABSTRACT

The plasma membrane and cell wall are essential structures for fungal architecture but also for physiology, interaction with extracellular substrates and stress response. Antifungals, and specifically azoles, target the plasma membrane by inhibiting the biosynthesis of ergosterol, one of its fundamental components. In addition, fungicidal effect of azoles has been linked to cell wall targeting. Since azole response and resistance in non-*fumigatus* species of *Aspergillus* remains poorly investigated, we aimed to characterize azole response with a phenotypic approach in fourteen strains from species of the section *Nigri* (*Aspergillus niger, A. tubingensis* and *A.* brasiliensis) displaying different VRC susceptibilities. We focused on their ergosterol content, plasma membrane and fungal cell wall composition. Results showed that strains with the lowest VRC susceptibility drastically reduced levels of ergosterol compared to the rest of strains, suggesting that hyper-susceptibility towards this antifungal might be connected to alterations in basal ergosterol content and membrane properties. Membrane and cell wall assays revealed increased permeability of the plasma membrane of the VRC susceptible strains as well as an increased content of chitin in the most resistant ones, which might affect azole import into the cell. Finally, to test whether conidia presented any hardiness differences among strains, we evaluated their resistance to mechanical stress, which showed no apparent relationship with VRC MICs. This study provides evidence, for the first time to our knowledge, on the relation between ergosterol content and azole susceptibility in *Aspergillus* section *Nigri* as well as the potential contribution of fungal membrane and cell wall properties to azole response.

INTRODUCTION

The fungal membrane is a fundamental component of the cell, showing a pivotal role in fungal structure and physiology. It acts as a protective barrier, and it is important for the secretion of virulence factors, cell wall synthesis, hyphal morphogenesis, endocytosis, and nutrient uptake (1, 2). Sterols are essential lipid molecules that carry out structural functions and maintain the fungal cell membrane integrity and fluidity. More specifically, while cholesterol is the major sterol in vertebrates and stigmasterol or sitosterol are majorly found in plants, ergosterol is the main sterol found in fungal membranes (3–5). In addition, fungal ergosterol participates in biological processes related to the activity of membrane-bound enzymes, cell growth and viability among others (3, 6, 7). Furthermore, lipid microdomains in fungal membranes consisting of sphingolipids and ergosterol have been associated to fungal virulence (2).

The fungal wall is another relevant structure of the cell, essential for viability, morphogenesis, pathogenesis and interaction with substrates and other organisms. Curiously, the composition of the wall slightly differs among fungal species. In *Aspergillus,* the cell wall core is composed of β-glucans, which are covalently attached to chitin forming the structural component of the wall with a layer of mannan on its outer part (8, 9).

Very interestingly, azole drugs have been described to affect *Aspergillus* fungal membrane by targeting the ergosterol biosynthetic pathway through the inhibition of 14 α-demethylase enzymes (Cyp51). This is translated into ergosterol depletion and accumulation of toxic sterol precursors (10, 11), reduction of membrane-bound enzymes activity, inhibition of fungal cell growth and disruption of the growth and proliferation stimulation (12–14). In addition, azoles have also been reported to affect the fungal cell wall.

This seems to occur after activation of the cell wall salvage system due to azole stress, which promotes an excessive synthesis of β-1,3-glucan within the cell wall. These carbohydrates penetrate the cell membrane forming invaginations and causing the final rupture of the cell membrane, which leads to the death of the fungal cell (15).

Although azoles continue to be the treatment of choice for invasive aspergillosis (IA) (16), the alarming increase of azole resistance incidence greatly impacts and impairs the clinical outcome of the disease (17, 18).

Since azole resistance has been poorly characterized in non-*fumigatus* species of *Aspergillus*, in this work we aimed to characterize azole resistance in a set of strains of species of section *Nigri* (*Aspergillus niger, A. tubingensis* and *A. brasiliensis*) with different VRC susceptibility using a phenotypic analysis approach. Due to the importance of membrane and cell wall in fungal viability and azole response, we have investigated and characterized the ergosterol content and the cell membrane and wall composition of such fungi.

MATERIALS AND METHODS

Strains. Fourteen strains belonging to different species of *Aspergillus* of the section *Nigri* (*A. niger, A. tubingensis* and *A. brasiliensis*) were obtained from mineral oil cultures of the Facultat de Medicina de Reus (FMR) collection. Nine strains were environmental (FMRs 14630, 14635, 14712, 15385, 15386, 15388, 15389, 15441 and 17207) and had been isolated from samples of different countries. The rest were clinical strains from sputum or skin lesions (FMRs 11254, 11894, 11898, 11900 and 11906). The VRC MICs of these fungi ranged from 0.25 to ≥ 16 μg/mL (19). On basis of the current VRC Epidemiological Cutoff Values (ECVs),

strains were categorized as wild type (wt; VRC MIC ≤ 2 µg/mL) or non-wild type (non-wt; VRC MIC > 2 µg/mL) (19, 20).

Media and growth conditions. Before their use, strains were grown on Potato Dextrose Agar (PDA) media (Conda-Pronadisa) for 3-5 days at 35 ºC twice.

For sterol analysis, conidia were collected by flooding the culture plates with 0.1 % Tween20 solution and gentle agitation. The conidial suspensions were adjusted by hemocytometer count to a final concentration of $1x10^7$conidia/mL in 50 mL of YG broth (0.5 % yeast extract, 2 % glucose) and germinated for 12 h at 37 ºC and 180 rpm. Mycelia were then harvested by filtration and aseptically transferred to either fresh new YG medium or new YG medium supplemented with 4 µg/mL VRC (Pfizer Inc). Cultures were further incubated at 37 ºC and 180 rpm for 8 h and mycelia were collected by filtration, washed with distilled water and lyophilized with the AdVantage Freeze Dryer (The VirTis Company Inc). Finally, mycelia were ground with a grinder and total dry weight was determined.

Sterol extraction and ergosterol quantification. Sterol extraction was performed as previously described with some modifications (21). Briefly, 60 mg of dry ground mycelia of each sample were placed into borosilicate glass tubes and 6 mL of 25 % alcoholic potassium hydroxide solution (25 g of KOH and 35 mL of sterile distilled water, brought to 100 mL with 100 % ethanol) was added to each tube. Samples were vortexed and incubated at 85 ºC in a water bath for 1 h. After tubes were cooled to room temperature, sterol extraction was carried by the addition of 2 mL of sterile distilled water and 6 mL of *n*-heptane. Samples were again vortexed for 3

min and the upper layer (heptane) was transferred to a clean borosilicate glass tube and stored at -20 ºC.

Sterol quantification was carried out as described elsewhere by spectrophotometric scanning between 240 and 300 nm with a NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific) (22). Presence of ergosterol and 24(28)DHE in the extracted sample resulted in a four-peaked curve.

Plate sensitivity assays. For membrane and cell wall assays, conidial suspensions were prepared and adjusted by hemocytometer count to 10^6 conidia/mL. Serial dilutions from the starting inoculum were carried out to obtain 10^5 and 10^4 conidia/mL, respectively. Next, each inoculum concentration was spotted onto Minimal Medium (SM) agar (0.2 g/L MgSO4·7H2O, 0.4 g/L KH2PO4, 0.2 g/L KCl, 1g/L NH4NO3, 0.01 g/L ZnSO4, 0.01 g/L MnCl2, 0.01 g/L FeSO4, 10 g/L glucose and 15 g/L agar) supplemented with the indicated agents when required. To qualitatively assess membrane fluidity and permeability, disturbing agents Sodium Dodecyl Sulfate (SDS) (0.01 %) and Ethylenediaminetetraacetic Acid EDTA (50 mM) were used as SM supplements. Additionally, analyses of cell wall composition and integrity in our strains were carried with SM supplemented with cell wall disturbing agents Calcofluor White (CFW) (200 µg/mL), Congo Red (CR) (400 µg/mL) and Caffeine (CAFF) (Sigma) (20mM).

All plates were incubated at 28 ºC and growth was assessed for every strain and condition.

In parallel, membrane and cell wall assays were quantitatively performed following a recently published method (23). Briefly, SM and SM supplemented with the previously indicated agents at the same

concentrations when required were placed in 96-wells plates. Afterwards, 30 μL of a 10^6 conidia/mL suspension of every strain were inoculated, and plates were incubated at 28 ºC to assess fungal growth. Growth quantification was carried by 620 nm absorbance reading at different time intervals.

Conidia resistance to disruption. Resistance of conidial walls to mechanical disruption was evaluated as elsewhere described (24). In brief, 500 μL of a 10^7 conidia/mL suspension of every strain were placed in Eppendorf tubes with 425-600 μm glass beads (Sigma). Then, conidia were disrupted for 1 min in a Fast-prep cell breaker and suspensions were plated on PDA and incubated at 30 ºC until visible colonies could be counted. Non-disrupted suspensions were used as controls to calculate percentage of conidia survival.

Statistical analysis. The Mann-Whitney test was used to compare ergosterol and 24(28)DHE content as well as conidial resistance to disruption. All statistical analyses were performed using GraphPad Prism 6.0 for Windows. *P* values ≤ 0.05 were considered statistically significant.

RESULTS

Ergosterol quantification. Contents of ergosterol and intermediate sterol 24(28) dehydroergosterol (DHE) were determined for all strains in control conditions and after VRC exposure (Figure 1). Basal ergosterol levels slightly fluctuated among strains, ranging from 0.053 % to 0.016 % (dry weight). Although no pattern or correlation to their VRC susceptibility was observed, the most susceptible strains (FMRs 11894 and 11898) showed the lowest ergosterol content, which was 3 to 5-fold lower than in the other strains (Figures 1A and B). Upon VRC exposure, all strains suffered a

significant ($P \leq 0.0290$) ergosterol depletion, with 0.0321 % to 0.0079 % (dry weight) of ergosterol (Figure 1).

Regarding 24(28)DHE, its basal levels were lower than those of ergosterol in all strains, ranging from 0.032 % to 0.008 % (dry weight), with strains FMR 11894 and FMR 11898 showing the lowest values (Figure 1C). After VRC exposure, 24(28)DHE content was not as altered as ergosterol and was only found significantly different than in the control condition in strains FMR 11900, 15386, 14630 and 11906 ($P \leq 0.0286$). Similar to the values obtained for the basal state, 24(28)DHE levels after VRC response ranged between 0.033 % and 0.006 % (dry weight).

Plate sensitivity assays. Qualitative assessment of cell wall and membrane disturbing agents' effects revealed diversity among strains and agents. Regarding membrane assays, wild type strains FMRs 11898 and 11254 showed the maximum growth at 0.01 % SDS, whereas FMRs 14630, 11906 and 11894 showed the most inhibited growth in SDS presence. Differently, results upon 50 mM EDTA showed the greatest inhibition for wild type (wt; *i.e.*: VRC MIC ≤ 2 μg/mL (20)) strains FMRs 15385, 15389, 11254, 15388 and 11898, while little or no effect was exerted by this substance towards non-wild type (non-wt; *i.e.*: VRC MIC ≥ 2 μg/mL (20)) FMRs 11900, 15441, 14712 and wild type FMR 11894 (Figure 2).

In relation to cell wall studies, presence 400 μg/mL of CR, had little and similar effect on all strains, showing slightly lower effect on strains FMRs 11900 and 11894. Growth under the medium supplemented with CFW (200 μg/mL) was more impaired in non-wt strains, since bigger colonies were obtained in the case of wt strains. Finally, CAFF (20 mM) completely inhibited growth in most of the strains, except for strains FMRs 11900, 14630, 15385 and 11894 (Figure 2).

A quantitative method was followed in parallel with the same media and supplements. In this case, OD_{620} measurements were taken to assess biomass accumulation at every condition, although results revealed no large differences among strains in every case of study (Figure 3).

The maximum OD_{620} value for growth on CFW-supplemented media was found to be 1.989 (wt strain FMR 11894), while minimum OD_{620} was 1.569, that of wt strain FMR 15388. In the case of CAFF-, SDS- and EDTA- supplemented media, maximum OD_{620} were also found to belong to wt strain FMR 11894, with values 1.848, 1.509 and 1.579, respectively. The lowest OD_{620} values observed in these media, belonged to diverse strains (*i.e.* OD_{620} 1.498 for wt strain 15385 in CAFF, OD_{620} 1.571 and 0.3525 for non-wt FMR 15386 in SDS and EDTA, respectively). Regarding CR, OD_{620} ranged between 2.778 (non-wt strain FMR 15386) and 1.8165 (non-wt strain FMR 17207) (Figure 3).

Conidia resistance to disruption. Conidia resistance to mechanical stress was assessed in terms of viability after physical disruption. Results showed that viability after disruption fluctuated between 75.0 and 34.1 % in the case of non-wt strains, with strains FMRs 14712 and 15386 displaying the maximum and minimum viability of the group, respectively. With regard to wt strains, viability ranged from 67.0 to 50.3 %, with the highest values for FMRs 15385 and 11898, and the lowest value for the strain FMR 11894 (Figure 4). Despite the fluctuations observed in the data obtained, no statistically significant differences were found among strains.

DISCUSSION

Due to the importance of membrane and cell wall in fungal viability and stress response, in this work we aimed to characterize potential differences

among these structures in a set of strains of species of *Aspergillus* section *Nigri* with different VRC susceptibility, for the first time to our knowledge. Within the fungal membrane, ergosterol is an essential component in terms of fluidity, permeability, microdomain formation as well as other important structural and signaling activities (25). It is also a very important target for antifungal therapies, such as those based on azoles activity, which are the preferred strategy when dealing with IA (16). These compounds directly block its biosynthesis leading to its depletion and the accumulation of other sterols, which triggers a severe membrane stress and results in the loss of membrane stability and discontinuation of fungal growth (26). In this context, we hypothesized that susceptibility differences among our set of section *Nigri* strains might be related to their ergosterol levels. Thus, we assessed the ergosterol and intermediate sterol 24(28)DHE contents of such strains as well as their adjustment upon VRC-induced stress. Importance of intermediate sterol 24(28)DHE relies on the fact that its structure and chemical properties are very similar to ergosterol, so it can replace it with no functional interferences in fungi (22, 27). The results we obtained, revealed that basal ergosterol and 24(28)DHE or their alteration upon VRC exposure failed to explain VRC resistance in section *Nigri*. Nevertheless, we found that strains with the lowest VRC MIC displayed drastically decreased levels of this sterol compared to the other strains, even after non-wt strains had been treated with VRC. These data imply that hyper-susceptibility towards VRC in the species of this section might be justified by alterations in the membrane fluidity due to the low ergosterol content. Some stable genetic modification in the ergosterol biosynthetic pathway of the most susceptible strains, although most probably not related to *cyp51*,as we have already discussed in another work (19), could be the reason of the

phenomenon. This contrasts to previous observations in *A. fumigatus*, since ergosterol analyses in strains with VRC MICs from ≥ 8 μg/mL to 0.5 μg/mL revealed that those isolates showing the highest VRC MICs were the ones with superior ergosterol content (28). However, minor and negligible changes in the basal sterol content were found among *A. fumigatus* isolates with diverse itraconazole susceptibility (29). This divergence within the same species evidences some degree of diversity among strains but might also be due to differences in the activity exerted by the specific azole used in every study.

In other fungi, such as *Saccharomyces cerevisiae,* lipid membrane composition has already been linked to differences in drug susceptibility (30). In *Candida albicans*, resistant isolates showed increased ergosterol content and altered membrane fluidity compared to the susceptible isolates tested (31, 32). In another study with filamentous fungi, such as some clinically relevant Mucorales and *A. fumigatus,* ergosterol and 24(28)DHE levels, were investigated among other sterols before and after posaconazole exposure . Not surprisingly, ergosterol was depleted by the azole treatment in all cases. However, although 24(28)DHE levels decreased after azole exposure in *Rhizopus arrhizus*, those of *A. fumigatus* were practically unaltered (33). Unexpectedly, 24(28)DHE showed no major alteration upon VRC treatment in the species of section *Nigri* tested here , and we could not observe a proportional tendency among its contents and VRC susceptibility. Furthermore, taking into account that 24(28)DHE has the ability to fulfill ergosterol functions (22), we would expect a notable increase of this sterol intermediate after azole exposure to compensate for the depletion of ergosterol. Nevertheless, our findings seem to indicate that little or no compensatory effect of 24(28)DHE exists in section *Nigri*, at

least in the conditions we tested. It remains unknown whether other sterol intermediates could functionally compensate ergosterol depletion and if their levels could be linked to azole resistance. Further and extensive studies are needed to characterize and profile sterol biosynthesis within section *Nigri* and alteration of its intermediates upon azole exposure.

Additionally, others have hypothesized that modifications on drug import might illustrate why some fungi are more resistant to azoles than others (34). In this context, we analyzed membrane fluidity and permeability in our set of strains by performing plate assays with synthetic compounds SDS and EDTA. While SDS is a well-known membrane perturbing agent (35), EDTA has been described to exert a fluidizing and permeabilization effect after intercalating lipid membranes and sequestering metal ions (36). In the case of SDS, our results generally show comparable effects of the agent against the whole set of strains. However, fluidizing agent EDTA more prominently impacted wt strains, which showed impaired growth when compared to the non-wt strains. This, points to enhanced permeability of the plasma membrane in the VRC susceptible strains, that could contribute to enhance azole activity against these strains by facilitating the entrance of VRC into the cell. In addition, it has been recently described that permeability increases in the plasma membrane of *A. fumigatus* are associated to an induction of oxidative stress within the cell (37). Most probably, activation of this pathway and imbalance in the production of free radicals and reactive oxygen species might entail oxidative damage and deleterious effects against the fungal cell, acting in synergy with VRC.

Another important structure indirectly targeted by azoles is the fungal cell wall (15, 38), thus, alteration in its composition could also contribute to the appearance of differences in azole response. In this work, we analyzed the

content of structurally important components of the fungal wall chitin and β-1,3-glucan. Specifically, we performed plate assays with both, chitin-binding agent CFW and β-1,3-glucan-binding agent CR, which have been proven to cause swelling or lysis of hyphal tips in *A. niger* (39). Another agent we used was CAFF, which has been reported to stimulate the phosphorylation of a few components in the cell wall integrity signal transduction (40) and thus presumed to activate and participate in stress signaling. Our results suggest similar β-1,3-glucan profile in all the strains tested, but slightly enhanced chitin content in the non-wt strains, as evidenced by a lightly lower growth of these as opposed to the wt strains. Finally, hypersensitivity to caffeine was observed in a few strains with no tendency related to VRC susceptibility and was exemplified with a complete inhibition of growth. As others have pointed out, this might be explained by modifications in the Target Rapamycin Complex (TORC) signaling pathway (41). It remains unknown if this could have any implication on antifungal response. It is worth mentioning that qualitative observations could not be exactly reproduced with the quantitative approach recently validated based on spectrophotometric analysis (23), suggesting optical density is not the best parameter to assess membrane or cell wall stress in this group of fungi.

Similarly, it has been described that physical properties and defects of rodlets on the conidial cell wall surface can impact fungal drug sensitivity (24). We aimed to test this hypothesis; however, conidia from the strains we analyzed here showed no hypersensitivity nor enhanced resistance to physical disruption, which implies similar hardiness and toughness among conidia, suggesting there is no physical or mechanical contribution to VRC response in conidia from species of section *Nigri.*

All considered, this study provides, for the first time to our knowledge, a connection between ergosterol content and azole susceptibility in *Aspergillus* section *Nigri*. We also contribute new data to the hypothesis that fungal membrane and cell wall properties could potentially be related to differences in azole response, even at the strain level within a same species or section. Extensive studies on this would be of great importance to elucidate the details behind this phenomenon.

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DECLARATION OF INTEREST

None to declare.

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FIGURES

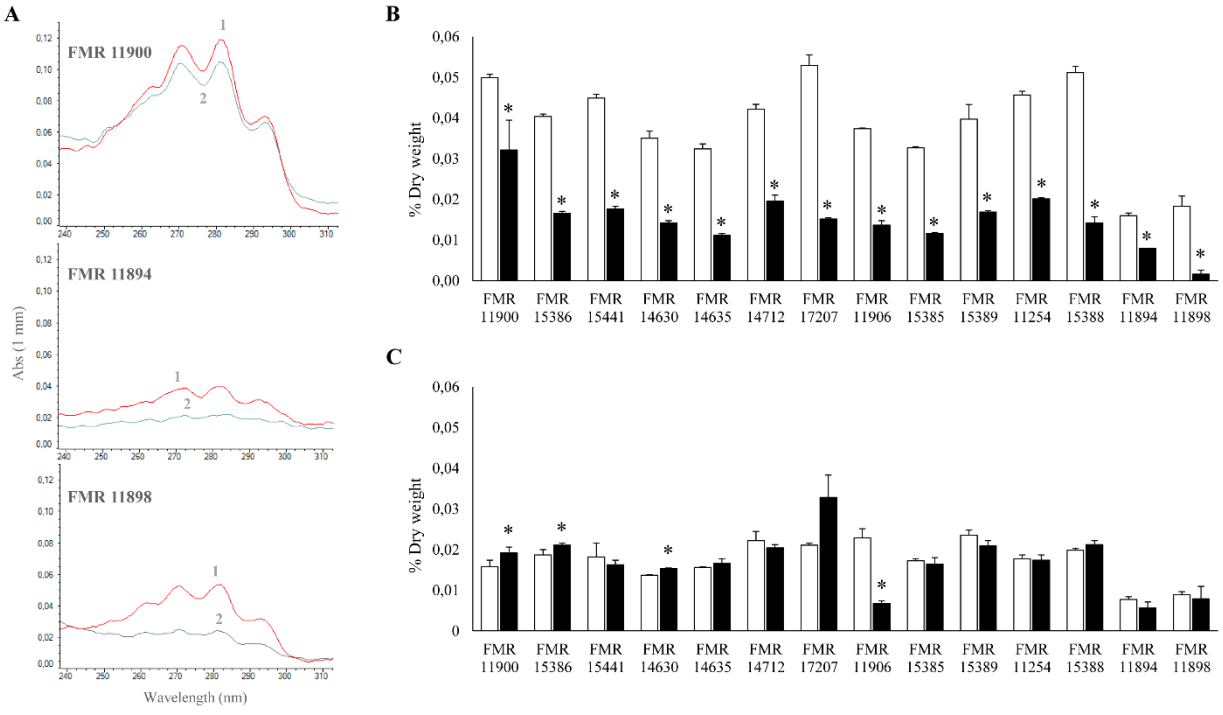

Figure 1. Ergosterol and intermediate sterol 24(28)DHE quantification. **(A)** UV spectrophotometric profiles of the most resistant and susceptible strains of the set tested (1) in basal conditions and (2) after 4 µg/mL VRC exposure for 8 h. **(B)** Ergosterol and **(C)** intermediate sterol 24(28)DHE content expressed as percentage of dry weight of the cell in control condition (white bars) and

after exposure to 4 µg/mL VRC for 8 h (black bars). Strains are ordered from left to right in a VRC MIC decreasing scale. Statistical significance ($P \leq 0.05$) is depicted by an asterisk (*)

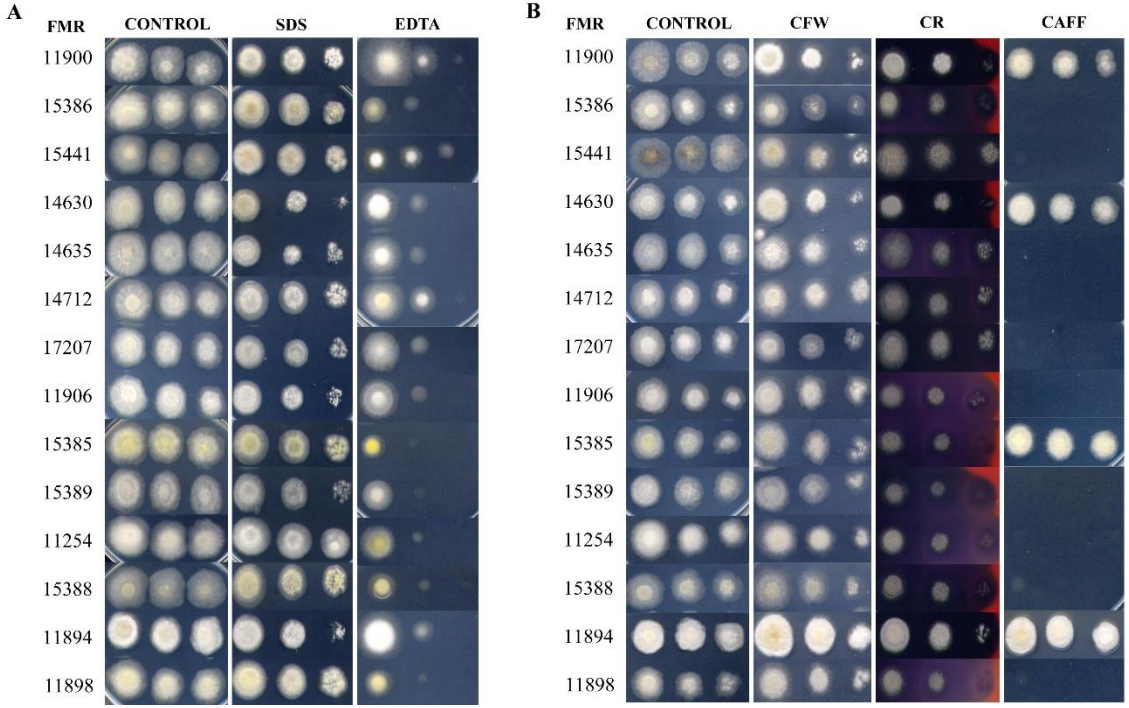

Figure 2. Plate sensitivity assays with 10^4, 10^3 and 10^2 conidia spots. **(A)** Qualitative assessment of membrane fluidity and permeability in SM supplemented with 0.01 % SDS and 50 mM EDTA. **(B)** Qualitative assessment of cell wall properties in SM

supplemented with 200 µg/mL CFW, 400 µg/mL CR and 20 mM CAFF. Strains are ordered from top to bottom in a VRC MIC decreasing scale.

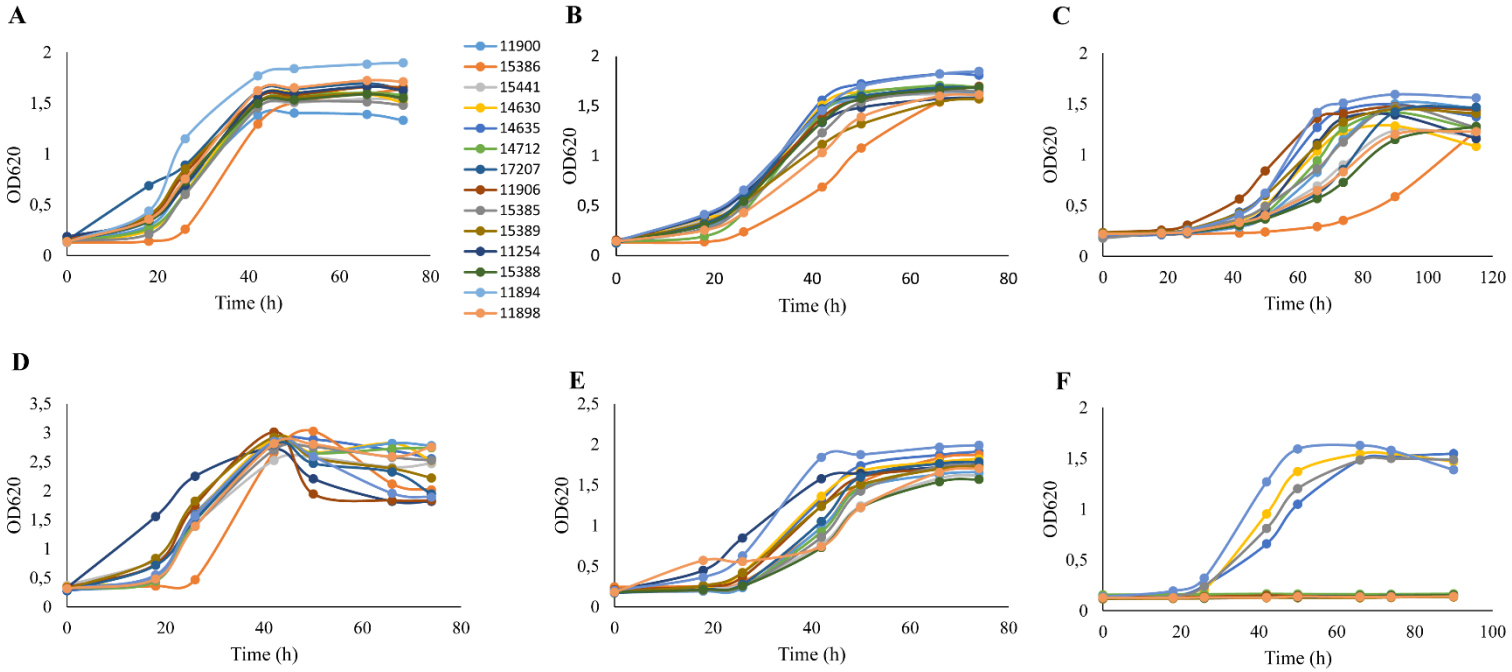

Figure 3. Quantitative assessment of membrane and cell wall properties through time. **(A)** control (non-supplemented SM); **(B)** 0.01 % SDS; **(C)** 50 mM EDTA; **(D)** 200 µg/mL CFW; **(E)** 400 µg/mL CR and **(F)** 20 mM CAFF. Legend represents FMR codes.

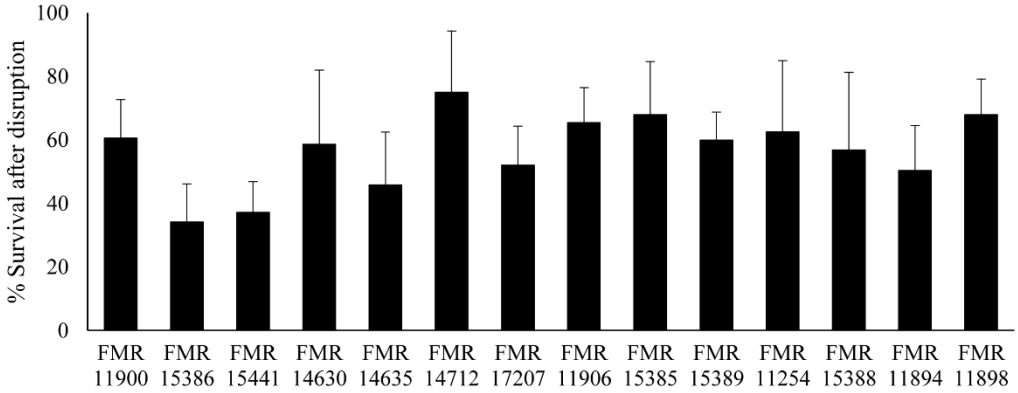

Figure 4. Percentage (%) of conidia survival after mechanical disruption. Strains are ordered from left to right in a VRC MIC decreasing scale

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5.5. Multicopper oxidase study in *Aspergillus niger* strains with different azole susceptibility: gene expression and laccase activity analyses

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UNIVERSITAT ROVIRA I VIRGILI
STUDY AND CHARACTERIZATION OF AZOLE RESISTANCE IN ASPERGILLUS SECTION NIGRI
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ABSTRACT

Multicopper oxidase (MCO) enzymes catalyze the oxidation of a large number of substrates. Among these enzymes, laccases, display elevated importance for industrial and biotechnological applications. Additionally, fungal laccases display an essential participation in biological processes, stress response, and degradation of various substrates, such as azoles or other drugs. In this work we aimed to analyze whether azole resistance in *Aspergillus niger* could be associated to laccase activity, by means of laccase gene expression and protein activity analyses in *A. niger* strains with diverse azole susceptibility. Our results show that gene expression of this group of enzymes is strain-dependent in both, basal conditions and variable patterns of expression were observed for every strain upon voriconazole (VRC) exposure. Interestingly, activity assays revealed that laccase activity is enhanced by VRC in a direct concentration-dependent manner, although no proportional relation could be established between laccase activity and the VRC MICs displayed by the tested strains. In summary, this work suggests for the first time to our knowledge, that laccase action is induced by azole stress in *A. niger*.

INTRODUCTION

Multicopper oxidases (MCOs) are a family of ubiquitous enzymes that catalyze the oxidation of a wide range of substrates. Specifically, they use oxygen as an electron acceptor, carrying out the reduction of molecular oxygen to water (1, 2). This group of enzymes include ferroxidases, ascorbate oxidases, bilirubin oxidases and laccases, which are found in numerous organisms (*i.e.* fungi, plants, insects, bacteria or humans) and take part in a wide variety of physiological roles (1, 3).

Within MCOs, the subgroup constituted by laccase proteins is the largest. These copper-containing enzymes display non-specific and diverse spectrum of substrates, which make them very useful for multiple biotechnological applications in different industries and fields such as textile, food, biofuel, organic synthesis, bioremediation, paper, pharmaceutical or cosmetic industries (4).

In fungi, laccases are involved in various processes such as fruiting body formation, pigmentation or pathogenicity, and they are essential for defense mechanisms, virulence and stress response. As a matter of fact, these enzymes facilitate the evasion of the fungus from the host's immune system and they promote stress adaptation to heavy metals, pro-oxidants and fungicides (5). In addition, fungal laccases are responsible for the degradation of a wide range of organic substrates and contaminants, such as aromatic compounds, some metals, bisphenol A, herbicides, pesticides and drugs. (6–8). Interestingly, fungal laccases have been associated to azole degradation, accomplished after the transformation of these drugs into other products with lower antifungal activity and reduced toxicity (9, 10).

On another note, phylogenetic analyses have demonstrated that fungal laccases are one of the most ancient metalloproteins. In fact, modern laccase gene families might have resulted from duplications of few ancestral enzymes and paralog diversification through evolution (2–4). The outcome of this genetic redundancy is a diverse group of laccase-like proteins that might play diverse physiological roles and display different regulation depending on environmental conditions (11).

In this sense, many genes coding for laccase-like MCO proteins can be found in the *Aspergillus niger* genome, which vary in a strain-dependent

manner from 13 to 16 different MCOs (11, 12). These are divided in four subgroups depending on their predicted functions. Accordingly, McoA, McoB, McoC, McoO and McoP are considered fungal pigment multicopper oxidases; McoD, McoF, McoG, McoI, McoJ, McoM and McoN belong to the laccase subgroup; McoE is a ferroxidase with weak laccase activity; and finally, McoH, McoK and BrnA are included in the fungal ferroxidases subgroup (11).

Considering that azole degradation is considered a potential antifungal resistance mechanism (13) and that fungal laccases can be involved in this process, we aimed to characterize the potential relationship between voriconazole (VRC) susceptibility and laccase activation. With this purpose, we analyzed laccase gene expression induction and protein activity in four *A. niger* strains with different azole susceptibility.

MATERIALS AND METHODS

Fungal strains and growth conditions. Four *A. niger* strains with different VRC susceptibility were retrieved from the Facultat de Medicina de Reus fungal collection (FMR) and used in this study. These strains had been previously categorized as non-wild type (non-wt) FMR 11900 (VRC MIC ≥ 16 µg/mL) and FMR 15441 (VRC MIC = 8 µg/mL) or wild type (wt) FMR 15388 (VRC MIC = 1 µg/mL) and FMR 11898 (VRC MIC = 0.25 µg/mL) (14) according to the current VRC Epidemiological Cutoff Value (ECV; *i.e.*: 2 µg/mL) established by the Clinical and Laboratory Standards Institute (CLSI) (15).

Before used, they were incubated and grown in Potato Dextrose Agar (PDA; Conda-Pronadisa) for 3-5 days at 35ºC twice. Then, conidia

suspensions were prepared after flooding the PDA culture plates with a solution of 0.1 % Tween20 and gentle agitation.

For gene expression analysis and extracellular protein obtention, conidia suspensions were adjusted by hemocytometer count to 1×10^7 conidia/mL and germinated in YG medium (0.5 % yeast extract, 2 % glucose) for 12 h at 37°C and 180 rpm. Afterwards, mycelia were harvested and transferred to fresh YG medium supplemented with 4 μg/mL of VRC (Pfizer, Inc.) when required. Cultures were further incubated at 37°C and 180 rpm for 8 h in the case of gene expression samples and mycelia were collected by filtration, quickly frozen in liquid nitrogen and stored at –80°C until use. Cultures for extracellular protein obtention were incubated in the same conditions for 24 h, culture media supernatant was sterile filtered and kept on ice.

Sequence retrieval and primer design. *A. niger* genomic *mco* sequences were identified and retrieved from the Aspergillus Genome (AspGD) and the Joint Genome Institute (JGI) databases (*A. niger* strains ATCC 1015 and CBS 513.88).

Primers were designed with the Oligo7 software (Molecular Biology Insights).

RNA isolation. For RNA extraction, frozen mycelia were ground in liquid nitrogen with mortar and pestle. Extraction of total RNA from ground mycelia was performed with TRIzol reagent (Thermo Fisher) following a previous protocol (16). Then, RNA was purified with the NucleoSpin RNA kit (Macherey-Nagel), which includes an on-column DNAse digestion step. The resulting RNA pellets were resuspended in DNase- and RNAse-free sterile water. RNA quantity and quality were checked by visualization after running aliquots in RedSafe-stained agarose gels as well as

spectrophotometric analysis in a NanoDrop2000 spectrophotometer (Thermo Fisher).

Retrotranscription and quantitative real-time PCR. For gene expression analysis, total RNA was reverse transcribed into first-strand complementary DNA (cDNA) using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's instructions. Real-time PCRs were carried in a StepOne Plus real-time PCR system (Applied biosystems) using 7.5 µL of TB Green Premix Ex Taq (Tli RNase H Plus) (Takara Bio), 6.6 µL of cDNA template, 0.3 µL of ROX and 300 nM of each gene-specific primer (Table 1) in a final reaction volume of 15 µL. Thermal cycling conditions were as follows: 95 ºC (30 s), and 45 cycles of 95 ºC (5 s), 60 ºC (30 s) and 72 ºC (s). For all reactions, a melting curve was obtained after PCR completion to check amplification specificity under the following conditions: 95 ºC (15 s), 60 ºC (15 s) and 95 ºC (15 s).
Expression levels were assessed after normalization of each mco gene cycle threshold (Ct) with those of the housekeeping gene actA (17).

Bromophenol plate assay. Laccase activity was assessed by spotting spotting $5x10^4$ conidia of every strain in the centre of PDA plates supplemented with 20 g/L of bromophenol blue and 0.25, 1 or 2 µg/mL of VRC when required. Plates were incubated for 5 days at 30 ºC. Laccase-produced yellowish and clear halos around colonies, indicative of bromophenol blue degradation by laccases (18, 19), were measured and normalized with colony diameter in every case with the ImageJ software v1.52a for Windows. Halos vs colony diameter were expressed as a ratio.

Protein concentration and quantification. Supernatants obtained and filtered from cultures for extracellular protein obtention were transferred to

dialysis tubing cellulose membranes with pore diameter of 14 kDa (Sigma). Samples were dialyzed at 4 ºC with cold distilled water and gentle agitation for 72 h. Afterwards, membranes were kept in external contact with polyethylene glycol (PEG) 35000 (Sigma) (20) for 6 - 8 h, carefully washed with cold distilled water and additionally dialyzed for 1.5 - 2 h to remove PEG leftovers. Then, 1 mM of phenylmethylsulfonyl fluoride (PMSF; Sigma) and 1 % of protease inhibitor cocktail (PanReac Applichem, ITW reagents) were added to the extracts, which were stored at -20 ºC until used. Proteins in the extract were quantified by the Bradford method using bovine serum albumin (BSA) as standard with the Bradford Protein Assay Kit (Takara bio) following the manufacturer's instructions. A NanoDrop2000 spectrophotometer (Thermo Fisher) was used .

Laccase activity assessment in extracellular extracts. Laccase activity was assessed in every extract as previously described with some modifications (21, 22). Briefly, assay mixtures with 5 µg of total protein and 2 mM of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma) were prepared in 0.1 M sodium acetate buffer at pH 3 and incubated at 30 ºC. ABTS oxidation by laccases was analyzed monitoring absorbance at 414 nm in a NanoDrop2000 spectrophotometer (Thermo Fisher).

Statistics. The Mann-Whitney T-test was performed to compare relative gene expression values. All statistical analyses were carried out with GraphPad Prism 6.0 for Windows. P values ≤ 0.05 were considered statistically significant.

RESULTS

Expression analysis of *mco* genes in azole-inducing conditions. Transcription levels of the 16 *A. niger mco* genes were investigated in control condition and after VRC exposure and normalized with those of the housekeeping *actA* in four strains with different VRC susceptibilities. Reverse transcription PCR results evidenced strain-dependent variations of *mco* gene expression in basal and azole-inducing conditions, although VRC addition affected *mco* levels in a negligible manner for most of the cases (Fig. 1). Specifically, *mco* expression in the strain with the lowest VRC susceptibility, FMR 11900 (Fig. 1A), ranged from 8.40×10^{-9} (*mcoI*) to 2.50×10^{-3} (*mcoH*) times fold with respect to the *actA* housekeeping gene in basal conditions. After VRC exposure, gene expression levels oscillated from 5.0×10^{-7} (*mcoK*) to 3.0×10^{-3} (*mcoH*). Significant upregulation by VRC was only observed for *mcoI* and *mcoM*, whereas *mcoK*, *mcoN* and *mcoO* levels resulted significantly downregulated ($P \leq 0.0424$). In the case of FMR 15441 (Fig. 1B), *mco* transcription levels comprised values from 1.6×10^{-5} (*mcoP*) to 1.5×10^{-2} (*mcoH*) in the control condition, and 1.8×10^{-5} (*mcoO*) to 4.2×10^{-2} (*mcoH*) when exposed to VRC. Transcription levels of *mcoH*, *mcoN* and *mcoM* were significantly increased upon VRC exposure ($P \leq 0.0303$). With respect to the wt strains (displaying VRC MICs below the VRC ECV), FMR 15388 (Fig. 1C) *mco* gene expression levels ranged from 1.1×10^{-5} (*mcoK*) to 1.2×10^{-5} (*mcoA*) in basal conditions, and from virtually 0 (*mcoK*) to 5.9×10^{-2} (*mcoB*) in VRC-inducing conditions. Significant increase in the gene expression due to VRC was found in *mcoJ*, whilst expression reduction was significant in *mcoI* and *mcoK* ($P \leq 0.0606$). Finally, in the case of the most susceptible strain in the study (FMR 11898; Fig. 1D), *mco* minimal expression in control condition was negligible for

mcoP, whilst maximum expression was that of *mcoH* (1.8×10^{-1}). Expression levels ranged between 6.1×10^{-6} (*mcoO*) and 2.5×10^{-2} (*mcoH*) after VRC exposure. Significant increase of the *mcoP* transcription levels and significant decrease of the *mcoH* and the *mcoO* gene expression were found in VRC exposure conditions ($P \leq 0.0220$).

Regarding *sensu-stricto* laccases, all strains showed a practically unaltered expression of *mcoD* and *mcoG* and a slight upregulation of *mcoF* upon VRC treatment. Variable patterns were observed for *mcoI, mcoJ, mcoM* and *mcoN* among strains in this condition, with significant upregulation of *mcoI* in FMR 11900, *mcoJ* in FMR 15388 and *mcoM* in FMRs 11900 and 15441.

Bromophenol blue plate assays. Screening of laccase activity in the four *A. niger* strains was performed by using bromophenol blue plates supplemented with different VRC concentrations. Basal laccase activity was positive in all strains, as indicated by yellowish clear halos around fungal colonies in the control plate (Fig. 2). Quantitative measurements revealed no important differences in the basal laccase activity within the four *A. niger* strains, with normalized ratios halo/colony areas ranging from 0.20 to 0.35 (Table 2). However, a notable increase in the laccase activity upon VRC addition in all cases, with the most prominent raise (compared to the control plate not supplemented with VRC) in the strains with the lowest azole susceptibility (FMRs 11900 and 15441) at a concentration of 2 μg/mL and 1 μg/mL VRC , respectively (Fig. 2 and Table 2).

Laccase activity. To establish whether laccase activity under VRC presence could be proportionally related to VRC resistance, we analyzed the laccase activity of extracellular extracts obtained from liquid cultures incubated in azole-inducing conditions. Oxidation of ABTS by laccases

was assessed by OD_{414} values (Fig. 3). Initial values (t = 0 h) were similar for the two strains displaying the lowest VRC susceptibility (FMRs 11900 and 15441), although they showed the lowest OD_{414} (0.248 ± 0.033 and 0.235 ± 0.016, respectively). Maximum OD_{414} was achieved after 30 min of incubation in all strains except FMR 15388, with values ranging between 0.308 ± 0.038 and 0.488 ± 0.061. From the set of strains in study, FMR 15441 exhibited the highest laccase activity increase from t = 0 h as evidenced by the most notable increase in absorbance. In the case of FMR 15388, laccase activity decreased after t = 0 h and OD_{414} was lower than the initial value at all the times tested.

DISCUSSION

Azole resistance in *Aspergillus* constitutes a global public health threat (23), since this phenomenon directly impairs clinical management and might worsen the final outcome of affected patients (24). Molecular mechanisms behind antifungal resistance have been extensively analyzed in *A. fumigatus;* however, they remain poorly characterized in other species of the genus, such as *A. niger*, which represents the second leading cause of invasive aspergillosis (25, 26). In previous works (14; Analysis of the *cyp51* genes contribution to azole resistance in *Aspergillus* section *Nigri* with the CRISPR-Cas9 technique, *manuscript in preparation* & Ergosterol quantification, membrane and cell wall characterization of *Aspergillus* section *Nigri* strains with different voriconazole susceptibility, *manuscript in preparation*), we have demonstrated that azole resistance in this and other species of section *Nigri* cannot be clearly explained by modifications or upregulation of the *cyp51* genes despite they are the central mediators in azole response (27).

Other described azole resistance mechanisms do not comprise *cyp51* genes, as could be increased azole efflux or import of host sterols. In this line, mechanisms such as intracellular drug sequestration and azole degradation have been proposed as potential key factors for azole resistance, despite they have not been observed in *Aspergillus* so far (13).

Laccases catalyze the removal of broad variety of substrates, including azole drugs (9, 10, 28), and thus they constitute possible candidates for the mediation of antifungal resistance due to azole degradation. In fact, laccase deletion in *Talaromyces mareneffei* has been linked to increased susceptibility to antifungals, oxidative stress and phagocytosis (29). Additionally, studies with *Cryptococcus neoformans* associated increased laccase activity with antifungal resistance (30) and phagocytosis protection (31). Taken together, these reports suggest a clear and important contribution of laccase proteins to azole resistance, stress responses and virulence.

In *A. niger*, laccases have been described to participate in the oxidation of phenolic compounds, decolorization and detoxification of textile dyes (32). Since the potential role of laccases in *Aspergillus* azole resistance has not been investigated, we have characterized laccase gene expression and enzymatic activity in four *A. niger* strains with different VRC susceptibilities.

Interestingly, our gene expression results revealed strain-dependent differences in the laccase-like genes we analyzed. In general terms, low transcript levels of *mco* compared to the housekeeping gene *actA* were observed in all cases. In addition, we found different tendencies and patterns of expression after VRC exposure among the set of studied genes. Contrasting to our initial hypothesis, a few genes showed reduces

expression levels after VRC exposure. Since laccase expression has been described to be upregulated by oxidative stress (as could be the exerted by azoles) (33, 34) we did not expect any downregulations upon VRC treatment. From the 16 *mco* genes analyzed , we found the *mcoH* gene to be the most expressed one in basal conditions and under the presence of VRC in all strains but one, although its upregulation was significant only in FMRs 15441 and 11898. This is not surprising considering that this laccase-like gene has been predicted to be an iron transporter located in the fungal plasma membrane of *A. niger* (11), and iron transport has been described to play an important part during azole-stress conditions (35). However, it contrasts with the low expression of another membrane-located iron transporter, *mcoK*¸ especially after VRC exposure, which might indicate *mcoK* is a functionally redundant gene.

In contrast, fungal pigments MCOs *mcoP* and *mcoO* (11) generally showed the lowest expression in control and under VRC presence, respectively.

With respect to the *sensu-stricto* laccase genes, variable patterns of expression were observed after VRC treatment, which complicates extrapolation of data. However, it is worth mentioning that the *mcoM* gene was significantly upregulated by VRC in the strains with low susceptibility, whereas VRC downregulated or did not affect *mcoM* expression in the most susceptible strains of the set. It remains unclear whether or how this gene could be contributing to azole resistance in *A. niger*.

Regarding plate activity assays with bromophenol as substrate, we observed that all the *A. niger* strains tested displayed laccase activity in basal conditions. Fascinatingly, laccase activity increased in a VRC concentration-dependent manner in all cases, although no proportionality along the VRC MIC scale could be established. Accordingly, increased

laccase activity upon azole exposure has also been described by others. Specifically, it has been reported that fungal laccase activity is stimulated and enhanced by the azole fungicide tebuconazole in a concentration-dependent manner (36). In addition, laccase activity was increased in *Thielavia* and *Chaetomium* cultures supplemented with the *cyp* inhibitor 1-aminobenzotriazole (37).

In order to examine whether VRC MIC differences among strains could be influenced by laccase activity, we investigated laccase activity in extracts from cultures supplemented with VRC. The results we obtained, reinforce the observations on strain-dependence laccase activity. Nevertheless, unexpectedly, the strains with the lowest susceptibility towards VRC were the ones with the lowest initial laccase activity. However, the most notable activity increase through time was displayed by one of them, suggesting differences in their regulation and response to azole stress. These phenomena could be explained by differences in the affinity for the VRC molecule due to the abundance or lack of specific groups of *mco* genes, which we observed to occur in a strain-dependent manner in the gene expression analyses.

Altogether, the data we present here suggest, for the first time to our knowledge, that laccase activity in *A. niger* is enhanced by VRC in a concentration-dependent manner as it happens in other fungi. However, gene expression and activity varied among the strains we tested and could not be correlated along the VRC MIC scale. In this sense, further and deep analyses with larger sets of strains could be useful to decipher the exact biological roles mediated by these versatile enzymes in *A. niger* and other species of the genus.

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DECLARATION OF INTEREST

None to declare.

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TABLES

Table 1. Primers designed with the Oligo7 software and used for gene expression analysis.

Primer	Sequence (5' – 3')	Gene
AnmcoA-F1	CCGACTGGCGATTCTTGAC	*mcoA*
AnmcoA-R1	ACATAGAAGGTGGCATAGCG	*mcoA*
AnmcoB-F1	CCTGAATGTCGAAAATGCCAA	*mcoB*
AnmcoB-R1	ACGGAGGACCAGATGAAGG	*mcoB*
AnmcoC-F1	GCGTGAACCAAGGCGACC	*mcoC*
AnmcoC-R1	TCTTGCTAAACGGTCTCTGC	*mcoC*
AnmcoD-F1	GCACAAACTCCATTACCCAAT	*mcoD*
AnmcoD-R1	GTTTCGTTGTTCTCCTTAACC	*mcoD*
AnmcoE-F1	AAAAGCTCTCCATAAACCACAA	*mcoD*
AnmcoE-R1	TACGGGTGCTTGGTAGGTC	*mcoE*
AnmcoF-F1	GAGACGGATGCCTTGAACAA	*mcoE*
AnmcoF-R1	AGGGGAGATGGATGATGCG	*mcoF*
AnmcoG-F1	TTGCTACTCCTTGGGCTTCT	*mcoG*
AnmcoG-R1	CCATTGTTTTCCATGCGATTC	*mcoG*
AnmcoH-F1	ACCGTGACCTATGACTGGAA	*mcoH*
AnmcoH-R1	GGTGAAGTTGTACGTGATGG	*mcoH*
AnmcoI-F1	GACCCCACTACCACGCAAA	*mcoI*
AnmcoI-R2	GAGGGGATTAGACGGATTAG	*mcoI*
AnmcoJ-F1	CAGTATCCGCTTCCACCAAA	*mcoJ*
AnmcoJ-R2	GAATTACGCCTGTGGGTGC	*mcoJ*
AnmcoK-F1	CACTTCCACGGCATGTACC	*mcoK*
AnmcoK-R2	TGGGATTATACAGCGACATGA	*mcoK*
AnbrnA-F1	ATGGAAAGCCCGCCTACAG	*brnA*
AnbrnA-R2	TTATCAGGATTCATTGCACGG	*brnA*
AnmcoM-F1	TGAACGATACTCTGTGGGTG	*mcoM*
AnmcoM-R2	TGCTGTTGTACTTGTCGGTG	*mcoM*
AnmcoN-F1	TCCTCATCAACGGGCAATTC	*mcoN*
AnmcoN-R2	CGCTGTAATGGGAATGGTAC	*mcoN*
AnmcoO-F1	CAACCCTCTCCTTCTCGATT	*mcoO*
AnmcoO-R2	GATGTCGATGTAAGGGGTAG	*mcoO*
AnmcoP-F1	AATCAGATCATCAACGGGACT	*mcoP*
AnmcoP-R1	AGAGCACAGGGGTCGATAG	*mcoP*

Table 2. Clear halos on bromophenol blue plates due to laccase activity. Values represent the normalized ratio between the halo and the colony areas in every case. Very poor or no fungal growth is indicated by the - symbol.

FMR strain	VRC concentration (µg/mL)			
	0	**0,25**	**1**	**2**
11900	0,28 ± 0,04	0,36 ± 0,08	0,90 ± 0,15	2,40 ± 0,33
15441	0,22 ± 0,13	0,39 ± 0,12	1,84 ± 0,23	-
15388	0,35 ± 0,11	0,49 ± 0,04	1,10 ± 0,02	-
11898	0,20 ± 0,06	0,44 ± 0,13	-	-

FIGURES

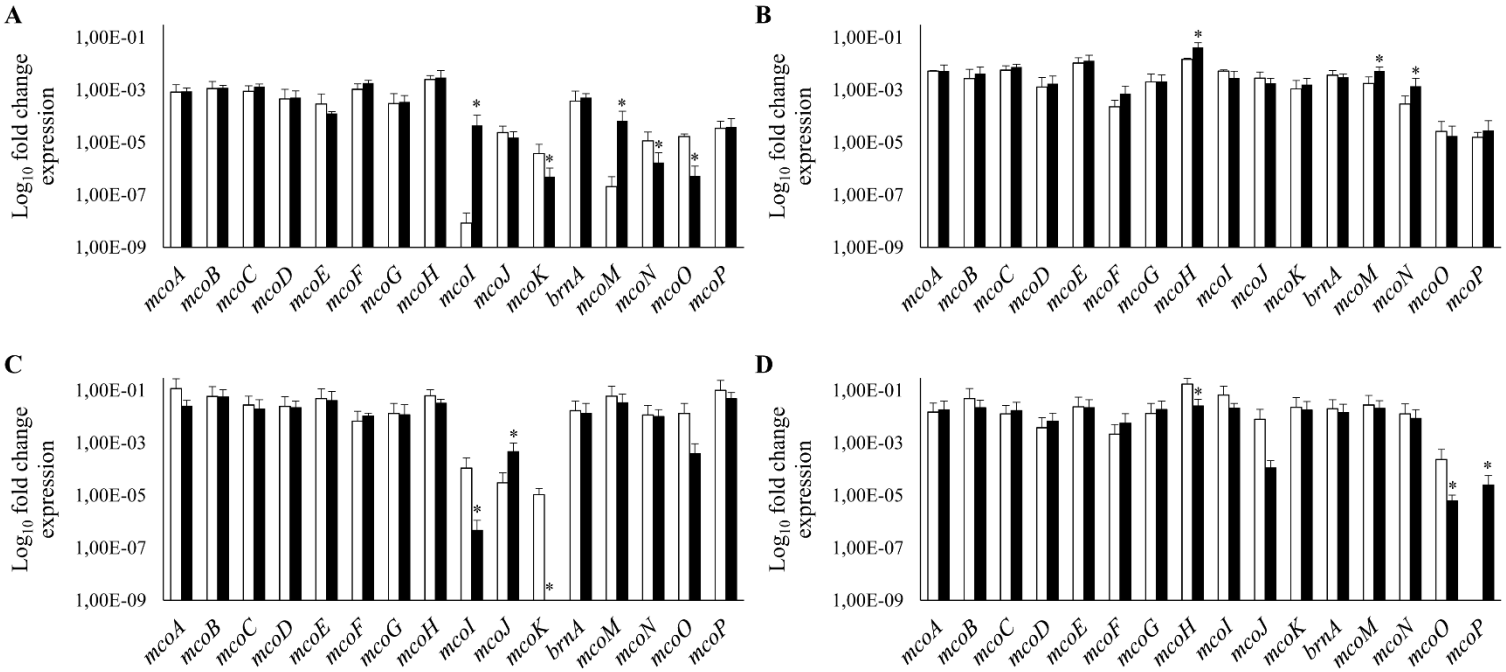

Figure 1. Expression results of *mco* genes in control condition (white bars) and after 4 µg/mL VRC exposure for 8 h (black bars) in strains FMR 11900 (A), FMR 15441 (B), FMR 15388 (C) and FMR 11898 (D). In all cases, *actA* was used as the housekeeping gene to normalize *mco* transcription levels. Statistical significance ($P \leq 0.05$) is depicted by an asterisk (*).

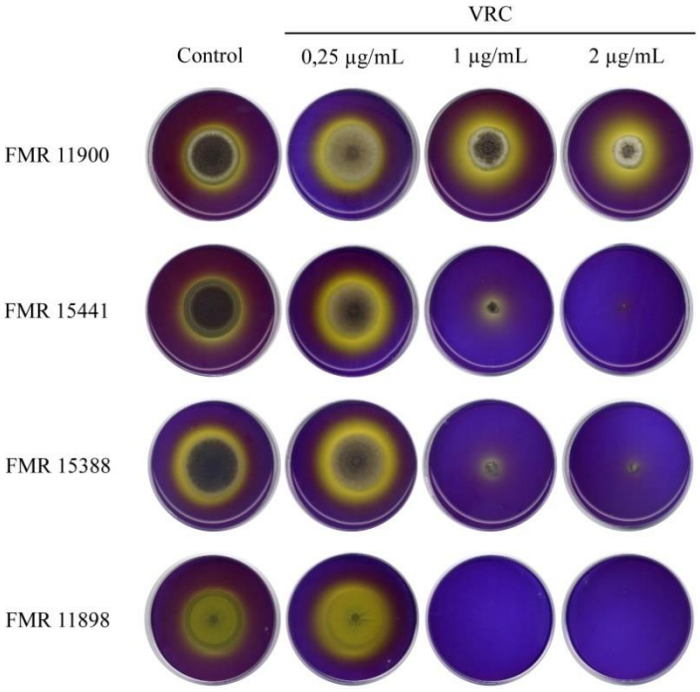

Figure 2. Laccase activity assessment in bromophenol blue supplemented agar plates in the absence and presence of different VRC concentrations. Clear halos around fungal colonies indicate laccase activity.

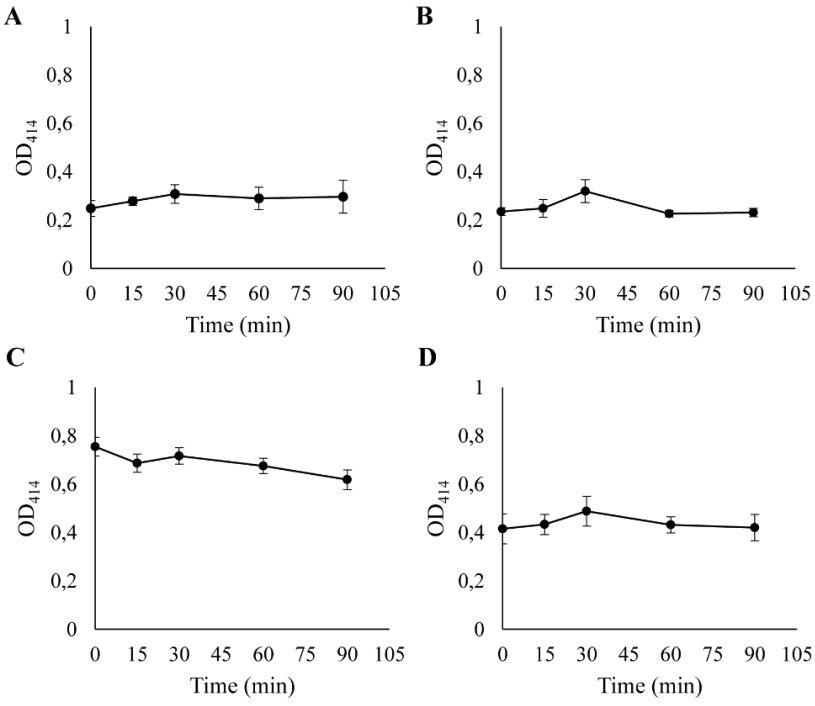

Figure 3. Laccase activity measured as OD_{414} at different times and 30 ºC with ABTS as a substrate. (**A**) FMR 11900; (**B**) FMR 15441; (**C**) FMR 15388 and (**D**) FMR 11898.

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5.6. *In vitro* assessment of phagocytosis in *Aspergillus* section *Nigri* strains with different azole susceptibilities

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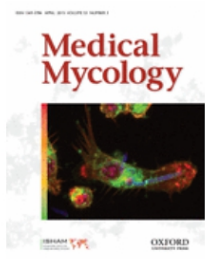

In vitro assessment of phagocytosis in _Aspergillus_ section _Nigri_ strains with different azole susceptibilities

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Keyword:	Aspergillus section Nigri, Azole resistance, Macrophage killing assay
Abstract:	*Aspergillus* remains responsible for a wide range of clinical conditions, especially in immunocompromised but also in immunocompetent individuals. Despite *Aspergillus fumigatus* is the most frequent species causing human disease, other species of the genus, such those belonging to section *Nigri* are also commonly involved in human infections. Interestingly, previous studies have suggested a connection between azole resistance and virulence. On this basis, and since macrophages are essential in the early response against fungal infections, we aimed to investigate possible differences in phagocytosis that could be correlated with voriconazole (VRC) resistance in section *Nigri*. To test this hypothesis, we conducted in vitro analyses on the macrophage phagocytic killing of conidia from section Nigri strains (species *Aspergillus niger* and *Aspergillus tubingensis*) displaying diverse VRC Minimal Inhibitory Concentrations (MICs). Our results show a proportional tendency between VRC susceptibility and conidia survival after macrophage infection for the strains tested, which resulted impaired after conidia were pre-exposed to VRC for the most-resistant and the most-susceptible strains. Conidial internalization in macrophages was also affected by pre-exposure to VRC in all cases, with a more noticeable effect on the strain displaying the highest VRC MIC,

ABSTRACT

Aspergillus remains responsible for a wide range of clinical conditions, especially in immunocompromised but also in immunocompetent individuals. Despite *A. fumigatus* is the most frequent species causing human disease, other species of the genus, such those belonging to section *Nigri* are also commonly involved in human infections. Interestingly, previous studies have suggested a connection between azole resistance and virulence. On this basis, and since macrophages are essential in the early response against fungal infections, we aimed to investigate possible differences in phagocytosis that could be correlated with voriconazole (VRC) resistance in section *Nigri*. To test this hypothesis, we conducted *in vitro* analyses on the macrophage phagocytic killing of conidia from section *Nigri* strains (species *A. niger* and *A. tubingensis*) displaying diverse VRC Minimal Inhibitory Concentrations (MICs). Our results show a proportional tendency between VRC susceptibility and conidia survival after macrophage infection for the strains tested, which resulted impaired after conidia were pre-exposed to VRC for the most-resistant and the most-susceptible strains. Conidial internalization in macrophages was also affected by pre-exposure to VRC in all cases, with a more noticeable effect on the strain displaying the highest VRC MIC, suggesting a clear impact of antifungal exposure onto phagocytosis success. In this sense, further analyses are needed to fully characterize the interdependence between azole resistance and virulence, especially regarding non-*fumigatus* species.

LAY SUMMARY

In vitro phagocytosis of *Aspergillus* section *Nigri* strains with different voriconazole (VRC) susceptibilities showed a direct relationship between

azole susceptibility and conidia survival after macrophage infection. Moreover, pre-exposure to VRC affected conidia internalization by macrophages.

INTRODUCTION

The genera *Aspergillus* is responsible of a broad spectrum of clinical manifestations including invasive life-threatening conditions such invasive aspergillosis (IA). IA is mainly caused by *Aspergillus fumigatus*, *Aspergillus flavus* or *Aspergillus niger* (1), and associated to mortality rates of 50-100 % (1, 2) depending on diagnosis, treatment and the host immune response (3). In particular, immunocompromised individuals are more prone to develop aspergillosis as well as any other fungal infection (4, 5), although aspergillosis has been increasingly reported to affect immunocompetent patients as well (6–9).

In immunocompetent individuals, macrophages and neutrophils constitute the key cellular components in fungal killing mediation. Specifically, macrophages are essential for the early response against fungal infections, as they contribute to the containment of pathogenic organisms and recruit other immune cells for further response (10). After conidia inhalation, macrophages recognize the fungal entry into the host by the pathogen-associated molecular patterns (PAMPs) displayed on the surface of the fungus, that are predominantly fungal cell carbohydrates. This recognition is carried by macrophage-associated pattern recognition receptors (PRRs), which promotes the engulfment and elimination of inhaled conidia by their degradation within the maturing phagosome compartments. If conidia are not cleared by the immune system at this stage, they develop invasive hyphae able to penetrate the pulmonary tissue, where neutrophils

effectively target them. In addition, dendritic cells also activate the adaptive immune response in order to mobilize the T-cell mediated defence (10–12). In all this process, the superficial structure of the *Aspergillus* conidia wall plays a crucial role, since it consists of a layer of highly hydrophobic and immunologically inert proteins, which impairs conidia recognition by macrophages. During conidia swelling and germination this layer becomes fragmented, proceeding with the exposure of underlying polysaccharides that show significant immunomodulatory activity and thus induce host immune response (12).

In parallel, voriconazole (VRC) is the current treatment of choice for IA affected patients(5), although the azole resistance incidence rise experienced in the last years has adversely affected aspergillosis therapeutic management (13, 14). In this context, a few studies have suggested the existence of a link between azole resistance and virulence (15–17), since the capacity of adaptation to any kind of stress, as could be the stress exerted by azoles, seems fundamental in *Aspergillus* pathogeny. As reviewed elsewhere, the deep understanding of the mechanisms behind these processes as well as their possible interdependence might contribute to the development of novel strategies to control fungal infections (18).

In this framework, we have hypothesized that fungal phagocytosis could depend on azole susceptibility and, therefore, it might occur differently between strains with different response to VRC.

Considering that virulence and azole resistance in non-*fumigatus* species such as section *Nigri* members remain obscure, we have focused this work towards the analysis of phagocytic activity against a few strains of *Aspergillus* section *Nigri* for the first time to our knowledge. Specifically, we have tested macrophage internalization and clearance of conidia from

strains with different VRC Minimal Inhibitory Concentrations (MICs). In addition, we have also addressed whether pre-exposing conidia to VRC could have any effect on phagocytosis.

MATERIALS AND METHODS

Fungal strains and growth conditions. Three strains of *Aspergillus* section *Nigri* belonging to species *A. niger* (FMRs 11900 and 11898) and *A. tubingensis* (FMR 14712) with different VRC susceptibility were used in this study. These were two clinical strains: FMR 11900 (VRC MIC ≥ 16 µg/mL), FMR 11898 (VRC MIC = 0.25 µg/mL) and one environmental strain: FMR 14712 (VRC MIC = 4 µg/mL) (19).

Strains were recovered from the Facultat de Medicina de Reus fungal collection (FMR). Before used, they were incubated and grown in Potato Dextrose Agar (PDA; Conda-Pronadisa) for 3-5 days at 35ºC twice.

Conidia swelling and germination time course. For conidia swelling assessment, $1x10^7$ conidia/mL of every strain were placed into the nutritious culture media YG broth (0.5 % Yeast extract, 2 % Glucose) and incubated at 37 ºC and 180 rpm for 7 h. During this incubation time, samples were taken at different times (0 h, 1 h, 2 h, 3 h, 5 h, 6 h and 7 h), placed on slides and images were taken with an optical microscope Zeiss Axio-Imager M1 light with a DeltaPix Infinity × digital camera. Analysis of conidia swelling was carried out by measuring conidia diameter with the ImageJ software (v1.52a for Windows).

Cell culture. The adherent murine macrophage J774A.1 cell line was incubated and maintained in tissue culture flasks at 37ºC under 5 % CO_2 in Dulbecco's Modified Eagle Medium (DMEM; Biowest) containing L-glutamine, 10 % (v/v) heat-inactivated fetal bovine serum and 1 %

penicillin/streptomycin. For the killing assay, murine macrophages were scraped from the tissue culture flask, seeded into 6-well culture plates with DMEM and incubated at 37 ºC, 5 % CO_2 (24 h approximately) until they reached 100 % confluence (*i.e.* 1,2x10^6 cells/well).

Killing assay. For the killing assay we followed previous protocols with some modifications (20, 21). Briefly, conidial suspensions of the three strains tested were adjusted by hematocytometer count to 6x10^5 conidia/mL in non-supplemented DMEM and incubated for 2 h at 37 ºC and 180 rpm for swelling.

In parallel, for infections with conidia pre-exposed to VRC, suspensions of 6x10^5 conidia/mL were inoculated in YG media and incubated for 1.5 h at 37ºC and 180 rpm. Then, VRC was added at a final concentration of half its MIC for every strain as follows: FMR 11900 = 8 µg/mL VRC, FMR 14712 = 2 µg/mL VRC and FMR 11898 = 0.12 µg/mL VRC. Samples were further incubated at 37 ºC and 180 rpm for 1 additional hour. Finally, cultures were centrifuged, washed with sterile distilled water and resuspended in non-supplemented DMEM.

For cell infections, growth medium from the cell culture plates was discarded in all cases, and macrophages were infected with the fungal suspensions prepared in non-supplemented DMEM at a Multiplicity of Infection (MOI) of 1. Infection was carried at 37 ºC and 5 % CO_2 for 4 h. Subsequently, DMEM was removed, wells were carefully washed with PBS twice to remove non-adherent macrophages and cells were lysed with 0.1 % triton in cold sterile deionized water. Lysates were diluted and plated on PDA for fungal cell count. All experiments were performed in triplicate.

Conidia internalization. In order to assess conidia internalization in macrophages, infections were carried as mentioned for the killing assay.

Afterwards, samples were taken from the cell culture plates and placed onto slides, which were analyzed by means of optical microscope observation and counting of 100 macrophages with 0 to 6 internalized conidia.

Statistical analysis. The two-way ANOVA test was used to assess differences in fungal viability after infection and conidia internalization according to strain differences and VRC exposure. All statistical analyses were performed using GraphPad Prism 6.0 for Windows. *P* values ≤ 0.05 were considered statistically significant.

RESULTS

Conidial swelling and germination. In order to determine the conidial swelling and germination process, a time-course growth analysis was performed. Monitoring the swelling process from resting conidia to germlings revealed a similar progression for all three strains in conidia size increase during the first three hours of the assay (Figures 1 and 2). Specifically, FMRs 11900, 14712 and 11898 conidia diameters increased 1.3, 1.2 and 1.4 times respectively for the first hour; 1.7, 1.5 and 1.5 times respectively for the second hour and 1.8, 1.6 and 1.6 times, respectively for the third hour, always relative to time 0. However, from the 5th hour on, the conidia diameter of the strain with the lowest VRC susceptibility, FMR 11900, increased in a more notable manner than the other two strains. Non-isotropic growth and germ tube formation mainly started after 6 h of incubation for the three strains and were completely visible at time 7 h (Figure 2). On this basis, infections were carried out with 2 h swollen conidia of all strains.

Killing assay. *In vitro* macrophage killing assays were performed for the assessment of virulence in our strains with and without VRC pre-exposure of conidia.

Regarding macrophage infections with non-exposed conidia to VRC, strain FMR 11900 displayed the highest intracellular survival after 4 h of infection, showing 66.1 % of the starting inoculum viable after phagocytosis. In contrast, the most susceptible strain of the set, FMR 11898, showed the lowest endurance against macrophages with only 19.8 % of intracellular survival. Finally, strain FMR 14712 showed 58.2 % of conidia survival after 4h of infection (Figure 3).

With respect to the results of VRC pre-exposed conidia, strains FMRs 11900 and 11898 displayed a higher survival when compared to that of non-exposed conidia, with 74.6 % and 38.0 % of viable conidia after infection, respectively. These results represented a survival increase of 8.5 % (FMR 11900) and 28.7 % (FMR 11898) in comparison to the infections with non-exposure condition. However, strain FMR 14712 showed lower viability after infection when conidia were VRC-exposed, with 48.5 % of conidia survival (20.2 % reduction of viability after infection).

Significant differences in fungal viability after infection were observed among strains when comparing non-exposed to VRC pre-exposed conidia ($P < 0.0001$), but no interaction effect was found between the two factors (VRC exposure and strain).

Conidia internalization. To decipher whether the results on fungal viability after infection could be affected by differences in the conidia uptake by macrophages, conidia internalization was assessed for every strain and condition.

When focusing on the swollen conidia uptake by the J774A.1 cell line without VRC pre-exposure, it was found to be similar in strains FMR 11900 and FMR 11898, since comparable percentage of macrophages with internalized conidia were found for the two cases (26.4 % and 27.6 %, respectively). Conidia uptake of FMR 14712 was higher, with a total of 39.4 % of macrophages with internalized conidia (Figure 4).

Regarding internalization of conidia that had been exposed to VRC before the infection, results greatly differed between strain FMR 11900 and the other two. Specifically, internalization of conidia remarkably dropped in FMR 11900 (10.3 %), while it slightly diminished in FMR 14712 (31.8 %) and resulted scarcely affected in FMR 11898 (32.2 %) when compared to the non-exposed conidia results (Figure 4).

Significant differences were found among strains when comparing non-exposed and VRC pre-exposed conidia ($P = 0.0111$).

DISCUSSION

The increment of azole resistance in *Aspergillus* has highlighted the importance on deciphering the factors contributing to it, since antifungal resistance impairs clinical management of the fungal infections and worsens patient outcomes (22, 23). In this context, many *A. fumigatus* stress-response pathways have been described to participate in fungal virulence and antifungal resistance (18), which suggests a clear connection between both factors. Insights on these mechanisms and their possible interdependence are essential to a better understanding of these processes as well as the development of new strategies to fight against *Aspergillus* infections, especially regarding non-*fumigatus* species known to cause infections that have been less studied so far (24). From this idea, the central

question tested in the study presented here was whether there might be azole susceptibility related differences in macrophage phagocytosis of *Aspergillus* section *Nigri*.

We showed that conidia swelling and germination at 37 ºC in the three strains of section *Nigri* analyzed (*A. niger* and *A. tubingensis*) followed the same progression from times 0 h to 3 h, with slight differences from time 3 h on and germination start at time 6 h. These results are in accordance to previous data on *A. niger* conidia germinated at 25 ºC (25). In this sense, our results revealed that both species, *A. niger* and *A. tubingensis* require very similar times for germination and display the same growth speed. This is of high importance, since in the context of immunity against *Aspergillus*, it has been reported that *A. fumigatus* conidia swelling is essential for macrophage-mediated phagocytosis (20). Interestingly, this seems also to be the case for *A. niger* and *A. tubingensis*, considering that in some initial experiments that we performed with resting conidia, inocula maintained 100 % viability after macrophage infection (data not shown). Nevertheless, there are exceptions on this behavior, such is the case of *A. terreus*, in which *in vitro* phagocytosis has been described not to be affected by the conidia swelling state (20). These relevant data imply potential differences in the disease establishment among species of *Aspergillus*, at least in the early stages of conidia attachment that lead to fungal invasion.

Next, in order to assess a possible relationship between phagocytosis and azole susceptibility, we performed *in vitro* macrophage infections and quantified the percentage of survival within the macrophage internalized conidia. Interestingly, the data we obtained suggests that fungal clearance might be more efficient in susceptible isolates, since our results show the highest post-infection survival for the strain with the lowest VRC

susceptibility of the set (FMR 11900) and the minimum post-infection survival for the most susceptible one (FMR 11898). This demonstrates an evident and proportional relationship between azole susceptibility and survival after *in vitro* infections, which have been potentially correlated in species of *Aspergillus* section *Nigri* for the first time to our knowledge.

Likewise, a similar tendency has been implied regarding *A. fumigatus*, since deletion of different genes involved in diverse fungal processes were reported to impact azole susceptibility and virulence with the same pattern of correlation we observed: as azole susceptibility increased, fungal virulence attenuated (17, 26–28). In addition, phagocytosis efficiency in *Aspergillus* has been shown to be species-dependent so far (29), but, despite our study included a limited number of strains, the results we obtained prove that phagocytosis is also extremely strain-dependent.

Regarding other fungi, connection between azole response and virulence has been also described to occur in *Candida albicans* after deletion of the ERG11 gene, which is involved in the ergosterol biosynthetic pathway and constitutes the gene target of azoles. However, in that case, gene deletion resulted in azole-resistant cells that were more efficiently killed by macrophages (30, 31), indicating that the ERG11 gene might also assist the host immune system in *Candida albicans* infections.

Another aim of our study was to ascertain whether exposing the conidia to subinhibitory concentrations of VRC before the infection could have any impact in the phagocytosis efficiency. When compared to the non-exposed conidia data, azole pre-exposure resulted in impaired fungal clearance for the two strains displaying the highest and the lowest VRC susceptibilities, which was translated into a post-infection survival increase. Curiously, the strain with intermediate VRC MIC displayed a slightly lower survival after

infection. These results might be explained by possible structural or physical conidia modifications exerted by the azole drug, which could contribute to their phagocytic escape or intracellular survival within the macrophage.

In contrast, divergent outcome in the virulence of azole pre-exposed *A. fumigatus* was reported by others. Specifically, exposure to either posaconazole (PSC) or VRC did not affect its virulence in an *in vivo Drosophila melanogaster* model (32). Extensive *in vitro* and *in vivo* studies on the matter will be essential to better contextualize these discrepancies.

On another note, since phagocytic fungal clearance depends on conidia uptake by macrophages, we also assessed conidia internalization for every strain and condition. As we would expect, VRC exposure clearly impaired conidia uptake of the strain with the lowest VRC susceptibility, while affected the other strains in a more subtle manner, suggesting different responses to azole stress by the three strains. Comparably, in a study regarding the effect of amphotericin B (AMB) towards macrophage phagocytosis, significant reductions in the uptake of conidia from *A. fumigatus* and *A. flavus* upon AMB exposure have been described (33). Our hypothesis is that the potential modifications exerted by antifungals on the superficial part of the cell might lead to poor fungal recognition by the macrophage.

With all, further analyses and *in vivo* studies to characterize the effect of azole pre-exposure in macrophage phagocytosis and fungal virulence are essential, since they could be useful to understand and prevent antifungal resistance acquisition and improve disease management.

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DECLARATION OF INTEREST

None to declare.

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FIGURES

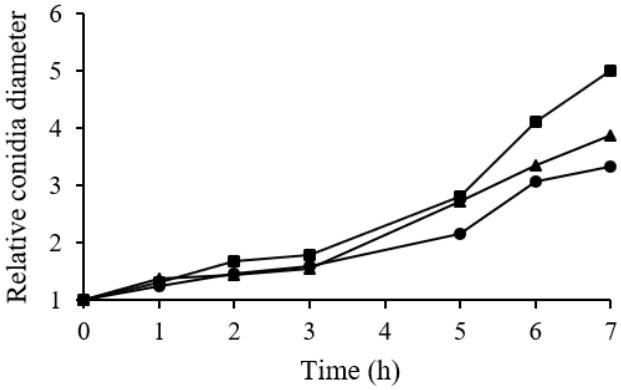

Figure 1. Section *Nigri* conidial swelling progression at 37 ºC and 180 rpm. Results are expressed as the relative conidia diameter at specific time points normalized to the measured diameter of every strain at time = 0 h. (■) FMR 11900, (▲) FMR 14712 and (●) FMR 11898.

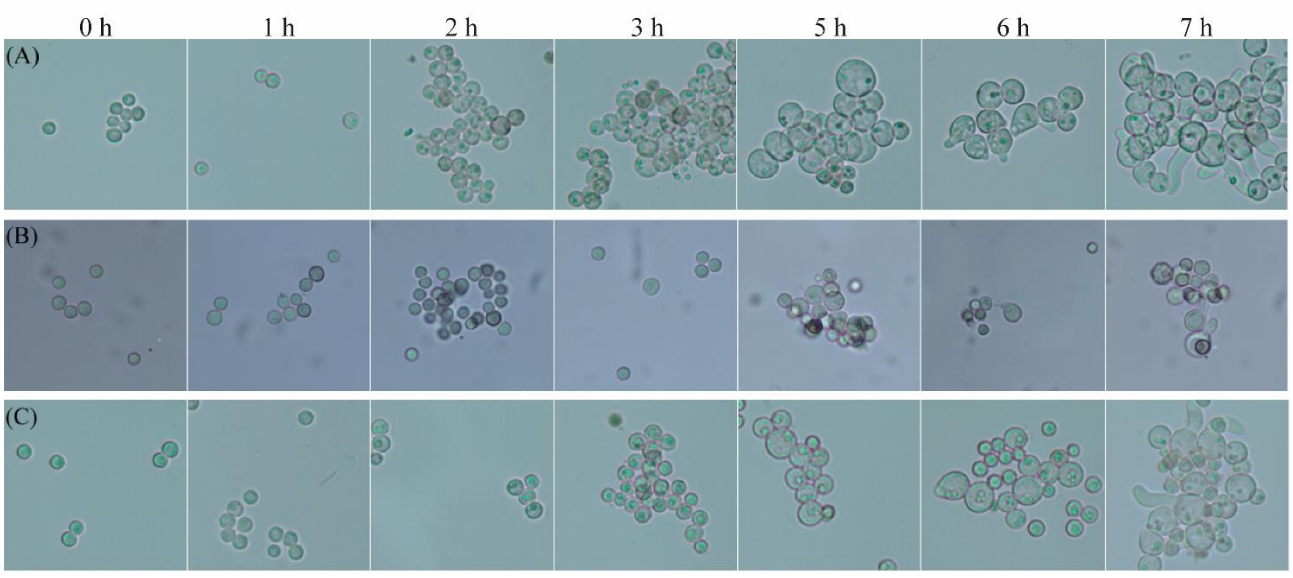

Figure 2. Assessment of conidial swelling and germination at different times of strains (A) FMR 11900, (B) FMR 14712 and (C) FMR 11898 in 37 ºC and 180 rpm liquid cultures.

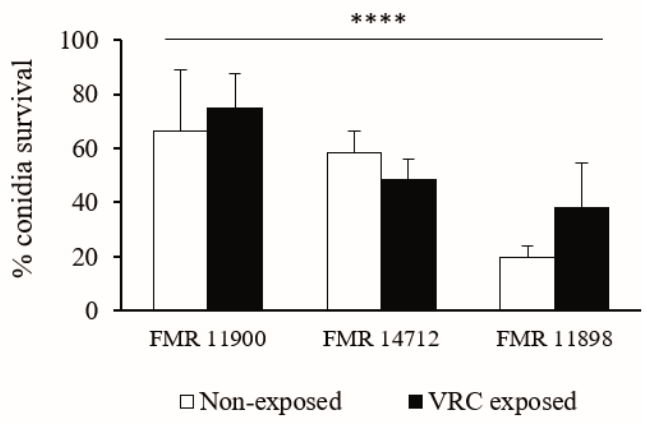

Figure 3. Percentage of conidial survival after 4 h of infection in J774.A1 cells. White bars correspond to results on macrophage infections with swollen conidia and black bars correspond to infections with swollen conidia exposed to VRC before infection. Statistical significance ($P \leq 0.001$) is depicted by asterisks (****).

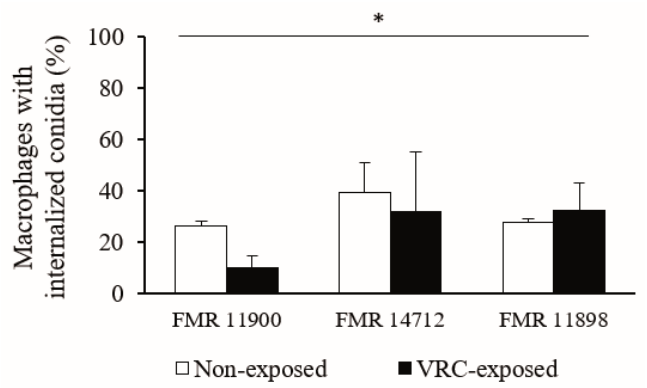

Figure 4. Total percentage of J774A.1 macrophage with internalized conidia (up to 6 conidia/macrophage). White bars correspond to results on macrophage infections with swollen conidia and black bars correspond to infections with swollen conidia exposed to VRC before infection. Statistical significance ($P \leq 0.05$) is depicted by an asterisk (*).

UNIVERSITAT ROVIRA I VIRGILI
STUDY AND CHARACTERIZATION OF AZOLE RESISTANCE IN ASPERGILLUS SECTION NIGRI
Alba Pérez Cantero

DISCUSSION

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Fungal infections are estimated to affect more than 1 billion people worldwide and are associated to cause 1.5 million deaths annually (Lee *et al.* 2019). The most common forms of fungal disease are superficial infections of skin and nails, and despite invasive fungal diseases have a much lower incidence, they are associated to elevated mortality rates. In fact, death rates due to the most significant invasive fungal infections exceed those of tuberculosis or malaria (Brown *et al.* 2012).

Interestingly, more than 5 million fungal species have been predicted to exist nowadays, but only a small number of species from four different genera (*Cryptococcus, Candida*, *Aspergillus* and *Pneumocystis*) have been reported to account for 90 % of all the fungal-related deaths. Nevertheless it is worth mentioning that fungal epidemiological data are scarce and probably biased due to misdiagnosis, which might imply an important underestimation of the invasive fungal disease burden (Blackwell 2011; Brown *et al.* 2012). In this context, it is estimated that 10 million people are at risk of infection by *Aspergillus*; however it is expected that the population at risk for IA and other invasive fungal infections will notably increase due to the rise in the predisposing factors (Thornton 2020), such as neutropenia, hematologic malignancies, hematopoietic stem cell or solid organ transplantation, prolonged glucocorticoid or immunosuppressive therapies and infections by the human immunodeficiency virus, cytomegalovirus or influenza (El-Baba *et al.* 2020).

Despite the most common *Aspergillus* species isolated from clinical samples is *A. fumigatus,* prevalence of cryptic species has incremented in the last years, as it is the case of section *Nigri* members *A. niger, A. tubingensis* or *A. brasiliensis*. As a matter of fact, prospective studies from different hospitals in Spain, Italy and Japan have demonstrated that section

Nigri is the second (Alastruey-Izquierdo *et al.* 2013; Prigitano *et al.* 2020; Toyotome *et al.* 2020) or the third complex most commonly isolated (Taccone *et al.* 2015). Regarding invasive disease, section *Nigri* members have also been acquiring clinical importance due to their increasing prevalence in IA patients. Specifically, they are the most frequently species involved in IA cases after *A. fumigatus* (Hagiwara *et al.* 2016b; Lass-Flörl & Cuenca-Estrella 2017).

The main concern about the emergence of cryptic species relies on the fact that they usually display reduced or variable susceptibility to azoles and other drugs, and thus negatively affect antifungal therapy. In the case of section *Nigri*, antifungal susceptibility analyses have evidenced its intrinsic lower susceptibility to ITC, VRC, PSC and AMB in comparison to *A. fumigatus* (Alastruey-Izquierdo *et al.* 2012; Alcazar-Fuoli *et al.* 2009; Wiederhold 2017) with especially high azole MICs against the cryptic species *A. tubingensis* (Hashimoto *et al.* 2017; Howard *et al.* 2011; Iatta *et al.* 2016). In this line, the data obtained from our work evidenced general good activity of VRC, PSC and ITC against *A. niger, A. tubingensis* and *A. brasiliensis*, with PSC exerting the most potent *in vitro* activity. Additionally, lower azole MICs were frequently associated to the *A. tubingensis* strains we tested (Pérez-Cantero *et al.* 2020). The intrinsic problematic of lower antifungal susceptibility within section *Nigri* is even more pronounced in strains harboring acquired resistance mechanisms. In the case of *Aspergillus*, azole resistance, and more specifically, VRC resistance, is of main interest, since this antifungal constitutes the treatment of choice for aspergillosis (Patterson *et al.* 2016). In recent years, azole resistance in *Aspergillus* has alarmingly increased, which is translated into a limitation of therapeutic options for affected patients and associated to

poor clinical outcomes (Bassetti *et al.* 2020; Perlin *et al.* 2017). This situation has been principally promoted by two factors: the widespread use of azole fungicides in agriculture and the escalation of azole use for clinical prophylaxis and therapy. In this sense, it has been described that evolution of resistance can occur in infecting strains through selection after fungicide exposure or during long-term antifungal treatment (Hendrickson *et al.* 2019; Thornton 2020).

Since the targets for azoles are the Cyp51 enzymes (encoded by the *cyp51* genes), these proteins have been suggested to be the main molecular mediators of azole response and resistance. In the case of *Aspergillus*, diverse number of *cyp51* paralog genes among species have been reported. Available data on the literature documented two *cyp51* paralogues (*cyp51A* and *cyp51B*) in the species *A. fumigatus, A. nidulans* and *A. niger,* and three paralogues (*cyp51A, cyp51B* and *cyp51C)* in *A. flavus* and *A. oryzae* (Hagiwara *et al.* 2016b; Mellado *et al.* 2001). From our *in silico* studies, we could confirm that species *A. terreus* and *A. carbonarius* also contain three Cyp51 proteins, which had not been reported. Moreover, phylogenetic analyses of this family of proteins throughout the genus, allowed us to corroborate that they share a common origin, from which they are evolutionarily differentiated into two separated lineages: the Cyp51A and the Cyp51B lineages. We also concluded that the Cyp51C protein might have its origins in duplications from either of the two main lineages (Cyp51A and Cyp51B) depending on the species. Interestingly, such evolutionary duplications have been connected to genetic adaptation, which suggests that the appearance of various *cyp51* paralogs might be directly related to azole tolerance and toxicity adaptation (Dudakova *et al.* 2017; Hawkins *et al.* 2014).

Although azole resistance mechanisms have been extensively characterized in *A. fumigatus*, up to date very few studies address this issue in non-*fumigatus* species. On this basis, our general objective was to characterize azole resistance in species of *Aspergillus* section *Nigri*, which comprises industrially and clinically relevant species.

Azole response and resistance in *A. fumigatus* has been mostly attributed to Cyp51A amino acid substitutions due to mutations in the *cyp51A* gene. These have been described to impair azole activity by reducing the affinity of the binding between the azole drug and the target enzyme. However, despite it has been revealed that Cyp51A and Cyp51B share the same substrate and display comparable functions, *A. fumigatus* Cyp51B has been categorized as a redundant protein with other potentially unknown functions. Accordingly, no amino acid substitutions in this enzyme have been found to confer azole resistance so far (Martel *et al.* 2010; Parker *et al.* 2014; Warrilow *et al.* 2015).

On the matter of Cyp51s in *Aspergillus* section *Nigri*, we shed light onto the Cyp51 the protein topology of *Aspergillus* section *Nigri*. Analysis of the conformation and structural domains of Cyp51A and Cyp51B in *A. niger*, *A. tubingensis* and *A. brasiliensis*, revealed the presence of two transmembrane domains in these species, contrasting to the only one described in other species of the genus such as *A. fumigatus*. Moreover, our predicted data also showed modest differences on the reported amino acids comprised in the transmembrane region of this latter species (Warrilow *et al.* 2010).

Regarding Cyp51A protein sequence examination, no obvious correlation could be established between amino acid substitutions and VRC

resistance. As a matter of fact, we detected a few non-wt strains without any amino acid substitutions, whereas some other modifications were present in both, wt and non-wt strains. Only substitutions H467Q, V377I and K64E, which had never been reported, were exclusively present in non-wt strains. Nevertheless, location of these mutations does not comprise azole binding sites residues, although they could possibly affect protein or binding pocket conformation. Further studies are needed to elucidate their potential contribution to azole resistance.

Another mechanism of azole resistance involving *cyp51* comprises the overexpression of these genes to overcome the inhibitory effects of the azoles. Noteworthy, transcriptional regulation of *cyp51A* notably differs from that of *cyp51B* in *A. fumigatus.* Specifically, gene expression of *cyp51A* is inducible by azoles, whereas *cyp51B* expression follows a constitutive tendency even after azole exposure (Hargrove *et al.* 2015). Accordingly, in the case of strains we tested belonging to section *Nigri*, gene expression results suggested that *cyp51A* leads the transcriptional response due to azole stress and it is upregulated upon VRC exposure in this section. In contrast, *cyp51B* expression was remarkably lower and somewhat stable despite VRC presence. However, *cyp51* transcription levels could not be proportionally correlated to VRC susceptibility in any case. Accordingly, variable expression patterns of *cyp51* have been described in strains with divergent azole susceptibility (Buied *et al.* 2013; Fattahi *et al.* 2015), which clearly suggests that *cyp51* overexpression might not be the main mechanism responsible for azole resistance in *Aspergillus.*

Furthermore, to assess the exact contribution of *cyp51A* and *cyp51B* to azole resistance in section *Nigri*, we generated, for the first time, *cyp51*

KOs with a CRISPR-Cas9 approach using strains from our set of study (with different genetic backgrounds) as parental strains (Al Abdallah *et al.* 2017). VRC MICs were minimized by both single deletions (Δ*cyp51*A and Δ*cyp51B*), although susceptibility was increased in a more remarkable manner by the *cyp51A* deletion. In addition, no transcriptional compensation was observed after deletion of either of the *cyp51* genes, although *cyp51A* expression (in Δ*cyp51B* strains) remained higher than *cyp51B* expression (in Δ*cyp51A* strains) in all cases. In line with our previous gene expression studies, VRC exposure in the KO strains caused a notable upregulation of the *cyp51A* gene expression, but mild fluctuations on that of *cyp51B.* Altogether these findings support the idea of *cyp51A* leading the fungal response towards azole stress, as previously suggested by us in section *Nigri*, and by others in *A. fumigatus* (Hargrove *et al.* 2015)

We also speculated that although variation of VRC susceptibility might not be straightforwardly explained by *cyp51* in section *Nigri*, it could still potentially be correlated to the ergosterol cell content. Assessment of ergosterol and 24(28)DHE intermediate sterol showed no differences in their respective basal levels that could be correlated to azole resistance in the set of strains we tested. Furthermore, intermediate sterol 24(28)DHE was slightly altered upon VRC exposure, with no proportional tendency to VRC resistance. Moreover, we also analyzed the levels of these sterols in the *A. niger* and *A. tubingensis cyp51A* or *cyp51B* KO strains we had generated. As expected, ergosterol levels were lower in the mutant strains than in their parental isolates, but basal ergosterol was quite similar between Δ*cyp51A* and Δ*cyp51B* mutants, which implies comparable activity of both enzymes in the ergosterol biosynthetic pathway. In the case of 24(28)DHE, we found equivalent amounts of this sterol in all strains and

slight alterations upon VRC exposure. Curiously, 24(28)DHE has the ability to fulfill ergosterol functions (Yang *et al.* 2015), and thus we would assume it to increase due to azole stress, but our data demonstrate that no compensatory effect between these two sterols occur in the strains we analyzed from section *Nigri.* Surprisingly, those strains displaying the lowest VRC MICs contained the most reduced levels of ergosterol, denoting that VRC hyper-susceptibility might be due to changes in the membrane fluidity and permeability caused by insufficient amount of ergosterol within plasma membrane. Divergence in the data acquired from *A. fumigatus* and other fungi in this regard has been reported by other authors (Dannaoui *et al.* 2001; Hagiwara *et al.* 2018; Müller *et al.* 2018), indicating significant diversity among species and strains.

Since *cyp51* failed to completely explain azole resistance in *Aspergillus* section *Nigri*, we aimed to elucidate which other mechanisms could be the contributors to this phenomenon.

Two fungal structures reported to be essential in stress response and cell viability are the plasma membrane and the fungal cell wall. Specifically, modifications on the membrane fluidity and permeability that could affect drug import have been suggested to affect drug susceptibility (Esquivel *et al.* 2015). Qualitative assessment of membrane fluidity and permeability on our section *Nigri* strains with different VRC susceptibility, suggested an increased permeability of the plasma membrane in the susceptible strains. This was especially evident with qualitative plate assays supplemented with EDTA, a synthetic compound that intercalates lipid membranes exerting a permeabilization effect (Prachayasittikul *et al.* 2007). We hypothesize that enhanced permeability might improve azole activity by facilitating the entrance of the drugs into the cell. Additionally,

the increment of membrane permeability has been reported to promote oxidative damage, which might also stimulate the inhibition of fungal growth (Ajdidi *et al.* 2020).

Regarding the fungal wall, it has been recently reported that azoles exert their fungicidal action by activation of the cell wall salvage system, which induces an excessive synthesis of its carbohydrates. This eventually causes the integrity loss of the plasma membrane and the final fungal cell death (Geißel *et al.* 2018). In this context, we hypothesized that potential modifications of its composition could cause the appearance of differences in azole susceptibility. Qualitative assessment of cell wall structural components chitin and β-1,3-glucan, showed analogous β-1,3-glucan contents in all the strains we studied. Unexpectedly, mildly enhanced chitin content in the non-wt strains was also observed; however, it is unclear if this could have any significance on azole resistance. Still in the same context, physical properties and modifications on the conidial cell wall surface have been connected to fungal drug sensitivity by others (Valsecchi *et al.* 2017). Therefore, to test whether a physical or mechanical component could be contributing to azole resistance, we analyzed conidial resistance to physical disruption, although no differences could be correlated to the azole susceptibility patterns of our section *Nigri* strains.

Parallelly, as it has been proposed but not described in *A. fumigatus*, we considered drug degradation as a potential azole resistance mechanism within section *Nigri.* For this, we estimated that the Multicopper Oxidase (MCO) protein family, and fundamentally its subgroup of laccase enzymes, could be perfect candidates to mediate azole degradation in *A. niger*. Interestingly, *A. niger* laccases are known to participate in the degradation of a wide range of organic substrates and 16 different laccase-like proteins

can be found in the genome of this species (Chen *et al.* 2016; Ferraroni *et al.* 2017; Soden *et al.* 2002; Tamayo Ramos *et al.* 2011; Weenink *et al.* 2006). Reports on the literature demonstrate that azoles (among other drugs) are indeed degraded by these enzymes, which also increase their activity upon azole fungicides exposure (Artigas *et al.* 2017; Yousefi-Ahmadipour *et al.* 2016). In accordance, our observations revealed an increase of the laccase activity in a VRC concentration-dependent manner, which suggests that this azole enhances laccase activity. Nevertheless, we could not proportionally correlate these results with *mco* transcription levels, since different patterns of expression among strains, in both, basal conditions and after VRC exposure were observed.

Another major mechanism of azole resistance not mediated by *cyp51* in *Aspergillus,* consists of the overexpression of multidrug efflux pumps, which results in insufficient intracellular azole concentration to carry out its fungicidal effect (Perlin *et al.* 2015). Particularly, there is a broad number of efflux pump protein-encoding genes in *Aspergillus,* suggesting a relevant function in multidrug resistance (Coleman & Mylonakis 2009; Meneau *et al.* 2016). Nevertheless, very controversial results regarding efflux pump overexpression and azole resistance have been illustrated in *A. fumigatus* and other species of the genus (da Silva Ferreira *et al.* 2004; Ferreira *et al.* 2006; Meneau *et al.* 2016; Natesan *et al.* 2013; Paul *et al.* 2013; Slaven *et al.* 2002). This evidences that efflux pump overexpression fails to explain differences in azole response and shows very poor correlation to azole MICs in *Aspergillus*.

On a different note, we aimed to investigate the potential correlation between azole resistance and macrophage phagocytosis, considering that these cells are essential players in the early immune response against fungal

infections. Among their duties, macrophages contribute to the containment of pathogenic organisms and recruit other immune cells for further response (Feldman *et al.* 2019). Of interest, some stress-response pathways of *A. fumigatus* have been reported to take part in both, fungal virulence and antifungal resistance (Brown & Goldman 2016), which is indicative of a clear relationship between both factors. On this basis, we carried out *in vitro* macrophage infections with three of our strains showing different VRC susceptibilities. The results that we obtained showed a direct proportional tendency between azole susceptibility and fungal survival after macrophage infection. In addition, pre-exposure of infecting conidia to subinhibitory concentrations of VRC resulted in alterations on fungal clearance by the macrophages, increasing the post-infection survival of the conidia in the most resistant and most-susceptible strains. An explanation to this phenomenon could be that azoles might exert structural or physical modifications onto the conidia surface that contribute to poor recognition by the macrophages or increase the conidia intracellular survival within them. Altogether these data could be extremely useful to characterize the early steps of *Aspergillus* infection establishment and the impact to this process caused by azole pre-exposure, which might be valuable to improve clinical management of the disease.

All things considered, this study provides new evidence onto the *cyp51* contribution to azole resistance in *Aspergillus* section *Nigri* and proposes potential new mechanisms that could promote azole resistance, especially in non-*fumigatus* species. However, further studies are needed to corroborate and expand our findings.

CONCLUSIONS

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1. Cyp51 proteins in *Aspergillus* share a common ancestor. Duplications through evolution resulted in two separated phylogenetic lineages of enzymes: the Cyp51A and the Cyp51B. A third paralog named Cyp51C has arisen from duplications of both lineages.

2. Protein topology of Cyp51 enzymes diverges among species within the genus *Aspergillus.* In species *A. niger*, *A. tubingensis* and *A. brasiliensis* belonging to section *Nigri*, these display two transmembrane domains in contrast to *A. fumigatus*, which displays only one.

3. Triazoles ITC, PSC and VRC display good activity against clinical and environmental strains of *Aspergillus* section *Nigri*, with PSC exerting the best *in vitro* activity.

4. Low susceptibility to triazoles is more frequent in *A. tubingensis* strains.

5. The Cyp51A protein leads azole response in *Aspergillus* section *Nigri* in contrast to Cyp51B, as evidenced by the higher expression of the *cyp51A* gene and its upregulation upon VRC exposure.

6. Amino acid substitutions in the Cyp51A protein are not clearly correlated to azole resistance in *Aspergillus* section *Nigri.* Only amino acid changes H467Q, V377I and K64E were exclusively detected in non-wt strains, although their location does not comprise azole binding sites residues.

7. Basal ergosterol and intermediate sterol 24(28)DHE levels do not correlate with azole susceptibility displayed by section *Nigri*. However, ergosterol is depleted after VRC exposure whereas minor alterations are observed in the 24(28)DHE contents.

8. Single gene deletion of either *cyp51A* or *cyp51B* impact azole susceptibility by reducing VRC MICs and decreasing ergosterol levels, with a more prominent effect due to the *cyp51A* deletion.

9. Composition and properties of the fungal plasma membrane and cell wall, such as their respective permeability or chitin content appear to be important for azole resistance.

10. Laccase activity is increased as a response to azole stress in a concentration-dependent manner in *A. niger*.

11. *In vitro* phagocytosis of conidia from species of *Aspergillus* section *Nigri* by macrophages is proportionally correlated to azole susceptibility.

12. Pre-exposure of conidia to subinhibitory concentrations of VRC results in an enhanced survival of conidia after *in vitro* macrophage phagocytosis.

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STUDY AND CHARACTERIZATION OF AZOLE RESISTANCE IN ASPERGILLUS SECTION NIGRI
Alba Pérez Cantero

SUPPLEMENTARY MATERIAL

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New insights into the Cyp51 contribution to azole resistance in *Aspergillus* section *Nigri*

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Table S1. Antifungal susceptibility results. In bold, MIC values above the stablished ECVs. Underlined strains were further studied by gene expression and sequencing analyses.

STRAIN	SPECIES	MICs (mg/L)		
		ITC	POS	VRC
FMR 6267	*A. niger*	1	0,06	1
FMR 7131	*A. japonicus*	0,03	0,03	0,03
FMR 9393	*A. niger*	1	0,06	2
FMR 11248	*A. niger*	2	0,12	2
FMR 11250	*A. niger*	1	0,06	1
FMR 11253	*A. niger*	2	0,25	2
FMR 11254	*A. niger*	2	0,06	1
FMR 11894	*A. niger*	0,25	0,03	0,25
FMR 11896	*A. niger*	2	0,12	2
FMR 11897	*A. niger*	0,12	0,03	0,25
FMR 11898	*A. niger*	0,12	0,03	0,25
FMR 11900	*A. niger*	**16**	0,25	**16**
FMR 11901	*A. niger*	1	0,06	1
FMR 11906	*A. tubingensis*	2	0,12	2
FMR 13537	*A. awamori*	1	0,12	1
FMR 13538	*A. awamori*	1	0,06	1
FMR 13539	*A. awamori*	0,25	0,06	0,5
FMR 13540	*A. awamori*	1	0,06	1
FMR 13541	*A. awamori*	1	0,06	1
FMR 14592	*A. japonicus*	0,12	0,03	0,12
FMR 14630	*A. tubingensis*	2	0,12	**4**
FMR 14635	*A. tubingensis*	4	0,12	**4**
FMR 14638	*A. carbonarius*	0,12	0,03	0,12
FMR 14640	*A. niger*	1	0,12	2
FMR 14712	*A. tubingensis*	**16**	0,25	**4**
FMR 14714	*A. niger*	1	0,06	1

FMR 15326	*A. neoniger*	2	0,25	2
FMR 15385	*A. niger*	1	0,25	2
FMR 15386	*A. brasiliensis*	**16**	0,12	**8**
FMR 15387	*A. niger*	2	0,12	2
FMR 15388	*A. niger*	1	0,5	1
FMR 15389	*A. tubingensis*	2	0,25	2
FMR 15396	*A. niger*	0,12	0,03	0,25
FMR 15413	*A. acidus*	1	0,25	2
FMR 15441	*A. niger*	1	0,5	**8**
FMR 17207	*A. tubingensis*	**8**	0,25	**4**

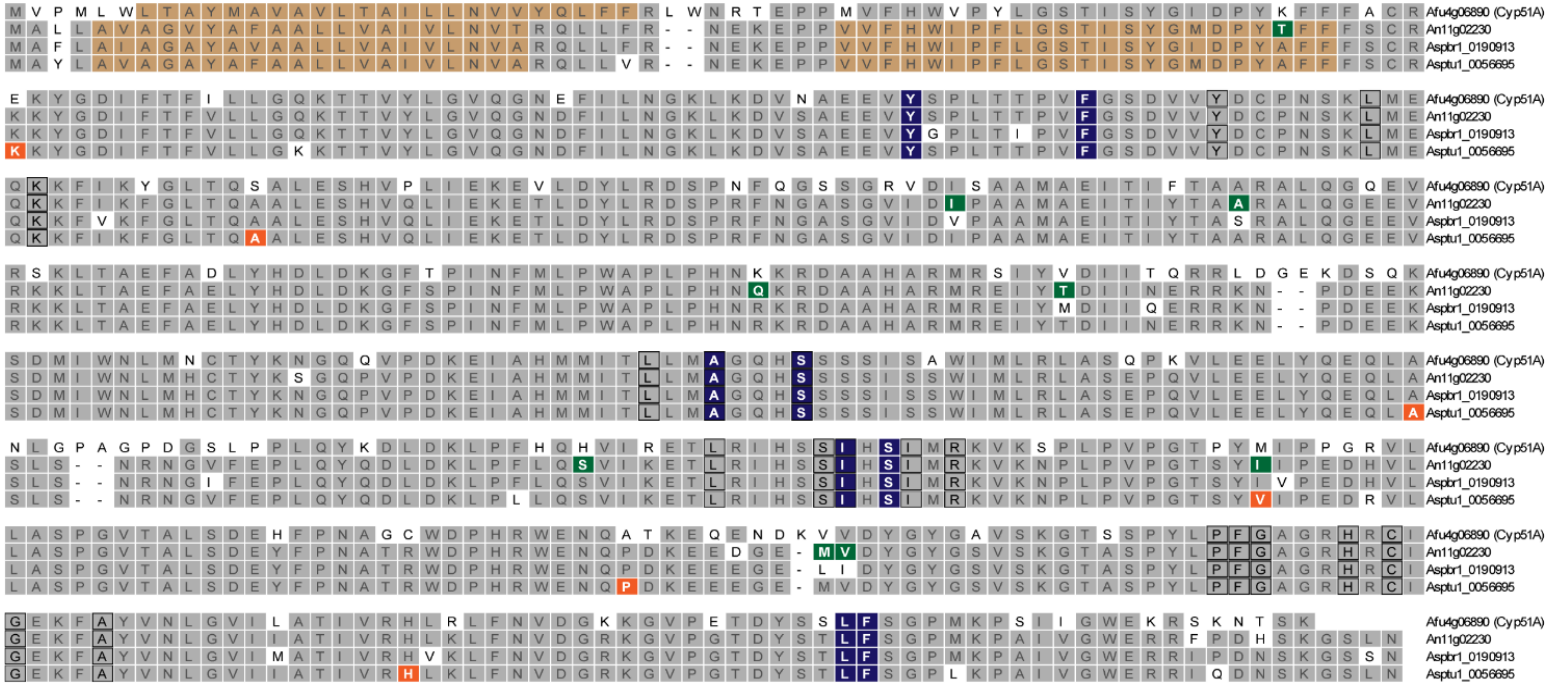


Fig S1. Sequence alignment of Cyp51A proteins from *A. fumigatus*, *A. tubingensis*, *A. niger* and *A. brasiliensis.* Identical residues are marked in grey shadow. Non-synonymous mutations found in the studied strains are highlighted in green (*A. niger*) or orange (*A. tubingensis).* Residues that constitute the putative azole binding pocket are marked in dark blue; the transmembrane domain is coloured in brown and the heme prosthetic group residues are squared

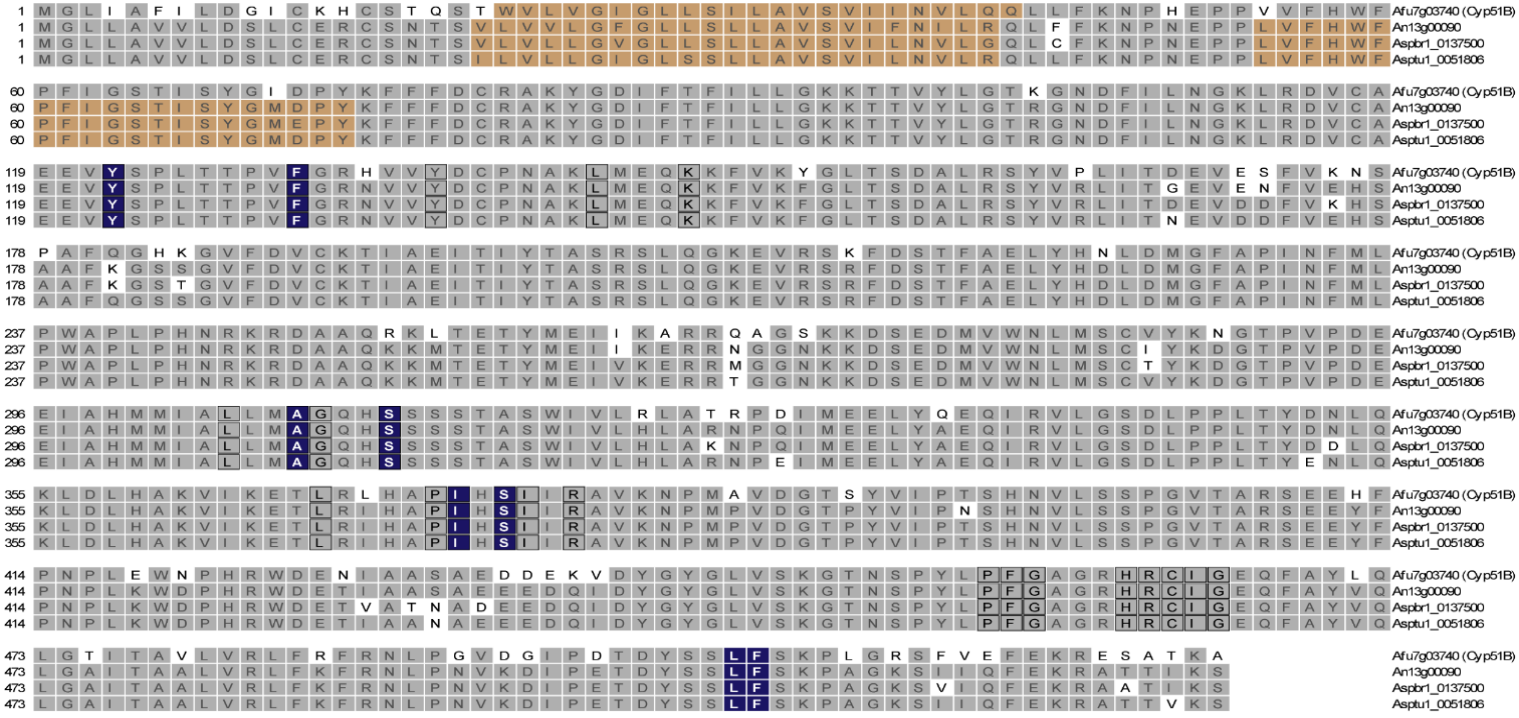
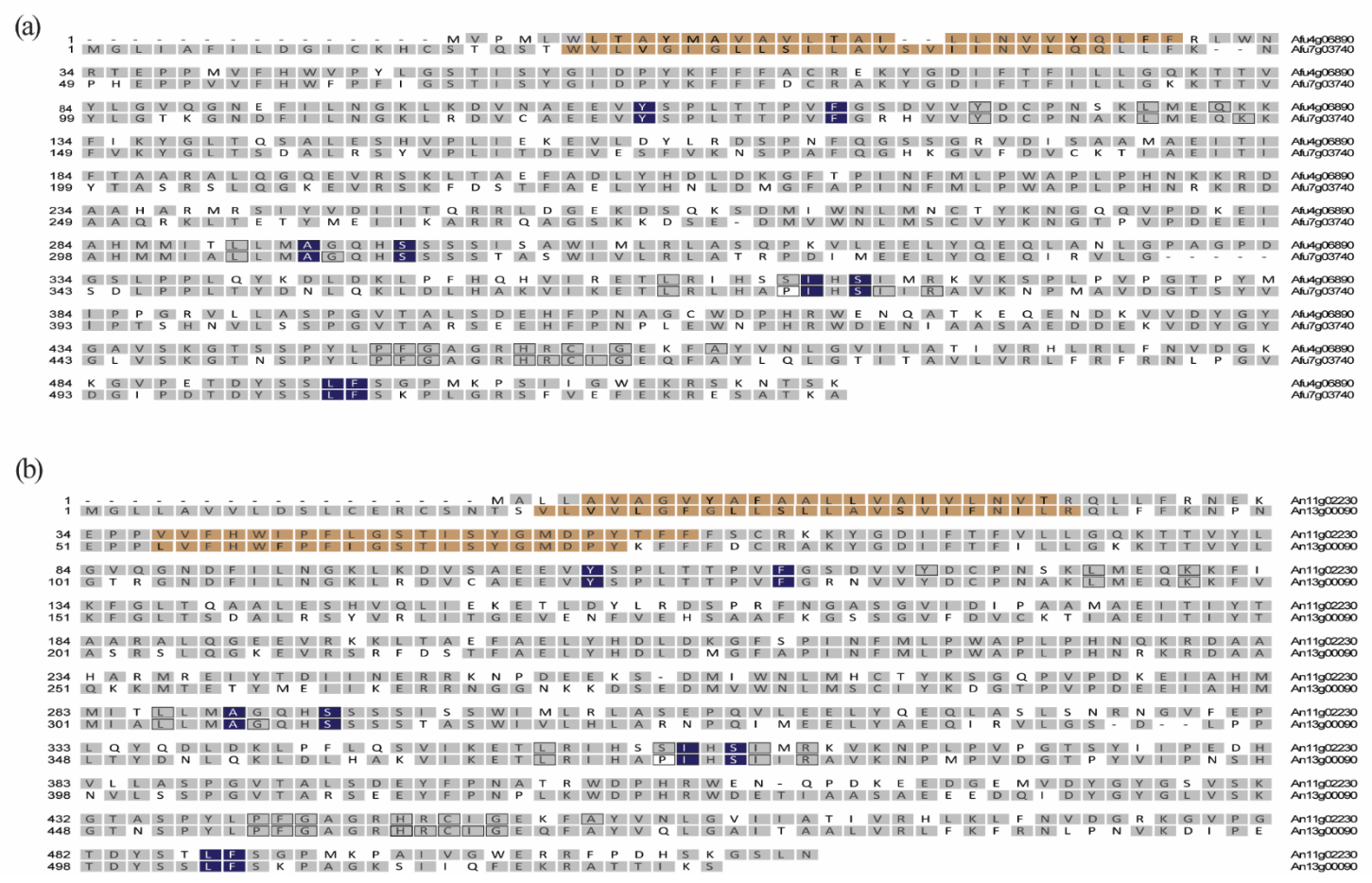
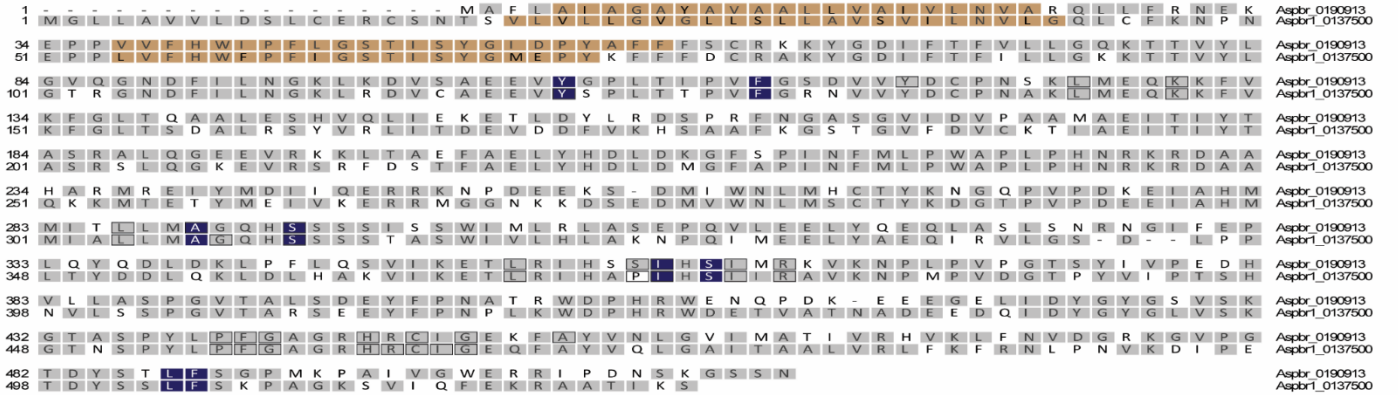


Fig S2. Sequence alignment of Cyp51B proteins from *A. fumigatus*, *A. tubingensis*, *A. niger* and *A. brasiliensis.* Identical residues are marked in grey shadow. Residues that constitute the putative azole binding pocket are marked in dark blue; the transmembrane domain is coloured in brown and the heme prosthetic group residues are squared.


(a)
Afu4g06890
Afu7g03740
(b)
An11g02230
An13g00090

(c)



(d)

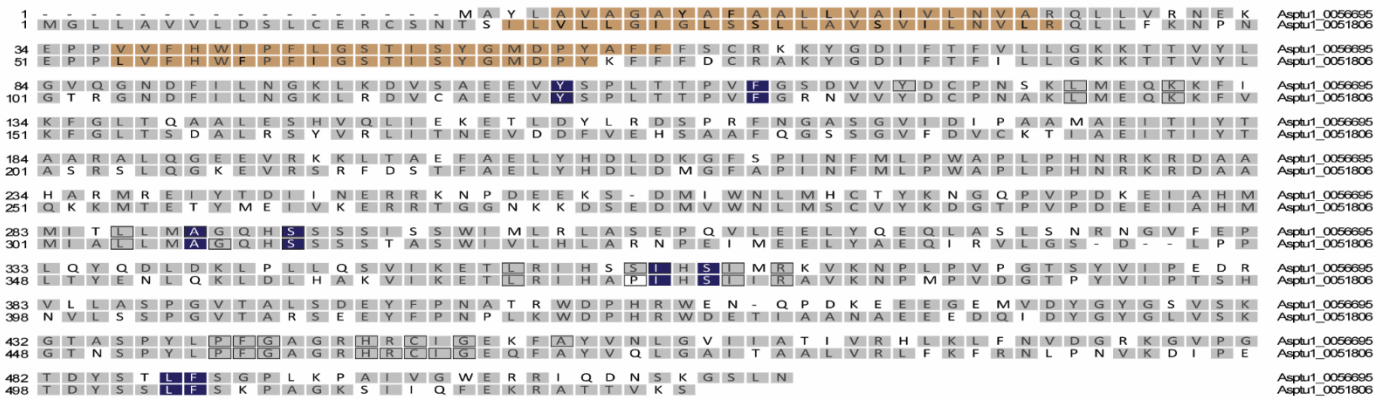


Fig S3. Sequence alignment of Cyp51A and Cyp51B proteins from (a) *A. fumigatus*, (b) *A. niger*, (c) *A. brasiliensis* and (d) *A. tubingensis.* Identical residues are marked in grey shadow. Residues that constitute the putative azole binding pocket are marked in dark blue; the transmembrane domain is coloured in brown and the heme prosthetic group residues are squared.

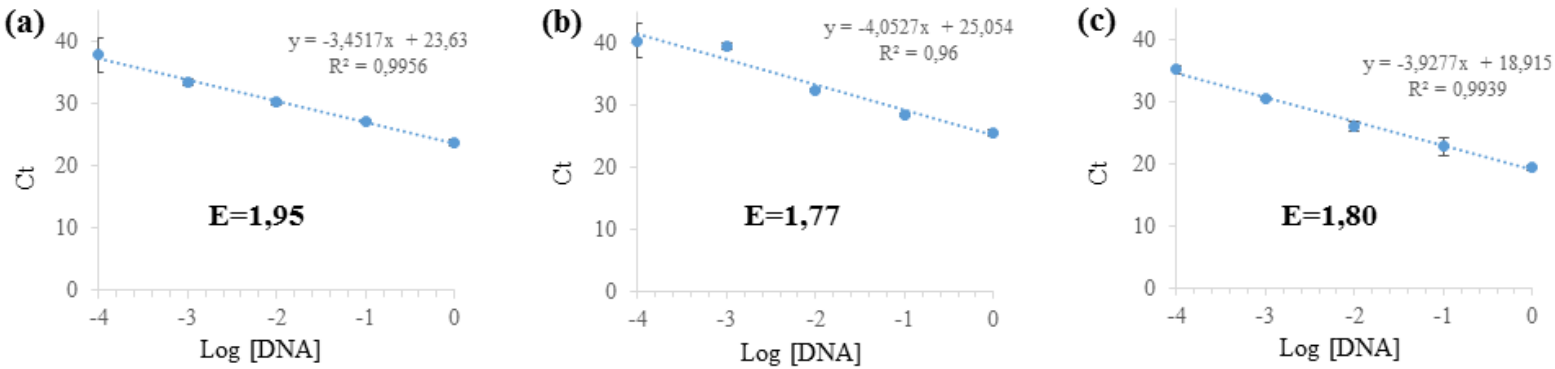


Fig. S4. RT-qPCR efficiency of *act* (**a**), *cyp51A* (**b**) and *cyp51C* (**c**). Amplification efficiency (E) was calculated according to $E=10^{-10/slope}$.

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