Emergence of multidrug cross-resistance between agricultural and human antifungals in clinically relevant species of *Aspergillus* and *Candida*.

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LIST OF PUBLICATIONS

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Chapter 2 **Faria-Ramos I**, Neves-Maia J, Ricardo E, Santos-Antunes J, Silva AT, Costa-de-Oliveira S, Cantón E, Rodrigues AG, Pina-Vaz C. Species distribution and in vitro antifungal susceptibility profiles of yeast isolates from invasive infections during a Portuguese multicenter survey. Eur J Clin Microbiol Infect Dis.2014. 33(12):2241-7.

Chapter 3 **Faria-Ramos I**, Tavares PR, Farinha S, Neves-Maia J, Miranda IM, Silva RM, Estevinho LM, Pina-Vaz C, Rodrigues AG. Environmental azole fungicide, prochloraz, can induce cross-resistance to medical triazoles in Candida glabrata.FEMSYeastRes.2014. 14 (7):1119-23.

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Chapter 5 Azevedo MM, **Faria-Ramos I**, Cruz LC, Pina-Vaz C, Gonçalves Rodrigues A. Genesis of Azole Antifungal Resistance from Agriculture to Clinical Settings. J Agric Food Chem. 2015. 63(34):7463-8.

Abstracts from international scientific congresses

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Faria-Ramos I, J. Santos-Antunes, S. Costa-de-Oliveira, A.G. Rodrigues, C. Pina-Vaz. Susceptibility profile of deep-seated yeasts isolates from a university hospital in the

northern region of Portugal. The 22nd European Congress of Clinical Microbiology and Infectious Diseases.ECCMID2012, London,UK.

Faria-Ramos I, S. Farinha, P. Tavares, J. Maia, E. Ricardo, C. Pina-Vaz and A.G. Rodrigues. Development of resistance to agricultural azole antifungal compounds - emergence of multidrug cross-resistance among human fungal pathogens *Candida*, *Cryptococcus* and *Aspergillus*. Interscience Conference on Antimicrobial Agents and Chemotherapy. ICAAC2012, Califórnia-USA.

LIST OF ABBREVIATIONS

5FC	5-Flucytosine
ABC	Adenosine Triphosphate Binding Cassette
ABPA	Allergic bronchopulmonary aspergillosis
AMB	Amphotericin B
AND	Anidulafungin
ATP	Adenosine Triphosphate
CLSI	Clinical Laboratory Standards Institute
CSF	Caspofungin
DMIs	Demethylation inhibitors
DMSO	Dimethysulphoxide
DNA	Deoxyribonucleotide Acid
dNTPS	Deoxynucleoside Triphosphates
ECDC	European Centre for Disease Control
ECV	Epidemiological Cutoff Values
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FLC	Fluconazole
FRAC	Fungicide Resistance Action Committee
IA	Invasive Aspergillosis
IE	Insufficient Evidence
IFI	Invasive fungal infection
ITC	Itraconazole

MALDITOF MS	Matrix assisted laser desorption ionization-time to flight mass spectrometry
MCF	Micafungin
MDR	Multidrug-Resistance
MFS	Major Facilitator Superfamily
MIC	Minimal inhibitory concentration
NA	Not applicable
PCR	Polymerase Chain Reaction
PCZ	Prochloraz
POS	Posaconazole
PPP	Plant Protection Products
QC	Quality Control
RT-qPCR	real-time quantitative Polymerase Chain Reaction
SPSS	Statistical Package for Social Sciences
VOR	Voriconazole
VRC	Voriconazole
YPD	Yeast extract-Peptone-Dextrose

LIST OF TABLES AND FIGURES

List of Tables

Chapter 1

Table 1. *Candida* habitat.

Chapter 2

Table 1. Distribution and characteristics of the isolated yeasts.

Table 2. Distribution of minimum inhibitory concentration (MIC) values comparing the Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocols.

Chapter 3

Table 1. Susceptibility profile of *Candida* species tested to PCZ, triazoles and anidulafungin.

Chapter 4

Table 1. Susceptibility pattern of tested *A. fumigatus* isolates to PCZ and clinical azoles.

List of Figures

Chapter 1

Figure 1. Macro and microscopic morphologic aspect of an *Aspergillus fumigatus.*

Figure 2. Schematic representation of the principal antifungal resistance mechanisms.

Chapter 3

Figure 1. Gene expression alterations triggered by PCZ exposure in *C. glabrata*.

Black bars represent the susceptible initial strain – day 0; gray bars represent the same strain after the induction assay, day 90. Comparative gene expression profile between the initial, day 0, susceptible strain and the strain after the induction assay, day 90. Gene expression is expressed as average with standard deviation of three independent experiments. Each mean value was normalized with the *ACT1* gene.

Figure 2. Single Nucleotide Polymorphisms (SNPs) found in *Candida glabrata PDR1* and *ERG11* genes. **(A)** *PDR1* missense mutation G727A results in the amino acid change D243N. **(B)** Several synonymous SNPs throughout the *ERG11* gene.

Chapter 4

Figure 1. Photographs of Sabouraud dextrose agar plates showing macroscopic morphological changes of colonies of *A. fumigatus* following exposure to subinhibitory concentration of PCZ. **A.** Initial morphological aspect (control). **B.** After fifteen days. **C.** Thirty days after

Figure 2. Photomicrographs of *A. fumigatus* colonies using the cellotape flag technique preparation with lactophenol cotton blue staining. Microscopic morphological changes in the development of conidiation of A. fumigatus following exposure to subinhibitory concentration of PCZ. **A.** Initial morphological aspect (control). **B.** After fifteen days. **C.** After thirty days.

Chapter 5

Figure 1. Chemical structure of (A) imidazole, (B) 1,2,4 triazole, (C) fluconazole, and (D) tebuconazole.

Figure 2. Sales of fungicides in Portugal between 2002 and 2012.

Figure 3. Sales of agricultural azole in Portugal between 2002 and 2012.

ABSTRACT

The spectrum of human pathogens and the infectious diseases they cause is continuously changing through evolution and changes in the way human populations interact with the surrounding environment. It is known that modern agriculture -very large scale, industrialized and fungicide dependent - was one of the turning points in the history of infectious diseases. Over the last three decades, clinically important fungi have become more prevalent, mostly due to medical advances that allow the survival of an increasing number of debilitated and immunocompromised patients. Fungicides with similar chemical structure and mode of action to those used in medicine are also widely used in agriculture. Thus, a few years ago a critical problem emerged – the development of cross-resistance between agricultural and human antifungals in clinically relevant species of *Aspergillus* and *Candida*.

The present study aims to provide insights whether the acquisition of resistance can arise from nature and impact on human health.

It is known that the epidemiological landscape of fungal invasive infections (IFI) is changing. Therefore, a national multicentric surveillance study was carried out. From this study important clinical and demographic data were obtained as well as information regarding the *in vitro* susceptibility profile of yeast isolates. *Candida albicans* remains the most frequent agent of IFI but the prevalence of non-*albicans* keeps increasing. The overall susceptibility rates ranged from 84 to 98% to azoles and 74 to 97% to echinocandins.

From this newly assembled collection, our next step was to determine its *in vitro* susceptibility to the most frequently used agricultural fungicides. Susceptible isolates of *C. albicans*, *C. parapsilosis* and *C. glabrata* to both clinical and agricultural antifungals were selected; an *in vitro* induction assay was started with one of the most frequently

used fungicide in Portugal – Prochloraz (PCZ), at sub-inhibitory concentrations. During the 90 days of induction, minimal inhibitory concentration (MIC) values were continuously evaluated and after 10 days of testing all *Candida* species revealed a 32 to 64 times higher MIC value to the inducer fungicide. *C. glabrata* was the only species developing cross-resistance to clinical azoles after 60 days of exposure to the agricultural fungicide. After screening the most common azole resistance mechanisms, we found that PCZ selective pressure triggered an upregulation of the ATP Binding Cassette multidrug transporter genes and the transcription factor, *PDR1*. Single point mutation previously associated with azole resistance was described in *PDR1* while in *ERG11* gene several synonymous single nucleotide polymorphisms were found. These results provide possible explanations for the worldwide increasing prevalence of *C. glabrata* and the associated poor clinical outcome associated with infections by this species.

The following work recreated a similar approach with another clinical relevant fungal pathogen – *A. fumigatus*. Two clinical and one environmental isolate were daily incubated with sub-inhibitory concentrations of PCZ. The fungicide exposure induced morphological changes and a significant increase of MIC value to PCZ, as well as cross-resistance to all the tested clinical azoles within the first 30 days of the assay.

In the last study we aimed to review the national and global origin of azole antifungal resistance from nature to clinical settings. After such state of art revision we were forced to conclude that a fungicide-based plant protection agriculture remains essential to maintain the needed high crop production. In order to prevent or delay the development of cross-resistance some efforts should be made by the agriculture communities such as rotation of fungicides with different modes of action during the crop campaign and making more efficient fungicide mixtures of multi-site inhibitors,

therefore excluding the use of monofungicides. Also, surveillance studies should be implemented in order to follow the variation in these fungal species distribution and resistance rate to both agricultural and clinical antifungals.

In summary, this dissertation highlights the occurrence of antifungal cross-resistance between agriculture and medicine involving two relevant species of *Aspergillus* and *Candida*. Both pathogenic fungi showed that agricultural fungicides are capable of inducing cross-resistance to medical antifungals.

RESUMO

A quantidade de agentes patogénicos e as doenças infeciosas que estes causam está continuamente a mudar e esta evolução ocorre devido às interações entre seres humanos e com o meio ambiente. A agricultura moderna, de larga escala, muito industrializada e altamente dependente de fungicidas, foi um dos pontos de viragem na história das doenças infeciosas. Ao longo das últimas três décadas, os fungos medicamente importantes tornaram-se mais prevalentes, sobretudo devido a técnicas médicas avançadas que permitem a sobrevivência de um número crescente de doentes muito debilitados e imunocomprometidos. Fungicidas, com estrutura química e modo de ação muito semelhantes aos antifúngicos médicos, são frequentemente usados na agricultura. Assim sendo, há alguns anos atrás surgiu um novo problema – o desenvolvimento de resistência cruzada entre antifúngicos agrícolas e clínicos em espécies clinicamente relevantes de *Aspergillus* e *Candida*.

O presente estudo procurou esclarecer se a aquisição de resistência pode surgir da natureza e sobre o seu impacto na saúde humana.

Dado que a epidemiologia destas infeções fúngicas invasivas (IFI) tem vindo a mudar, foi inicialmente realizado um estudo multicêntrico a nível nacional. Foram obtidos dados clínicos e demográficos e, resultados importantes sobre o perfil de suscetibilidade *in vitro* destes isolados clínicos. A levedura *Candida albicans* continua a mais frequentemente encontrada como agente causal de IFI; contudo a prevalência de leveduras não-*albicans* continua a aumentar. As taxas globais de susceptibilidade aos antifúngicos clínicos variaram entre 84 a 98% para a classe dos azoles e de 74 a 97% para a classe das equinocandinas.

A partir desta recém formada coleção de leveduras determinámos a susceptibilidade *in vitro* aos fungicidas mais frequentemente utilizados na agricultura.

Alguns isolados de *C. albicans*, *C. parapsilosis* e *C. glabrata* suscetíveis aos antifúngicos clínicos e agrícolas foram selecionados para o estudo seguinte. Foi efetuado um ensaio de *indução in vitro* com um dos fungicidas mais utilizados em Portugal – Prochloraz (PCZ). Durante os 90 dias de indução os valores da concentração inibitória mínima (CIM) foram continuamente avaliados. Após os primeiros dez dias, o perfil de susceptibilidade de todas as espécies de *Candida* avaliadas revelou um valor de CIM entre 32 a 64 vezes superior ao inicial relativamente ao fungicida indutor. *Candida glabrata* foi a única espécie a desenvolver resistência cruzada aos antifúngicos clínicos após 60 dias de exposição ao fungicida agrícola. Estudámos os mais comuns mecanismos de resistência aos azoles e descobrimos que a pressão seletiva de PCZ desencadeou uma sobreregulação de genes que codificam para transportadores multidrogas, designados por ATP Binding Cassette, e o factor de transcrição, *PDR1*. Foi descrita uma mutação associada à resistência a azoles no factor de transcrição enquanto no gene *ERG11* foram detectados vários "single nucleotide polimorfisms". Estes resultados podem explicar a prevalência crescente de *C. glabrata* a nível global e o pior prognóstico associado a infecções causadas por esta espécie. O estudo seguinte recriou uma abordagem semelhante com *A. fumigatus.* Dois isolados clínicos e um ambiental foram expostos ao PCZ e esta exposição induziu alterações morfológicas e um aumento concomitante do valor de CIM ao PCZ. Após 30 dias de ensaio de indução, verificou-se para todos os isolados a aquisição de resistência cruzada aos antifúngicos de uso médico.

Por último, procedemos a uma revisão acerca da origem da resistência aos azoles, desde a agricultura até ao contexto clínico, a nível nacional e internacional. Concluímos que a agricultura moderna, altamente dependente de fungicidas, se mostra essencial para manter a produção de alimentos nas quantidades necessárias. A fim de

prevenir ou retardar o desenvolvimento da resistência cruzada, a comunidade agrícola deveria tomar algumas medidas, tais como rotação de fungicidas com diferentes modos de acção durante do cultivo, efectuar misturas de fungicidas mais eficientes, com múltiplos locais de inibição e excluir a utilização de monofungicidas. Além disso, estudos de vigilância deveriam ser implementados para acompanhar e monitorizar a distribuição de espécies e taxas de resistência a antifúngicos agrícolas e de uso médico.

Em resumo, esta dissertação destaca o efectivo desenvolvimento de resistência cruzada aos antifúngicos entre a agricultura e a medicina em espécies clinicamente relevantes de *Aspergillus* e *Candida*. Ambos os fungos patogénicos mostraram que os fungicidas agrícolas são capazes de induzir resistência cruzada aos antifúngicos de uso médico.

Contents

Acknowledgments / Agradecimentos xi

List of Publications xvii

List of Abbreviations xxi

List of Tables and Figures xxiii

Abstract xxvii

Resumo xxxi

Contents xxxv

Chapter 1 Introduction

General considerations **3**

Aspergillus fumigatus as a human pathogen **4**

Candida as a human pathogen **8**

Bridging *Aspergillus* and *Candida* **10**

Antifungals and susceptibility testing **11**

Antifungal resistance **12**

Dilemmas in clinical management **17**

The use of fungicides in anthropogenic activities **19**

The impact of agricultural antifungals upon clinical antifungals **22**

Aims and outline of this thesis **28**

Chapter 2 Species distribution and *in vitro* antifungal susceptibility profiles of yeast isolates from invasive infections during a Portuguese multicenter survey. **32**

Chapter 3 Environmental azole fungicide, prochloraz, can induce cross- -resistance to medical triazoles in *Candida glabrata*. **46**

Chapter 4 Development of cross-resistance by *Aspergillus fumigatus* to clinical azoles following exposure to prochloraz, an agricultural azole. **60**

Chapter 5 Genesis of Azole Antifungal Resistance from Agriculture to Clinical Settings. **73**

Chapter 6 Overall discussion and conclusions **90**

Chapter 7 References **101**

Chapter 8 Publications **124**

1 Introduction

General considerations

From our contemporary perspective it is difficult to grasp the magnitude of achievements of our ancestors, mainly because we take the power of experimentation for granted. It is not easy, in the context of our modern scientific methodology, to imagine a time when information was sought largely by speculation guided by religious fantasy.

Fungi were most probably discovered from early struggles to understand and overcome plant diseases and crop losses. There are several stories about early neolithic farmers that pointed out black dust over plant cultures and even noticed black-streaked cereal leaves. The first description of fungi was given by Giambattista della Porta as "tiny black seeds" over a plant named *Phytognomonica* in 1588. This vague information was only reviewed more than a century afterwards, and with the help of the microscope and by the illustrations of Hooke, Malpighi and Leeuvenhoek. However, only in 1729, a priest and botanist, Pier Antonio Micheli, describes a fungus in "Nova Plantarum Genera", that he named *Aspergillus* due to its microscopic morphologic similarity to an aspergillum - a brush or instrument used for sprinkling holy water [1].

On the other hand, yeasts can be considered man's oldest industrial microorganism. It's likely that man used yeast before the development of a written language. Hieroglyphics suggest that ancient Egyptians used yeast and the process of fermentation to produce alcoholic beverages and to leaven bread over 5,000 years ago. The biochemical process of fermentation that is responsible for these actions was not understood and undoubtedly looked upon by early man as a mysterious and even magical or miraculous phenomenon. It is believed that these early fermentation systems for alcohol production and bread making were formed by natural microbial

contaminants of flour, other milled grains or from fruit. Such microbial flora would have included wild yeasts and lactic acid bacteria that are found associated with cultivated grains and fruits. Also, it was not until the invention of the microscope followed by the pioneering scientific work of Louis Pasteur in the late 1860's that yeast was identified as a living organism and the agent responsible for alcoholic fermentation and dough leavening [2-4].

Nowadays, the importance of *Aspergillus* and *Candida* as human pathogens is undeniable. Based on population and disease demographics, it is estimated that globally over 1 billion people are afflicted with a fungal infection and 25 million are at great risk severe organ damage or death due to an invasive fungal infection (IFI) [5-8]. IFI caused by *Aspergillus* spp. and *Candida* spp. represent the most frequent life-threatening fungal infections in Europe [9-11]. Global models estimate that invasive aspergillosis affects more than 300.000 people and invasive candidosis more than 700.000 people per year, with mortality rates above 40% for both [8, 9].

***Aspergillus fumigatus* as a human pathogen**

Aspergillus species are widespread in the environment, growing on plants, decaying organic matter, in soil and in fresh or marine waters. Aspergilli are also found in indoor environments, in drinking water and dust. The diverse species belonging to *Aspergillus* genus are able to use a wide variety of organic substrates and adapt very well to a broad range of environmental conditions. They produce asexual conidia that readily become airborne and are highly stress tolerant, but can also produce environmentally persistent sexual ascospores [11].

The genus *Aspergillus* contains 339 species divided into eight subgenera – Aspergillus, Fumigati, Circumdati, Candidi, Terrei, Nidulantes, Warcupi and Ornati – which in turn are subdivided into several sections or species complexes [12]. Only a few species, less than 40, are known to cause diseases in humans. Most invasive infections are caused mainly by members of *A. fumigatus* complex followed by *A. flavus*, *A. terreus* and *A. niger* species complex. Recently, the taxonomy of *A. fumigatus* has been revised by incorporating sequence-based information. In fact, *A. fumigatus* designation actually represents a section or complex of closely related species also referred as cryptic species that cannot be distinguished morphologically. *A. fumigatus* sensu stricto is the leading human pathogen while the sibling species of the complex have only been recognized as occasionally causing invasive aspergillosis, from 3 to 6% of the cases [13]. However, their actual prevalence may be underestimated because of the lack of recognition by conventional diagnostic approaches [14, 15]. Apart from *A. fumigatus*, the species of the complex *Fumigati* that are most frequently recovered in clinical specimens and associated with invasive fungal diseases are *A. lentulus*, *A. udagawae*, *A. viridinutans*, *A. thermomutatus*, *A. novofumigatus* and *A. hiratsukae*. Their limited pathogenic role compared to *A. fumigatus* may be explained by a lower thermotolerance and different profiles of secondary metabolites with decreased production of micotoxins, such as gliotoxin. Interestingly, these cryptic species exhibit decreased susceptibility to azoles and other medical antifungal agents [16, 17].

A. fumigatus is a saprophytic fungus that plays an important role in recycling environmental carbon and nitrogen in the composting procedure. It has thermotolerant properties which made it one of the few fungi able to handle with the high temperature during composting. *A. fumigatus* spp. exhibit typical morphological features that allows differentiation from other fungi. Macroscopically, they are characterized by rapid

growth, with a powdery texture and colored with various shades of green-blue, depending on the fungal strain and type of agar used. Microscopically, they are characterized by the presence of narrow, dichotomously branched septate hyphae. The conidiophores are a branch of the vegetative hyphae originating from the foot cell and ending with a vesicle. *A. fumigatus* sensu stricto exhibits columnar conidial heads with a flask-shaped vesicle, named phialides, and has rough conidia that are macroscopically green (**Figure 1**) [14, 18].

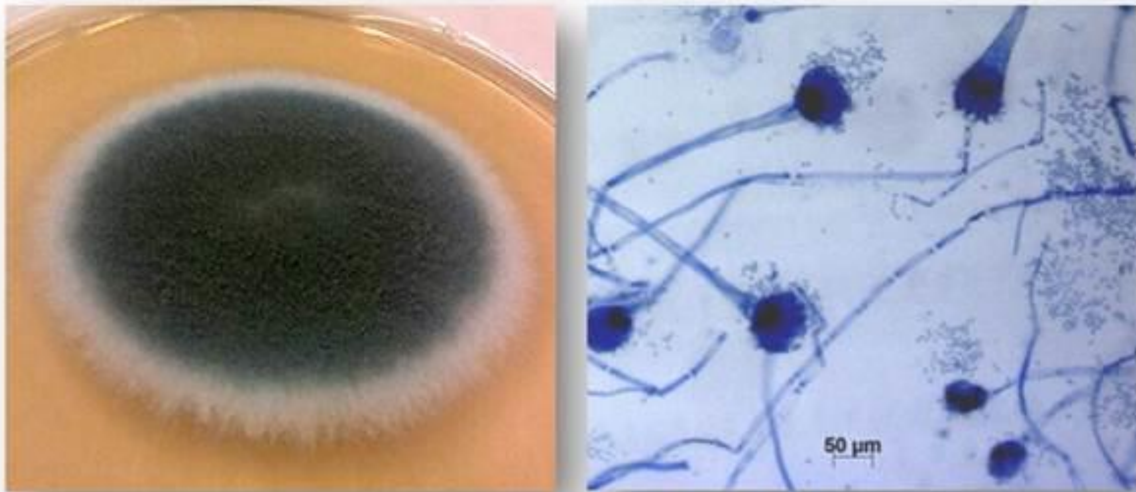

Figure 1. Macro and microscopic morphologic aspect of an *Aspergillus fumigatus.*

Conidia of *A. fumigatus* that are inhaled by humans are usually eliminated by the innate immune system (neutrophils and macrophages) in immunocompetent individuals. However, depending on the virulence of the fungal strain, immunological status, and the host's pulmonary structure and function, *A. fumigatus* may cause a spectrum of diseases that may range from allergic bronchopulmonary aspergillosis, chronic pulmonary aspergillosis to invasive aspergillosis (IA), which is a disseminated disease with a mortality rate of up to 90% in high-risk populations.

Allergic diseases caused by *Aspergillus* can be associated with asthma, sinusitis and alveolitis and occur following repeated exposure to conidia and/or *Aspergillus* antigens [19, 20]. In such cases, there is usually no mycelial colonization, and removal of the patient from the environmental source results in clinical improvement. Allergic bronchopulmonary aspergillosis (ABPA) is considered as an extreme form of *A. fumigatus*-induced asthma. In this case, the fungus grows saprophytically in the bronchial lumen, resulting in bronchial inflammation. The conidia trigger an IgE-mediated allergic inflammatory response, leading to bronchial obstruction. Symptoms are recurrent fever, cough, wheezing, pulmonary infiltrates and fibrosis [21]. ABPA is observed in a small but numerically significant fraction of patients with asthma or cystic fibrosis (1–2% or 8–9% of the total, respectively) [22]. A locally invasive version of the disease, chronic necrotizing pulmonary aspergillosis, is mainly observed in humans with mild immunodeficiency or with a chronic lung disease [23]. Non-invasive forms of *Aspergillus*-induced lung disease include aspergilloma and ABPA [24]. IA currently constitutes the most common cause of pneumonia-related mortality in patients undergoing hematopoietic stem cell transplantation and is an important cause of opportunistic respiratory and disseminated infections in other immunocompromised patients [25, 26]. Globally, *Aspergillus* is estimated to cause health issues in millions of people annually, with IA accounting for approximately 200.000 annually [18, 27]. Early diagnosis as well as severity of underlying diseases impact the mortality rate of IA, which is acknowledged to be in the range of 30-50% [27], but such numbers are alarmingly high (>80%) when diagnosis is delayed or the causative agent is resistant [28].

***Candida* as a human pathogen**

Candida organisms are present not just in the usual human microbioma of mucosal oral cavity, gastrointestinal tract and vagina as commensals but are also ubiquitous in the environment [29, 30], **Table 1**.

Table 1. *Candida* habitat. (Table from Schauer and Hanschke [30]).

SOURCE(S) OF ISOLATES	% OF TOTAL [a]
Plants, leaves and flowers	21
Fruits and juices	9
Beer and wine	4
Other food items	8
Soil and sludge	11
Water	6
Air	1
Insects	12
Animals	8
Humans	16
Machines	3

a Frequency of isolation from source(s) relative to other sources.

The genus includes a very large heterogeneous group of organisms, while only a few different *Candida* species are known to be aetiological agents of human infection. Only five species account for 92% of cases of candidemia: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei*. However, its distribution varies in population-based studies conducted in different geographical areas. *C. albicans* is the most frequent species, but considerable differences are found between the number of cases caused by *C. glabrata* and *C. parapsilosis*. Studies from Northern Europe and the United States of America (USA) reported a high number of cases caused by *C. glabrata*, whereas studies from Portugal, Spain and Brazil demonstrated a lower number of cases caused by *C.*

glabrata and a higher number of cases attributed to *C. parapsilosis* [31]. Globally, the frequency of *C. albicans* is decreasing, while that of *C. glabrata* and *C. krusei* is stable, and *C. parapsilosis* and *C. tropicalis* is increasing. In most individuals, *C. albicans* resides as a lifelong, harmless commensal [32]. However, under certain circumstances, *C. albicans* can cause infections that range from mucocutaneous overgrowth to life-threatening systemic infections [33]. The growing population of immunocompromised patients receiving intravenous catheters, total parenteral nutrition or invasive procedures and the increasing use of broad-spectrum antibiotics, cytotoxic chemotherapies and transplantation contribute to the increase of such infections [34]. The pathogenicity of *Candida* species is attributed to certain virulence attributes, such as the ability to evade host defenses, adherence, biofilm formation (on host tissue and medical device surfaces) and the production of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and haemolysin [33].

Candida spp. remain the most common cause of IFI, and the incidence of candidemia, which is estimated at 72.8 cases per million inhabitants/year, clearly exceeds that of invasive aspergillosis and mucormycosis [35]. Indeed, *Candida* spp are the fourth most common agent of hospital-acquired systemic infections in the United States, with crude mortality rates of up to 50% [34]. *C. albicans* can cause two major types of infections in humans: superficial infections, such as oral or vaginal candidosis, and life-threatening systemic infections. Candidemia has an attributable mortality of 15–35% for adults and 10–15% for neonates and early and late mortality (7 days and 30 days after diagnosis) which is 13% and 30%, respectively [36]. Whereas early mortality is associated with factors such as appropriate antifungal therapy and early removal of central venous catheters, late mortality is associated with factors related to the baseline condition of the host [31]. The mortality rate is closely correlated with a delay in the

initiation of appropriate antifungal treatment. Inappropriate treatment includes absence of antifungal treatment, a delay in initiation, but also the use of an inactive agent due to antifungal resistance, which is a growing problem [37-39].

Bridging *Aspergillus* and *Candida*

The total number of eukaryotic species on Earth was recently estimated in 8.7 million, with fungi making up approximately 7% (611.000 species) [40]. Among fungi, nearly 600 species are human pathogens [5, 41]. This relatively small group encompasses fungi that cause relatively mild infections of the skin (e.g., dermatophytes and *Malassezia* species), fungi that cause severe cutaneous infections (e.g., *Sporotrix schenkii*) and fungi that have the potential to cause life-threatening systemic infections, being *Aspergillus* and *Candida*, by far, the most significant pathogens. It is recognized that most invasive candidosis episodes are related to concomitant colonizing *Candida* organisms; although, it remains unclear what is the extent of colonizing fungal microbiota that is influenced by exogenous *Candida* [34]. Only a few studies have investigated the potential concern of an exogenous source and described the occurrence of antifungal resistant *Candida* organisms on fruit and vegetables from plantations displaying cross-resistance to clinical azoles [42, 43]. In contrast, the current situation of resistant clinical infections caused by *A. fumigatus* potentially originating from the environment is now a worldwide concern [28, 44, 45].

Antifungals and susceptibility testing

The development of antifungals is challenging since fungi are eukaryotes like the human hosts they infect. Therefore, there are few distinct targets that can be used for drug development. There are three major classes of antifungal drugs available to treat invasive fungal infections. The first generation of antifungals was implemented in the late 1950s with intravenous formulations of polyenes such as Amphotericin B (AMB). Polyenes target ergosterol - a component that is analogous to cholesterol in mammalian cell membranes. Therefore, these compounds exhibit high toxicity in humans [46]. A key breakthrough occurred two decades later with the advance of azoles as intravenous and oral formulations. The azoles target the biosynthetic pathway of ergosterol by inhibiting an early-phase enzyme called lanosterol 14α-demethylase, leading to the accumulation of sterol intermediates, making cells vulnerable to membrane damage due to its toxicity. The fungistatic nature of azoles and its interaction with the cytochrome P450 enzymes make them less appealing in patients undergoing multidrug therapies. The development of echinocandins tried to overcome azole limitations in 2001. These drugs are semi-synthetic derivatives of a cyclic lipophilic peptide isolated from *Glarea lozoyensis*. Echinocandins are fungicidal drugs that block cell-wall synthesis by inhibiting β-(1, 3)-D-glucan synthase [47].

Several *in vitro* assays have been developed and standardized in order to test the susceptibility of a microorganism, and the result is expressed as the minimal inhibitory concentration (MIC) of a determined antifungal – conveying the concentration of a drug which is required to kill or inhibit the growth of the fungus [48]. Two major protocols are currently used, brought by the two major antifungal susceptibility testing subcommittees: CLSI, Clinical Laboratory Standards Institute [49-51] and EUCAST,

European Committee on Antimicrobial Susceptibility Testing. EUCAST has set forth a species-specific approach for the most prevalent pathogenic yeasts (E.Def 7.2) [52] and moulds (E.Def 9.2) [53] and established clinical breakpoints. The EUCAST broth dilution method is similar to that of CLSI with modifications concerning some of the test parameters such as inoculum preparation and inoculum size. The MIC reading method is visual in CLSI assay and spectrophotometric in EUCAST guidelines. Such breakpoints can be used to determine whether an organism is susceptible (S), intermediate (I) or resistant (R) to a drug, which in turn may be translated to clinical susceptibility. No official breakpoints exist for agricultural fungicides. Consequently, to achieve a clear perspective of what is more or less susceptible to a determined fungicide, a large number of *in vitro* susceptibility tests is necessary.

Antifungal resistance

Some fungal species are not disturbed by specific antifungal agents, independently of the concentration of the drug. The absence of drug activity in a species that was not pre-exposed to that agent is known as intrinsic resistance. Taking as example the response of *Candida* and *Aspergillus* spp. to fluconazole, it is known that wild type *C. albicans* is susceptible to fluconazole, whereas *A. fumigatus* and *C. krusei* are intrinsically resistant to this drug [54].

Mechanisms of antifungal resistance fall into distinct categories, including decrease of effective drug concentration (A), drug target alterations (B), and metabolic bypasses (C) – representative illustration in **Figure 2**.

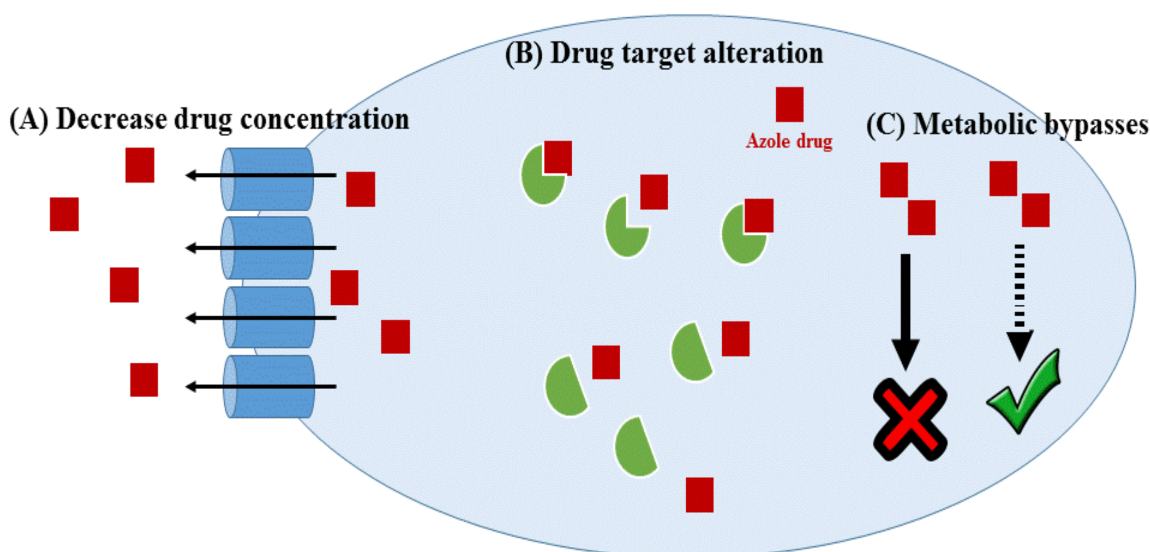

Figure 2. Schematic representation of the principal antifungal resistance mechanisms.

Cells can resist to the drug activity simply by pumping the drug out of them (**Figure 2 (A)**). These resistance mechanisms can be mediated by several efflux transport systems, including adenosine triphosphate-binding cassette (ABC) transporters and transporters of the major facilitator superfamily (MFS). Genome sequencing has allowed the identification of several different types of transporters: *C. albicans* is predicted to contain 28 ABC proteins and 96 potential MFS transporters, whereas *C. glabrata* displays at least 18 ABC transporters and 33 MFS transporters [55]. Larger numbers of ABC and MFS proteins are found in *A. fumigatus* (45 and 275, respectively) [56]. Despite the differences among the ABC transporters sub-families, all of them contain membrane spanning domains and use adenosine triphosphate (ATP) hydrolysis

for drug transport. MFS transporters are transmembrane proteins, which use the electrochemical proton-motive force to mediate drug efflux. MFS are co-players in multidrug resistance (MDR) (MFS–MDR transporters) as proton antiporters and are classified into two groups: H+ antiporter-1 DHA1 family and H+ antiporter-2 DHA2 family [57].

Only ABC transporters of the pleiotropic drug resistance (PDR) class are relevant for antifungal drug resistance. In *C. albicans*, the PDR class comprises the major transporters involved in azole resistance, including *CDR1* and *CDR2* - the upregulation of both mediates azole resistance by enhanced drug efflux and reduces azole accumulation in some *C. albicans* clinical strains. For *C. glabrata* the ABC transporters known to be involved in azole resistance by their upregulation are *CgCDR1*, *CgCDR2*, *CgSNQ2* genes [55]. In *A. fumigatus*, it is still not clear the association between azole resistance and transporter upregulation. Slaven and colleagues report the upregulation of the ABC transporter *atrF* in an azole-resistant clinical isolate but this could not be definitely attributed as the cause of resistance [58]. The only known transporter gene with a direct role in azole resistance in *A. fumigatus* is *abcA* gene, renamed *cdr1* [59]. Also, *AfuMDR3* - a MFS transporter was found to be upregulated also in a collection of *A. fumigatus* itraconazole (ITC)-resistant laboratory-derived mutants [60].

On the other hand, MFS involved in the development of azole resistance in clinical isolates are restricted to *MDR1* from *C. albicans* and *C. dubliniensis*. *MDR1* is upregulated in specific strains, which results in enhanced azole efflux [55]. Upregulation of ABC and MFS transporters is mediated by specific regulators in resistant fungal pathogens. In *C. albicans*, *CDR1* and *CDR2* are known to be regulated by a zinc cluster finger transcriptional regulator called *TAC1* and *MDR1* by another regulator called *MRR1* [61, 62]. Gain of function mutations (GOF) in these regulators

have been described, which confer a hyperactivation state that does not require additional stimulation, thus explaining the inherent high expression levels of the transporters in drug-resistant isolates [62]. Other transcriptional regulators of drug transporters relevant to azole resistance, such as *PDR1*, have been described in *C. glabrata* [63]. Another resistance mechanism can be the increasing of the number of drug targets, making the effective drug concentration insufficient to saturate all target molecules. For example, *ERG11* upregulation has been associated with azole resistance in *C. albicans*. This transcriptional regulation is mediated by a zinc cluster finger transcription factor called *UPC2*. As in the case with other drug resistance transcriptional regulators, GOF mutations in *UPC2* have been described and result in upregulation of various genes, among which is *ERG11* [62]. Upregulation of *Cyp51A* is also recognized in azole-resistant *A. fumigatus* isolates; however, the upregulation is mediated by duplication of 34- and 42-bp elements (*trans*-regulation) in the *Cyp51A* promoter. This duplication is associated with specific Cyp51A mutations (L98H, Y121F/T289A) [64].

Drug target alterations have been reported for azoles and echinocandins (**Figure 2 (B)**). The targets of these two drugs are a 14α-lanosterol demethylase and a β-1,3 glucan synthase, respectively. Lanosterol demethylase is encoded by *ERG11* in *C. albicans* and by *Cyp51A* and *Cyp51B* in *A. fumigatus*. Mutations in *ERG11*, resulting in non-synonymous amino acid substitutions that are present in azole-resistant *C. albicans* isolates, are numerous and were shown to decrease the affinity of the target to azoles [55]. Mutations in lanosterol demethylase genes from azole-resistant *A. fumigatus* isolates have been only reported in *Cyp51A* until now. Single *Cyp51A* mutations are sufficient to confer high level resistance to azoles in this species. As in the case of *ERG11*, *Cyp51A* mutations have different impact on MICs that depend on the azole

structure [64, 65]. Decreased affinity to the target is also recognized in the case of echinocandins. β-1,3 glucan synthases are encoded by *FKS* genes in different fungal species. *Candida parapsilosis* family, including *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* exhibit intrinsic low susceptibility to echinocandins. *FKS1* genes in these species exhibit a natural polymorphism, enabling decreased affinity of the β-1,3 glucan synthase to echinocandins. However, this natural *FKS1* polymorphism of these species has less impact than those resulting from mutations, since these *Candida* species still respond to echinocandin therapy [66].

Metabolic bypasses occur when some metabolic pathways are impaired by loss or significant decrease of specific functions (**Figure 2 (C)**). For example, resistance to azoles can be mediated by loss-of-function mutations in the gene *ERG3* that encodes a sterol $\Delta^{5,6}$ desaturase. If active, the gene product converts 14α-methylated sterols that arise from azole exposure into a toxic 3,6-diol derivative. Fungi unable to produce this metabolite acquire azole resistance. Several studies have reported *ERG3* loss-of-function mutations to account for azole resistance**.** Due to a deficiency in ergosterol biosynthesis, these isolates can be, however, less competitive than wild type isolates. As a result of loss-of-function of *ERG3* in specific mutants, ergosterol is absent from cell membranes - this way, the mutants escape the toxic effect of AMB [61, 62]. Until now it is quite clear that while the variety of resistance mechanisms may be equally complex as for *Candida* species [31], about 90% of azole resistance cases in *A. fumigatus* have so far, been linked to genetic changes in *CYP51A* gene [65, 67, 68].

Dilemmas in clinical management

The susceptibility profile of the fungal pathogen is only one of the factors that contributes to the clinical outcome. Several factors, as site of infection, fungal burden, strain acquisition with virulence attributes and underlying disease - have been identified as key determinants [69]. However, it is expected that patients infected with a fungal isolate for which an antifungal MIC is higher than average, or within the MIC range categorized as resistant for that organism, will fail antifungal treatment [5]. This has partly been attributed to the growing population of pathogenic fungi with emerging resistance to antifungal therapy, which potentially compromises the management of infected patients [70, 71]. In most cases, a reduced *in vitro* fungal susceptibility to azoles and/or echinocandins can be a sign of the acquisition of a particular resistance mechanism. Strategies using antifungal combinations or adjunctive agents that maximize the efficacy of existing antifungals may somewhat limit treatment failures [72, 73] . New therapeutic approaches, including antifungal agents with novel mechanisms of action are under investigation [74-78].

Thus, clinical management of antifungal-resistant *Candida* and *Aspergillus* diseases remains difficult. Multi-azole resistance among *A. fumigatus* is a current medical problem and, indeed, the high number of international reports addressing azole resistance among *A. fumigatus* reflects a particular focus on this emerging threat [28, 44, 45, 64]. This is also verified with the increasing number of co-resistant *C. glabrata* to both azoles and echinocandins [79].

Another very relevant dilemma responsible for a very high mortality and morbidity in IFI can be attributed to the delay in diagnosis and institution of appropriate therapy [73]**.** Diagnosis of a fungal pathogen deep-seated in the tissues or

organs is difficult and is often driven by clinical suspicion. The detection of IFI is only apparent following the dissemination of cells from the invaded tissues, organs or biofilms. Current diagnostic practices involve histopathology analyses and cultures. Histology of biopsy samples lacks specificity, sensitivity and taxonomic information [80]. Fluid samples such as blood, alveolar lavage, and sputum or tissue counterparts are usually first cultured on non-selective medium to maximize microbial counts for 24 hours and later using genus-specific agar plates [36, 81]. Novel molecular methods on antigen detection do not add much in terms of diagnostic efficacy [80, 82]. However, in patients suffering from life-threatening fungal infections, institution of appropriate (type and dose) antifungal therapy becomes of critical importance for the outcome. In such circumstances, the clinician has to choose between time-consuming confirmatory tests that may delay therapy and broad spectrum antifungals institution. Considerable challenges also arise when a clinician deals with non-sterile samples, which pose difficulties in differentiating colonization from contamination. Such scenarios demand a deep knowledge in recognizing disease signs and symptoms [47, 54, 69, 73]. Even owing to such uncertainties, clinical samples are often not subjected to *in vitro* antifungal susceptibility testing as a part of the diagnosis. Despite that techniques being time consuming and "low-throughput", it is known that early diagnosis and concomitant targeted therapy has proven to significantly improve patient outcome [54]**.**

The last and most recent clinical dilemma arises from advances in phylogenetic analyses and molecular methods that revealed the important diversity of *Aspergillus* species within the section Fumigati [13, 16]. Some clinical isolates may actually be misidentified as *A. fumigatus* by conventional diagnostic methods, which may result in inappropriate antifungal therapy because of the decreased susceptibility of those cryptic species to many antifungal agents [17]. Azole resistance among *A. fumigatus* isolates is

an emerging problem, which has been highlighted by multiple recent reports throughout the world, but its prevalence is still low [83-85]. Therefore, in the case of an azole-resistant IA, clinicians should suspect that a sibling species of *A. fumigatus* may be the causal agent. Slow or poor sporulation is usually the first hint that should alert the microbiologist. Sequencing of the ITS region, followed by targeted sequencing of the beta-tubulin or calmodulin genes seems to date the most appropriate method for reliable species identification. However, these procedures are not available in most institutions. Matrix assisted laser desorption ionization-time to flight mass spectrometry (MALDITOF MS) may be a very convenient alternative approach for the rapid detection of *A. lentulus* and other *A. fumigatus*-related species, but further investigations for standardized sample treatment procedures of filamentous fungi and spectra characterization are required. Antifungal susceptibility testing may also be useful because of the unpredictable susceptibility profile of these species. However, results should be interpreted with caution because of the lack of clinical breakpoints and the absence of data correlating MICs with clinical outcomes [13, 16, 17].

The use of fungicides in anthropogenic activities

Fungal diseases have a significant economic impact on plant yield and quality. Thus, managing such diseases is essential for most crops. A fungicide is a specific type of pesticide that controls fungal disease by specifically inhibiting or killing fungi that cause disease. Fungicides are used also to prevent or to ease the problem of postharvest spoilage of plants and fruits [86, 87]. In agriculture, various compounds have been described for fungicide activity against a broad range of fungi. Among them, azoles are

widely applied, besides dithiocarbamates, strobilurins and benzimidazoles [88-90]. Azoles are used in wood and textile preservation and human and animal health products, but their major use is in agriculture, horticulture and prevention of post-harvest losses. The imidazole imazalil was introduced in the late 1970s and is still used today in wax formulations applied as post-harvest treatments to citrus and pome fruits, bananas and seed potatoes. Another imidazole, PCZ), was introduced a few years later, primarily for control of cereal eyespot (*Tapesia* sp.) and is still widely used today. Several triazoles were introduced in the 1980s, especially for control of cereal and fruit diseases, and although they are still in use in some markets, they have largely been replaced by more effective products, including propiconazole (1990), tebuconazole (1992), difenconazole (1994) epoxiconazole (1994) and bromuconazole (2000) [91]. All azole fungicides are of synthetic origin and are characterized by the presence of an aromatic five-membered heterocycle. These include triazoles (two carbon atoms and three nitrogen atoms), imidazoles (three carbon atoms and two nitrogen atoms), and triazoles (three carbon atoms, one nitrogen atom and one sulfur atom). The characteristics of the azole rings, which are distinguished by the number of nitrogen and sulfur atoms, change the physical and chemical properties, toxicity, and therapeutic efficacy of these compounds [90]. Therefore, the addition of different substitutes to the pristine 1,2,4-triazole molecule influences its fungicide or fungistatic effect. Around a third of all fungicides used for the protection of crop yields include triazoles, among which more than 99% are demethylation inhibitors (DMIs) [92]. Triazoles affect the biosynthesis of ergosterol, a fundamental component of the fungal cell plasma membrane. The main target of antifungal azole drugs is lanosterol 14-demethylase (Erg11 protein), a cytochrome P450 enzyme that is involved in the conversion of lanosterol to 4,4-dimethylcholesta-8(9),14,24-trien-3-ol. The azole agents link to this

enzyme using the aromatic five-membered heterocycle and thereby inhibit the cytochrome P450 catalytic activity. The absence of ergosterol and the increase of intermediate compounds alter fungal membrane integrity as well as cell morphology, which inhibits fungal growth [93].

Thousands of tons of azoles are sold and applied annually in crop fields [87, 91, 94]. According to the instructions of manufacturers, the quantities applied should be about 10 mg of azoles on 1 m^2 of plant surface. Usually, multiple applications per year are needed if, as might be the case during rainy seasons [91]. Azoles exhibit several advantages when compared with other fungicides. They are not expensive and show a broad spectrum of antifungal activity. They are effective against mildews and rusts of grains, fruits, vegetables, and ornamentals; powdery mildew in cereals, berry fruits, vines, and tomatoes; leaf spots and flower blights in flowers, shrubs, and trees [95, 96]. They are used not only in preventing but also for the treatment of plant infection. However, some azoles could remain active during several months in certain ecological niches, as in soil and water, over months with only slight changes in their chemical structures, like the loss of some side chains [91]. Azole residues have been detected in fruits and plants for human consumption, for example, in strawberry [97], grapes [98], and peppermint [99], with values up to 0.5-0.8 mg/kg. The most considerable amount of azole residues has been reported in apples, reaching up to 2.16 mg/kg [100].

The Fungicide Resistance Action Committee (FRAC) is a technical group maintained by the industry, which was developed at an industry seminar in Brussels in 1981. Nowadays, FRAC has several delegations not only in Europe but also in USA, Latin America (Brazil) and Asia (Japan). Its aim is to provide guidelines for the management of fungicide resistance, trying to estimate the baseline resistance level of a determined fungicide prior to its commercial use. Also, during fungicide

commercialization, this committee provides periodically information concerning the risk of resistance development [101, 102]. The acquisition of resistance to azoles by plant pathogenic fungi is complex and it is generated slowly and in small steps. Although it has been considered that the risk of selecting azoles-resistant strains is low, there have been reports claiming that some plant pathogenic fungi have indeed acquired azole resistance [90]. It is anticipated that the excessive use of azoles in agriculture would not only influence the plant pathogenic species but also would certainly attack susceptible species of the saprophytic flora [103].

One possible consequence of such a disequilibrium in the ecology of the fungal flora is that some naturally existing human fungal pathogens may survive and multiply. Those strains which have acquired azole resistance will particularly benefit from the selective pressure. This could increase the risk of the contact of humans with such resistant fungal organisms.

The impact of agricultural antifungals upon clinical antifungals

Triazole fungicides, in particular, are used globally for the control of fungal diseases both in humans and plants. Also, the resistance to triazoles among fungi is an emergent issue both in agriculture and medicine. The non-rational use of fungicides with site-specific mechanisms of action, such as the triazoles, may increase the risk of antifungal resistance [92]. In the medical field, the emergence of resistant fungi has been related to the intensive and recurrent therapeutic use of a limited number of triazoles for the treatment and prophylaxis of many mycoses. Similarities in the mode of action of triazole fungicides used in these two fields may lead to cross-resistance, thus

expanding the spectrum of resistance to multiple fungicides and contributing to the perpetuation of resistant strains in the environment.

The number of antifungals available in the medical field for the treatment of systemic infections is relatively limited compared to those used for controlling diseases in plants, which is mainly due to problems related to erratic efficacy, drug toxicity, and intrinsic resistance. The first generation of triazoles for human therapy included fluconazole (FLC) and ITC. The second generation is represented by voriconazole (VRC) and posaconazole (PSC), which proved to be less toxic, safer, and with a broader spectrum of activity, including activity against fungi that were resistant to the previous generation [76, 104]. Presently, isavuconazole, ravuconazole, and albaconazole are being investigated in phase III clinical trials as extended-spectrum triazoles with fungicidal activity against a wide number of clinically important fungi [105].

In the medical field, the first report of resistance of *A. fumigatus* isolates was almost three decades ago. In 1989, the first ITC resistant isolate was recovered from two American patients with invasive aspergillosis that were under ITC therapy [106]. Afterwards, two studies in Europe also found ITC resistant isolates after prolonged azole therapy: in 2002 a Dutch study assessing the *in vitro* susceptibility of isolates recovered from 1945 to 1998 found three ITC resistant isolates recovered in 1997 from a lung transplant recipient [107]; a French study found 4 ITC resistant isolates in 1999 [108]. Later, in 2007, a Dutch study found 4 azole resistant isolates from patients who had never received azole therapy [109].

It is known that, sometimes, the emergence of resistance has some kind of repercussion upon other capabilities of the cell corresponding to the sacrifice of an adaptive feature – impairing the so called fitness of the fungal cell [110]. Nevertheless, concerning *A. fumigatus* the ability to survive in very different environmental

conditions, the widespread asexual sporulation, the hydrophobicity of spores and the easy airborne dispersibility, were not affected and altogether probably empower the global spread of resistance from its centers of origin [54]. Therefore, we have witnessed several reports claiming that exposure of environmental fungi to triazole fungicides may cause shifts from susceptible to resistant populations, which especially in the absence of adaptive costs, may facilitate its spread into diverse environments and geographical areas [111]. Subsequently, the emergence of fungi in the medical field otherwise harmless to humans may become a reality – for instance, the fungus *Colletotrichum graminicola* that causes anthracnose of corn plants is now an emerging pathogen in humans - such as the zygomycetes and other hyaline filamentous fungi [112]. In fact, the initial hypothesis suggested by studies conducted in the Netherlands [67, 113] that the mechanism that may be leading to resistance is the selective pressure of fungicides used in agriculture is now corroborated by studies conducted a little all over the world [114]. The development of cross-resistance to triazoles and the low number of triazoles recommended for human therapy in comparison to the higher number of triazoles used in agriculture may impair triazole efficacy for human therapy [115]. Cross-resistance to triazoles may spread either way: from resistant plant pathogenic yeast and moulds to clinical antifungals and/ or from clinical isolates of yeasts and filamentous fungi resistant to fungicides [42, 116-119].

The increasing reports that the use of azoles in the environment is driving the development and spread of resistance to medical antifungals is so strong that, in 2013, the European Centre for Disease Control (ECDC) published a risk assessment report about this topic and also summarized several evidences for the environmental origin of azole resistance [92]:

1) Azole resistant *A. fumigatus* isolates have been recovered from naïve patients (i.e. with no history of previous azole exposure) [120];

2) Azole resistant *A. fumigatus* isolates with a dominant resistance mechanism and only two variants TR34/L98H or TR46/Y121F/T289A [28];

3) From this dominant mechanisms, both variants TR34/L98H and TR46/Y121F/T289A are stable and are the most commonly found mechanism among clinical and environmental isolates. In other studies involving a high proportion of azole treated patients with chronic forms of aspergillosis, as in the United Kingdom (UK), a more heterogeneous population of *CYP51A* variants exists. Therefore, it is assumed that the *in vivo* selection of resistance may result in a more diverse panel of resistance mutations in *CYP51A* [121];

4) Both TR34/L98H and TR46/Y121F/T289A resistance mechanisms associate to two independent genetic events (mutations and a tandem repeat), which have not been found previously in any case of *in vivo* resistance development but are present in azole resistant plant pathogenic filamentous fungi [67, 122];

5) Both environmental and clinical isolates harboring TR34/L98H cluster genetically together and are distinct from any other susceptible wild-type isolate [123, 124];

6) The TR34/L98H isolates are cross-resistant to several triazole fungicides which were introduced in the agriculture just few years before the first detection of a TR34/L98H isolate in 1998 (tebuconazole, propiconazole, difenoconazole, epoxiconazole and bromucazole) [125, 126];

7) Triazole fungicides display similar molecular structure and mode of action as clinical azoles. Moreover, they have been shown to induce tandem repeats in *CYP51A in vitro*, conferring reduced susceptibility to clinical azoles [125].

The use of azoles in the environment will be difficult to restrict, unless scientists raise better public and political awareness about this problem. Vulnerable patients can become exposed and, if these hosts develop *Aspergillus* disease, the medical azoles are no longer effective due to the structural similarity of the molecules. Still, it is unclear how and where resistance develops in the environment and which form of application of fungicides are inducing the development of resistance most successfully [127].

It has not been routine practice in microbiological laboratories to carry out *in vitro* susceptibility testing of filamentous fungi. However, this has started to change in the last few years and presently many centers now test *A. fumigatus* for resistance to azoles. As a result, it has become evident that azole resistance has a potentially global distribution, and is a worldwide problem [115]. Isolates harboring one of these two dominant mutations associated with the environmental route are being found in Europe, already including a considerable number of countries like Belgium, France, Spain, Denmark, Italy, The Netherlands, Norway, Germany, Ireland, UK, Poland, Romania and Austria [114, 115]. Reports about these resistance mechanisms are also found outside Europe: in Turkey, Iran, Kuwait, Japan, China, Taiwan, Pakistan, India, Tanzania and Australia, mainly associated with TR34/L98H but increasingly also with TR46/Y121F/T289A [114]. Also, recently, in USA and Colombia, azole resistance owing to environmental mutations was reported recently [128-130]. In Portugal, in 2014 a study from Lago *et al*, tried to the answer the question: "Does fungicide application in vineyards induce resistance to medical azoles in Aspergillus species?"; however, they did not have a conclusive response since there was not any decrease in the susceptibility of the *Aspergillus* isolates to the tested azoles tested during the period when vineyards were treated with fungicides. They have found a few environmental isolates of *Aspergillus* spp. resistant to azoles but they could not prove that the use of fungicides

were responsible for it [117]. Recently, Sabino *et al* provided a study upon the clinical antifungal susceptibility of 175 *Aspergillus* isolates from both clinical and environmental sources, describing that species identity and site of isolation influences the clinical antifungal susceptibility profiles. Differences were found in clinical isolates showing higher susceptibility to AMB and POS while isolates recovered from the hospital environment showed to be significantly less susceptible to the same clinical antifungals [131]. A similar study previously published by Araújo *et al.* some years ago, involving 446 isolates belonging to the *Aspergillus* genus; in contrast, they found that resistant isolates to clinical antifungals from both environment and clinical settings were very uncommon. Only a few non-*fumigatus* clinical isolates revealed significantly higher MIC values to AMB, ITC and CSF. In fact, neither clinical nor environmental isolates of *A. fumigatus* showed to be resistant to any of the tested clinical antifungals [132]. To the best of our knowledge, there is no study in Portugal assessing the genetic mechanisms in *Aspergillus fumigatus* responsible for azole resistance from both clinical and environmental sources nor a study revealing the prevalence of infections caused by resistant filamentous fungi.

Outline of this thesis

The main aim of this thesis was to investigate whether agricultural antifungals may have an impact upon these ubiquitous organisms (*Aspergillus* and *Candida*) namely whether resistance may develop and jeopardize the susceptibility to clinical antifungals. In order to know the clinical yeasts isolate distribution and susceptibility to clinical antifungals a national surveillance study was conducted; the results are described in **chapter 2**. Clinical *Candida* isolates obtained from chapter 2 were screened and a few were selected to assess the *in vitro* induction of resistance to an agricultural azole and the hypothetical development of cross-resistance to clinical azoles; this study is detailed in **chapter 3**. A similar approach was followed with isolates of *Aspergillus fumigatus*; the results are shown in **chapter 4**. In **chapter 5** we reviewed the origin of azole resistance, from agriculture to clinical settings.

2 Species distribution and *in vitro* antifungal susceptibility profiles of yeast isolates from invasive infections during a Portuguese multicenter survey.

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ABSTRACT

This is the first Portuguese multicenter observational and descriptive study that provides insights on species distribution and susceptibility profiles of yeast isolates from fungemia episodes.

Ten district hospitals across Portugal contributed by collecting yeast isolates from blood cultures and answering questionnaires concerning patients' data during a twenty-four month-period. Molecular identification of cryptic species of *Candida parapsilosis* and *C. glabrata* complex was performed. The susceptibility profile of each isolate, considering eight of the most used antifungals, was determined. Both CLSI and EUCAST protocols were applied.

The incidence of 240 episodes of fungemia was 0.88/1000 admissions. Fifteen different species were found, with *Candida albicans* (40%) as the most prevalent followed by *C. parapsilosis* (23%) and *C. glabrata* (13%). Most isolates were recovered from patients admitted at surgical wards or Intensive Care Units with 57% being males and 32% being of the age between 41 to 60 years. For both CLSI and EUCAST, the overall susceptibility rates ranged from 74% - 97% for echinocandins and 84% - 98% for azoles. Important resistance rate discrepancies between protocols were observed in *C. albicans* and *C. glabrata* for echinocandins and in *C. parapsilosis* and *C. tropicalis* for azoles. Death associated with fungemia occurred in 25% of the cases with more than half of *C. glabrata* infections being fatal.

The great number of *Candida* non-*albicans* is noteworthy despite a relatively low antifungal resistance rate. Studies like this are essential to improve empirical treatment guidelines.

INTRODUCTION

The increased incidence of fungemia represents a clinical problem and its impact has significantly risen during the last twenty years [133]. Fungemia is an important cause of morbidity and mortality, being related to longer hospital stays and high economic costs. *Candida albicans* still remains the leading cause of fungemia worldwide [134]; *Candida parapsilosis, Candida glabrata* and *Candida tropicalis* occupy places two to four, varying according to region. Due to the medical relevance of fungemia and its strong association with unfavorable outcomes, epidemiological surveillance studies are urgently needed in order to evaluate species' geographic distribution and potential changes in susceptibility profiles. In addition, there is a limited number of therapeutic options, with the main class of drugs (azoles) being controversially used for prophylaxis, possibly leading to decreased susceptibility and the selection of non-*albicans* species [10, 38, 135]. Regarding the susceptibility testing there are currently two independent organizations suggesting standard protocols: the CLSI and EUCAST [49, 50, 136]. Their protocols differ not only on the laboratory execution and interpretation but also with respect to the breakpoints proposed for each species / drug combination, leading to discrepancies on the categorization of the isolates as susceptible or resistant [137]. The aim of this study was to provide an overview of blood-borne yeast infections in Portugal and to evaluate the susceptibility profiles provided by both protocols, CLSI and EUCAST.

MATERIALS AND METHODS

Study design Ten hospitals from northern (four), central (two) and southern (four) regions of Portugal accepted to participate in this study, providing isolates collected from patients with fungemia from January 1st 2010 and December 31st 2011. All hospitals enrolled in this study were able to admit adults, children and neonates. From those, two hospitals had more than 1000 beds, 3 had between 600 and 1000 beds and 5 had less than 600 beds. All were asked to collect and send the strains to the Microbiology Department of the Faculty of Medicine of the University of Porto, where the study was conducted. In addition, a questionnaire regarding patient's clinical and demographic data was also sent.

Definitions The start of an episode of fungemia was defined at the first isolation from a blood culture of a yeast strain from a patient with related signs and symptoms. Nosocomial fungemia was defined whenever the yeast isolate was obtained more than 48h after hospital admission. Patients were grouped into five age categories: less than 19 years, 19-40 years, 41-60 years, 61-70 years and more than 70 years old. For each individual patient, the outcome of the fungemia episode was evaluated at 30 days after the first yeast isolation. Death associated with fungemia was defined as death within 30 days after recovery of the first yeast isolate, without any other concomitant cause of death, like intra-cerebral or gastrointestinal bleeding or pulmonary embolism.

Identification and in vitro antifungal susceptibility testing of yeast isolates Yeasts were identified using Vitek 2 YST cards from bioMérieux (Marcy l'Etoile, France). The characterization of *Candida glabrata* sensu stricto*, Candida bracarensis* and *Candida*

nivariensis was confirmed as previously described by Romeo *et al* [138]. *Candida parapsilosis* sensu stricto, *Candida orthopsilosis* and *Candida metapsilosis* isolates were identified as previously described by Tavanti *et al* [139]. Furthermore, the identities of these species were confirmed by deoxyribonucleotide acid (DNA) amplification and further sequencing of *ITS1* and *ITS4* regions of ribosomal ribonucleic acid (RNA) operons. MIC was considered as the lowest concentration showing a 50% reduction on growth compared with control after 24h incubation for azoles and echinocandins. For AMB and 5FC the MIC was the lowest concentration showing a complete absence of growth compared to the control and 50% reduction of growth compared to the control, respectively – both after 48h incubation. MICs were determined for Fluconazole (FLC), Voriconazole (VOR), Posaconazole (POS), Anidulafungin (AND), Caspofungin (CAS), Micafungin (MCF), Amphotericin B (AMB) and 5-Flucytosine (5FC) by broth microdilution, regarding two different protocols: CLSI M27-A3 (S4) and EUCAST EDef 7.2 document [49, 50, 136]. Also, being aware of the inter-laboratory variation of CAS MIC values [57] we tried to minimize it by using a single drug powder lot and stock solution solvent. In order to compare both protocols we assigned Categorical Agreement (CA), expressing the level of discrepancy on the number of resistant isolates when categorized by one protocol or another. Reference powders for each antifungal agent were obtained from their respective manufacturers. FLC, CAS and MCF were dissolved in water and the other drugs in DMSO. Antifungal resistance rates were calculated regarding both protocols although for CLSI, several times, the Epidemiological Cutoff Values (ECV) were used; for EUCAST many breakpoints are still undefined. For AMB, yeasts inhibited by ≤1 mg/L were considered to be susceptible according to suggestions by Pfaller *et al* [140].

The quality control (QC) strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included in all assays [49, 50, 136].

Statistical analysis A descriptive revision of the collected data was performed using Statistical Package for Social Sciences (SPSS) v21.0 (SPSS Software, Chicago, USA). Chi-squared tests were used to compare proportions and analyze differences in the species distribution and in antifungal susceptibility profiles. A P value below 0.05 was considered significant.

RESULTS

Patient's data A total of 240 fungemia episodes were reported in the current survey (corresponding to 240 different patients) and included in the study. The mean incidence of fungemia was 0.88 per 1000 admissions, ranging from 0.15 to 2.4 depending on the hospital. The mean incidence of nosocomial fungemia was 0.74 per 1000 admissions, ranging from 0.14 to 2.1. This corresponds to approximately 86% of all episodes of fungemia. In Portugal, Pediatrics admits neonates and children aged 18 years or under. However, often, these patients – especially adolescence - are admitted by other departments due to numerous and unspecified issues (**Table 1**). Fifty-seven per cent of the patients with fungemia were males and the most affected group was the one aged 41-60 years (32%), closely followed by the group of patients over 70, together accounting for 62% of cases (**Table 1**). Most patients enrolled in this study were admitted at Intensive Care Unit (ICU) (39%) and surgical wards (30%). The mortality rate due to fungemia was 25%. The deadliest species was *C. glabrata*, with more than 50% of patients dying within 30 days of fungemia detection (**Table 1**).

Species distribution and identification Table 1 shows the most important characteristics regarding the top five recovered species, the total of the others species and an overall perspective. Fifteen different yeast species were identified and nearly 60% of fungemias were caused by non-*albicans* species. The five most prevalent were *C. albicans* (40.4%), *C. parapsilosis* (22.9%), *C. glabrata* (13.3%), *C. tropicalis* (6.3%) and *C. krusei* (5%). *Cryptococcus neoformans* was responsible for eight (3.3%) episodes of fungemia followed by *C. lusitaniae* (2.5%), *Candida guilliermondii* (1.7%), *Candida dubliniensis* (1.3%) and *Candida famata* (1.3%). Single cases due to *Candida*

sake, *Candida inconspicua*, *Candida haemulonii*, *Candida kefyr* and *Trichosporon mucoides* were also found, as a whole accounting for 1.7% of all cases. Regarding the molecular identification of the cryptic species, from 55 isolates of *C. parapsilosis* sensu lato, 49 corresponded to *C. parapsilosis* sensu stricto, 4 to *C. orthopsilosis* (3 isolated from Medicine Department and 1 from ICU) and 2 to *C. metapsilosis* (both from the Medicine Department). The 32 isolates primarily identified as belonging to the *C. glabrata* group all corresponded to *C. glabrata* sensu stricto. Two cases of fungemia in neonates were observed, one infection caused by *C. parapsilosis* and another one by *C. glabrata*. We report three cases of fungemia in children under two years old, all caused by *C. albicans*. Very distinct geographic areas were covered by these hospitals, although no significant differences in species distribution were found. *C. glabrata* appears in more than half of the cases in ICU and Medicine Departments while *C. tropicalis* was mostly found in Surgery wards.

Table 1. Distribution and characteristics of the isolated yeasts.

	No. of isolates (%)						
	C. albicans	*C. parapsilosis*	*C. glabrata*	*C. tropicalis*	*C. krusei*	Other species	Overall
Gender							
Male	59 (61)	26 (47)	21 (66)	11 (73)	4 (33)	16 (55)	137 (57)
Female	38 (39)	29 (53)	11 (34)	4 (27)	8 (67)	13 (45)	103 (43)
Total	97 (40)	55 (23)	32 (13)	15 (6)	12 (5)	29 (12)	240 (100)
Age group (years)							
<15	4 (4)	5 (9)	-	-	-	2 (7)	11 (3)
15-19	4 (4)	8 (15)	1 (3)	-	-	10 (35)	23 (10)
20-40	10 (10)	4(7)	3(9)	2(13)	2(17)	6 (21)	27 (11)
41-60	35 (35)	13(24)	9(29)	7(47)	6(50)	7 (24)	77 (32)
61-70	15 (16)	7(13)	3(9)	3(20)	1(8)	2 (7)	31 (13)
>70	29 (30)	18(32)	16(50)	3(20)	3(25)	2 (10)	71 (30)
Hospital Department							
ICU	42(43)	21(38)	12 (38)	4(27)	5(42)	10 (35)	94(39)
Surgery	22 (23)	18(33)	8(25)	8(53)	3(25)	11 (38)	70(30)
Medicine	28 (29)	13(24)	11(34)	3(20)	4(33)	6 (21)	65(27)
Pediatrics	5 (5)	3 (6)	1(3)	-	-	2 (7)	11 (5)
Outcome							
Death 30 days	23 (24)	12 (22)	17 (53)	3 (20)	-	4 (14)	59 (25)

Species susceptibility profiles Table 2 details all the data for susceptibility testing using CLSI and EUCAST protocols. Readings were made after 24h of incubation for azoles and echinocandins. For AMB and 5FC 48h readings were registered. Similar MIC results were obtained despite differences arising from both protocols (one dilution discrepancy above or below for each protocol was not been taken into account). Generally, CLSI breakpoints categorized less isolates as resistant (R) than EUCAST. Among the group of azoles, the highest percentage of resistance (9%) coupled *C. glabrata* to FLC, according to both protocols. Concerning the echinocandins, several differences were observed with the application of specific breakpoints for each protocol. Resistance to AMB and 5FC was rare. Regarding simultaneous resistance to antifungals of different classes, applying both protocols and excluding strains with intrinsic resistances such as *C. krusei* for FLC and *C. neoformans* for the echinocandins, we detected one isolate of *T. mucoides* (resistant to FLC, AND, CAS and MCF), one of *C. krusei* (resistant to AMB and CAS) and one of *C. parapsilosis* (resistant to FLC and CAS) (data not shown). Resistance to drugs within the same class was more frequent, with 8 strains of *C. albicans* and 1 of *C. tropicalis* showing resistance to the three azoles and 6 strains of *C. parapsilosis* being resistant to the three echinocandins. With the exception of three isolates (one each of *C. glabrata*, *C. krusei* and *C. kefyr*), which corresponded to a high MIC value (2 mg/L), all the other strains were susceptible to AMB with low MIC values, ranging from 0.03 to 0.125 mg/L. The 4 *C. orthopsilosis* and the 2 *C. metapsilosis* strains were found to be susceptible to all antifungals tested. Regarding the CA, half of the possible comparisons between CLSI and EUCAST had a CA of 100% while the other half was ranging from 42% - the lowest CA obtained confronting both protocols – in the case of *C. krusei* isolates to 5FC - to 97% in the case of *C. albicans* to VOR (see **table 2**).

Table 2. Distribution of minimum inhibitory concentration (MIC) values comparing the Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocols.

Species (no.isolates)	Drug	R breakpoint or ECV		No. of isolates with MIC (mg/L)														No (%) of R		CA (%)
		CLSI	EUCAST	0,016	0,03	0,06	0,125	0,25	0,5	1	2	4	8	16	32	64	128	CLSI	EUCAST	
C. albicans (97)	AND	>0.5	>0.03	88	2	3				1	2		1	-	-	-	-	4 (4)	8 (8)	96
	CAS	>0.5	a		75	1	1	5	10	3			2	-	-	-	-	5 (5)	-	-
	MCF	>0.5	>0.016	82	1	3	2	3	3	1	1		1	-	-	-	-	3 (3)	15 (15)	88
	FLC	>4	>4	-	-	-	27	53	6	7	1	1	1			1		2 (2)	2 (2)	100
	VOR	>0.5	>0.125	-	75	7	11	1	2					1	-	-	-	1(1)	4 (4)	97
	POS	>0.06*	>0.06	-	81	5	5	5						1	-	-	-	11(11)	11 (11)	100
	AMB	>1	>1	11	21	54	1	2	4	4				-	-	-	-	0	0	100
	5FC	>0.5*	> 0.5	-	-	-	61	18	10	5	2					1	-	8 (8)	-	-
C. parapsilosis (55)	AND	>4	>4	17			1	1	4	1	6	25			-	-	-	0	0	100
	CAS	>4	a	20	1	2	1	1	3	12		3	10	2	-	-	-	12 (22)	-	-
	MCF	>4	>2	16	4		8	2	4	9	3	9				-	-	0	9 (16)	84
	FLC	>4	>4	-	-	-	10	16	17	4	2	4	2					2 (4)	2 (4)	100
	VOR	>0.5	>0.125		17		23	8	7						-	-	-	0	13 (24)	76
	POS	>0.25*	>0.06		11	20	12	3	9						-	-	-	9 (16)	24 (44)	73
	AMB	>1	>1		42	8	4	1							-	-	-	0	0	100
	5FC	>0.5*	>0.5	-	-	-	49	3	2	1								1 (2)	-	-
C. glabrata (32)	AND	>0.25	>0.06		19	7	3	2	1					-	-	-	-	1 (3)	6 (19)	84
	CAS	>0.25	a		8	6	3	4	10	1				-	-	-	-	11 (34)	-	-
	MCF	>0.12	>0.03	6	21		5							-	-	-	-	0	5 (16)	84
	FLC	>32	>32	-	-	-			2	1	8	7	11			3		3 (9)	3 (9)	100
	VOR	>0.5*	IE		5	10	3	9	3	1	1				-	-	-	2 (6)	-	-
	POS	>2*	IE		4	4	7	5	3	9					-	-	-	0	-	-
	AMB	>1	>1		4	9	7	6	3	2	1							1 (3)	1 (3)	100
	5FC	>0.5*	>0.5	-	-	-	22	2	5	3								3 (9.4)	-	-
C.tropicalis (15)	AND	>0.5	>0.06		7	3	4	1						-	-	-	-	0	5 (33)	67
	CAS	>0.5	a		5		2	4	3	1				-	-	-	-	1 (7)	-	-
	MCF	>0.5	IE		3		5	3	4					-	-	-	-	0	-	-

	FLC	>4	>4	-	-	-	5	3	3			4						0	0	100
	VOR	>0.5	>0.125		1	3	1	5	3	1	1				-	-	-	2 (13)	10 (67)	47
	POS	>0.12*	>0.06		2	1	3	4	2	3					-	-	-	9 (60)	12 (80)	80
	AMB	>1	>1		12	3												0	0	100
	5FC	>0.5*	>0.5				6	4	4				1					1 (7)	-	-
	AND	>0.5	>0.06		8		2	2						-	-	-	-	0	4 (33)	67
	CAS	>0.5	a				3	2	2	3	1	1		-	-	-	-	5 (42)	-	-
	MCF	>0.5	IE				3	6	3					-	-	-	-	0	-	-
C. krusei (12)	FLC	>64*	b											2	7	3		-	-	-
	VOR	>1	IE		6	5	1								-	-	-	0	-	-
	POS	>0.5*	IE		3	1	1	7							-	-	-	0	-	-
	AMB	>1	>1		9	1		1			1							1 (8)	1 (8)	100
	5FC	>32*	>8									2	3	6	1			0	7 (58)	42
	AND	>2	IE	2	3	1	7	1	1	3	1	1						1 (3)	-	-
	CAS	>2	IE	4	1	3	2	1	5	1	2	1						1 (3)	-	-
	MCF	>2	IE	2	1	3	2	3	2		4	2	1					3 (10)	-	-
Other *spp*.(20)	FLC	>64	>4			1	4	3	4	3		1		3		1		1 (3)	4 (14)	90
	VOR	>4	IE		10	2	3	4	1									0	-	-
	POS	>4	IE		10	3	3	2	1			1			-			0	-	-
	AMB	>1	IE		4	2	1	7	4	1	1							1 (3)	-	-
	5FC	>32	IE			3	6	3	6	1	2				1			1 (3)	-	-
	AND	NA	NA	107	39	14	13	7	6	5	9	26	1					6 (3)	23 (10)	93
	CAS	NA	NA	24	90	12	12	17	33	21	3	5	12	2				35 (15)	-	-
	MCF	NA	NA	106	30	6	25	17	16	10	8	11	2					6 (3)	29 (13)	90
Overall (231)	FLC	NA	NA			1	46	75	32	15	11	17	14	5	7	8		8 (4)	11 (5)	99
	VOR	NA	NA		114	27	42	27	16	13	2			1				3 (1)	27 (12)	89
	POS	NA	NA		111	34	31	26	15	12		1	1					29 (13)	47 (20)	93
	AMB	NA	NA	11	92	77	13	17	11	7	3							3 (1)	2 (1)	100
	5FC	NA	NA			3	144	30	27	10	4	2	4	6	2	1		14 (6)	7 (3)	97

ECV – Epidemiological Cutoff Value; IE – Insufficient Evidence; NA – not applicable.

[a] Due to significant interlaboratory variation in MIC ranges for caspofungin, EUCAST breakpoints have not yet been established.

[b] Isolates of *Candida krusei* are assumed to be intrinsically resistant to fluconazole (FLC).

[c] Except *Cryptococcus neoformans* and *Trichosporon mucoides*.

DISCUSSION

Our study reports a mean incidence of fungemia of 0.88/1000 admissions (ranging from 0.15 to 2.4), which is comparable to recent data from other European countries [141, 142], but being somewhat lower than in Brazil [143]. A previous study in Portugal, based on a single university hospital, found a higher incidence of 2.7/1000 admissions [144]. Healthcare-associated fungemia remains the vast majority, representing more than 80% in most studies [43, 145]. Thus, it is not surprising that most of the isolates were recovered from ICU, surgery and internal medicine departments, since most of the patients admitted to these wards are in poor health conditions and are submitted to invasive procedures and aggressive antibiotic and immunosuppressive drug regimens, which both are associated with increased rates of fungal infections [39, 146]. We report a 30-day mortality rate of 25%, with more than half (53%) of *C. glabrata* fungemias being lethal. Costa-de-Oliveira *et al*. reported five years ago even higher values, with a 78% mortality rate for *C. glabrata* fungemia and 46%, 30% and 53% for *C. albicans*, *C. parapsilosis* and *C. tropicalis*, respectively [144]. On the reasons behind this decrease one can only speculated. In other European studies, rates of fungemia ranged from around 30% [43, 141] to 40% [37, 145], but *C. glabrata* did not carry such a death burden.

C. albicans appears as the leading causative agent of fungemia in our study (40%), as reported in other European studies [9, 37, 43, 141, 147]. However, the second position is not occupied by *C. parapsilosis* in most European countries; instead, *C. glabrata* appears repeatedly as the most common non-*albicans* species isolated from blood cultures [134], reaching almost a third [9, 145]. The causes of the difference in incidence are unknown but, curiously, our data parallel those of Spanish and Brazilian studies, where *C. parapsilosis* is responsible for 27% and 37% of cases of fungemia

[142, 148]. *C. orthopsilosis* and *C. metapsilosis* represented 7.3 and 3.6%, respectively, of the *C. parapsilosis* sensu lato isolates, while *C. nivariensis* and *C. bracarensis* were not identified, coinciding with results obtained in other Mediterranean countries [142, 149, 150] and Brazil [148].

Concerning our susceptibility results, comparing CLSI and EUCAST protocols implies limitations because several breakpoints are undefined and some are discordant, with EUCAST being predisposed to lower breakpoint values. However, in recent years, great efforts have been made to overcome those differences and to harmonize both protocols [137, 151, 152]. The similar MIC values obtained by both protocols, despite the differences on practical detail and test execution, are in agreement with those obtained by Pfaller and colleagues [151, 152]. This study represents another step towards a unified categorization of resistant isolates, strengthened by the concordant results. Overall, it is important to highlight the relative small size of our sample and point out that more isolates (either through greater participation or by extending the period of the study) would boost our results.

This is the first multicenter study carried out in Portugal on this topic. These studies provide valuable data on the species distribution and antifungal susceptibility. This information is important since it can guide clinicians in the treatment of fungal infections and, ultimately, reduce the mortality due to fungemia, which still remains at unacceptably high values.

3 Environmental azole fungicide, prochloraz, can induce cross--resistance to medical triazoles in *Candida glabrata*.

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ABSTRACT

Acquisition of azole resistance by clinically relevant yeasts in nature may result in a significant, yet undetermined, impact in human health. The main goal of this study was to assess the development of cross-resistance between agricultural and clinical azoles by *Candida spp*. An *in vitro* induction assay was performed, for a period of 90 days, with Prochloraz (PCZ) – an agricultural antifungal. Afterwards, the induced molecular resistance mechanisms were unveiled.

MIC value of PCZ increased significantly in all *Candida spp*. isolates. However, only *C. glabrata* developed cross-resistance to fluconazole and posaconazole. The increased MIC values were stable. *C. glabrata* azole resistance acquisition triggered by PCZ exposure involved the upregulation of the ATP binding Cassette multidrug transporter genes and the transcription factor, *PDR1*. Single mutation previously implicated in azole resistance was found in *PDR1* while *ERG11* showed several synonymous single nucleotide polymorphisms.

These results might explain why *C. glabrata* is so commonly less susceptible to clinical azoles, suggesting that its exposure to agricultural azole antifungals may be associated to the emergence of cross-resistance. Such studies forward potential explanations for the worldwide increasing clinical prevalence of *C. glabrata* and the associated worse prognosis of an infection by this species.

INTRODUCTION

Although *Candida albicans* is still the most frequently isolated *Candida* organisms, *C. glabrata* and *C. parapsilosis* have recently emerged as the second or third most common agent of invasive candidosis [34, 141, 144]. In an attend to understand the growing clinical relevance of *Candida* species, several facts were considered, namely: the fact that antifungal agents used for crop protection of the azole class, (e.g. propiconazole, prochloraz, imazalil), very similar to those used in human therapy, are extensively used in agriculture within the EU [87]; *Candida* organisms are human commensals, colonizing places like the skin or the gastro-intestinal tract, but they are also ubiquitous in the environment [153]. Such long antimicrobial pressure is recognized to lead to drug resistance. Fungal diseases are problematic for both human health and agriculture, azole drugs representing the core therapy for both; such circumstance may represent an initial step in the emergence of clinical resistant fungal isolates. Therefore, antifungal resistance may perhaps be driven by the extensive use of azole fungicides in agriculture, similar to azoles used in humans. Resistance of yeast clinical isolates to azole antifungal agents can result from either overexpression or mutations in *ERG11* gene. Alternatively, the cells can fail to accumulate azole antifungal agents due to enhanced drug efflux, a consequence of transcriptional activation of drug efflux pumps. At least two families of multidrug transporters, the ABC transporter family and the MFS, are involved in azole resistance [63] . Therefore, the purpose of this study was to evaluate the potential development of cross-resistance by the extensive use of azole fungicides in agriculture, similar to azoles used in humans.

MATERIALS AND METHODS

Strains One clinical isolate each of *Candida albicans*, *C. parapsilosis* and *C. glabrata* were selected from the fungal collection of the Microbiology Department of Faculty of Medicine, University of Porto. They were chosen according to the corresponding clinical information and susceptibility profile to clinical azoles, i.e., strains should have been isolated from patients without previous exposure to any clinical azole and be categorized as susceptible to them. Strains were kept in a YPD medium (0.3% yeast extract, 1% peptone and 2% dextrose) broth supplemented with 10% glycerol, stored at -80ºC; after thawing, strains were subcultured twice and incubate at 35ºC, for 48 hours on Sabouraud dextrose agar (Difco) to assess the purity of culture.

Antifingal agents PCZ was used as a representative of agriculture azoles; Fluconazole (FLC), Voriconazole (VCZ) and Posaconazole (POS) as clinical azoles. Anidulafungin (AND) was included as representative of another class of antifungals – echinocandins. PCZ was ressuspended in 80% acetone solution at a final concentration of 5 mg/L. Clinical azoles were dissolved in dimethysulphoxide (DMSO) to obtain a stock solution of 10 mg/L. All drugs were stored at -20ºC until use.

Antifungal susceptibility testing Broth microdilution susceptibility assay was performed in order to evaluate the initial minimal inhibitory concentrations (MIC) of the isolates to PCZ and to confirm results for all the clinical azoles, according to the Clinical and Laboratory Standards Institute M27-A3 protocol (applying species-specific breakpoints, as advised in supplement S4) [50]. Drug concentration ranged from 0.125 to 64 mg/L of FLC and PCZ; from 0.0313 to 16 mg/L of POS, VCZ and AND; MIC

determination was repeated at least twice. *C. albicans* and *C. parapsilosis* strains for which FLC MIC was ≤2mg/L were categorized as susceptible and resistant at MIC >4 mg/L. *C. glabrata* was considered FLC susceptible-dose dependent (S-DD) at MIC ≤32 mg/L and resistant at MIC >32 mg/L. VRC MIC ≤0.125 mg/L for *C. albicans* and *C. parapsilosis* was considered as susceptible and ≥1 mg/L as a resistant phenotype. Since no specific breakpoints are available for *C. glabrata* regarding VRC or for any of the three species to POS, we used the older breakpoints for both antifungals: susceptible, MIC ≤ 1 mg/L; intermediate, MIC = 2 mg/L; resistant MIC ≥ 4 mg/L. For AND, the three different species have three different breakpoints: *C. albicans* was classified as susceptible whenever MIC ≤0.25 mg/L and resistant whenever MIC >0.5 mg/L; *C. parapsilosis* was susceptible or resistant according to a MIC value ≤2 mg/L or >4 mg/L, respectively; *C. glabrata* was categorized as susceptible whenever AND MIC ≤0.12 mg/L and as resistant if >0.25 mg/L.

***In vitro* induction assays** A single, randomly selected colony from each strain was incubated in 10 mL of YPD medium overnight in a shaker at 180 rpm, 35 ºC. Aliquots of this culture, containing 10^6 blastoconidia, were transferred to different vials, each one containing 10 mL of same culture medium with MIC of PCZ for each strain (0.25 µl, 1 µl and 0.5 µl of PCZ for *C. albicans*, *C. parapsilosis* and *C. glabrata*, respectively) and incubated overnight as described above [154, 155]. The following day, aliquots from each culture containing 10^6 blastoconidia were again transferred into fresh medium containing the same antifungal concentration and reincubated as described. Each day, during a 90 days period, a 0.75 mL aliquot from each subculture was mixed with 0.75 mL of 80% glycerol and frozen at -70ºC for later testing. The induction assay was carried out for 90 days keeping daily constant the concentration of PCZ, corresponding

to the minimal inhibitory concentration (MIC) value for each species. MIC of PCZ was determined every ten days along the 90 days of assay. Whenever a marked MIC increase was observed (four fold the initial PCZ MIC), the MIC values of clinical antifungals were determined as well.

Stability of *in vitro* developed resistance phenotype In order to assess the stability of the developed MIC increment to PCZ and of the developed cross-resistance to clinical azoles, the induced strains were afterwards sub-cultured for an additional ninety days in the absence of antifungals and MIC values re-determined, as previously described in the induction assay.

RNA isolation RNA was extracted from a 50 ml volume of a *C. glabrata* culture that had been grown overnight in YPD broth (without antifungal drugs) at 150 rpm and 35°C to an optical density of approximately 1.0 at 600 nm. Total RNA was extracted using the hot acid phenol method, as described by Köhrer & Domdey [156]. RNA concentration was assessed by Nanodrop ND-1000 and the RNA quality and integrity levels were controlled by capillary electrophoresis in the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), according to the manufacturer's instruction. Only samples yielding a 28S rRNA/18S rRNA ratio ranging from 1.6 to 2.2, and showing the absence of degradation were used for further testing.

cDNA synthesis and RT-qPCR For each real-time quantitative PCR (RT-qPCR) analysis, three replicates for each species, of the initial and final strain, were included: analysis involved the search of the most important genes for each species, reported as responsible for azole resistance. for *C. albicans CDR1*, *CDR2*, *MDR1* and *ERG11*; For

C. parapsilosis MRR1, *MDR1*, *UPC2*, *NDT80*, *ERG6* and *ERG11*; and for *C. glabrata PDR1*, *CDR1*, *PDH1*, *YOR1*, *SNQ2* and *ERG11* [93]. The PCR protocol used included a first minute hot start at 95°C, followed by a 35-cycle program composed of a 15 seconds denaturation step at 95°C, a 30 seconds annealing step at 60°C, and a 30 seconds extension step at 60°C, followed by a 5-minutes final extension step at 60°C. PCRs were performed using PerfeCTa SYBR green Fast Mix (Quanta Biosciences, Gaithersburg, MD, USA) in a Realplex Mastercycler instrument (Eppendorf, Madrid, Spain). The signal obtained for each gene was normalized with the *ACT1* for *C. albicans* and *C. glabrata*; and with *TUB4* for *C. parapsilosis.*

RESULTS

***In vitro* induction assay** *In vitro* induction assays were performed with three isolates of each species with similar results within the same species – one isolate representative of each species was shown in the results. The three species developed a progressive increment of PCZ MIC value comparatively to the initially determined value. As no breakpoints exist for PCZ, we abstained from using the terms “susceptible” or “resistant”. After ten days of induction, all *Candida* species developed a higher MIC value to PCZ; this increase was 32 times in *C. albicans* and *C. parapsilosis* and 64 times in *C. glabrata*. In addition, a concomitant increase of the MIC of FLC, VCZ and POS was observed, but only for *C. glabrata*. VCZ was the less affected clinical azole. Cross-resistance in *C. glabrata* was well established after sixty days of induction with PCZ, for FLC and POS. No cross-resistance was registered regarding AND, for any species (**Table 1**).

Table 1. Susceptibility profile of *Candida* species tested to PCZ, triazoles and anidulafungin.

Species	Time of exposure to PCZ (days)	MIC (mg/L)				
		PCZ	**FLC**	**VRC**	**POS**	**AND**
C. albicans	**0**	0.5	0.25	0.03	0.03	0.015
	10	16	0.25	0.06	0.5	0.03
	30	16	0.5	0.06	0.5	0.03
	60	16	0.5	0.06	0.5	0.03
	90	16	1	0.06	0.5	0.03
	∅90	16	0.5	0.06	0.5	0.03
C. parapsilosis	**0**	2	0.5	0.03	0.03	1
	10	64	1	0.06	1	1
	30	64	1	0.06	1	1
	60	64	1	0.06	2	2
	90	64	1	0.06	2	2
	∅90	64	1	0.06	2	2
C. glabrata	**0**	1	1	0.25	0.03	0.06
	10	64	16	0.5	16	0.125
	30	64	16	0.5	16	0.125
	60	64	32-64	1	16	0.125
	90	64	32	1	16	0.125
	∅90	64	2	0.25	16	0.125

PCZ=Prochloraz; FLC=Fluconazole; VCZ=Voriconazole; POS=Posaconazole; AND=Anidulafungin;
∅= MIC after 90 days of culture in the absence of PCZ.

Stability of the *in vitro* developed resistant phenotype The *in vitro* developed high MIC values of PCZ maintained stable following removal of the selective pressure of the drug, for all species. Also, the cross-resistance observed in *C. glabrata* did not revert in the absence of the inducer antifungal, PCZ.

Gene Expression Profile No significant difference was obtained between the initial and final strain, regarding gene expression in *C. albicans* or *C. parapsilosis. C. glabrata* was the only species that consistently overexpressed genes previously associated with azole resistance due to upregulation of efflux pumps [157]. Meanwhile, *ERG11* was found to be dowregulated (0.141 fold and p=0.775). PCZ exposure triggered overexpression of ATP Binding Cassette (ABC) multidrug transporters *PDH1*, *YOR1*, *CDR1* and *SNQ2*. The first two genes were found to be 48.5 (p<0.001) and 66.8 (p<0.001) fold overexpressed, respectively; *CDR1* and *SNQ2* showed an expression level of 2.9 (p=0.008) and 1.3 (p=0.193) fold respectively. These multidrug transporters are regulated by *PDR1* encoded transcription factor, which was also found to be overexpressed – 6.8 fold (p<0.001) (**Figure 1**). Therefore, both *C. glabrata* transcriptional factor and related efflux genes were upregulated following the *in vitro* induction assay. To determine whether resistance was associated with mutations: *ERG 11* and *PDR1* genes were sequenced for a single *C. glabrata* isolate. DNA products were sequenced in an ABI Prism 3130 genetic analyzer (Applied Biosystems). A G727A point mutation in *PDR1* gene was found, leading to an aspartic acid to asparagine amino acid substitution at codon 243. *ERG11* analysis revealed several synonymous single nucleotide polymorphims (SNPs) (**Figure 2**).

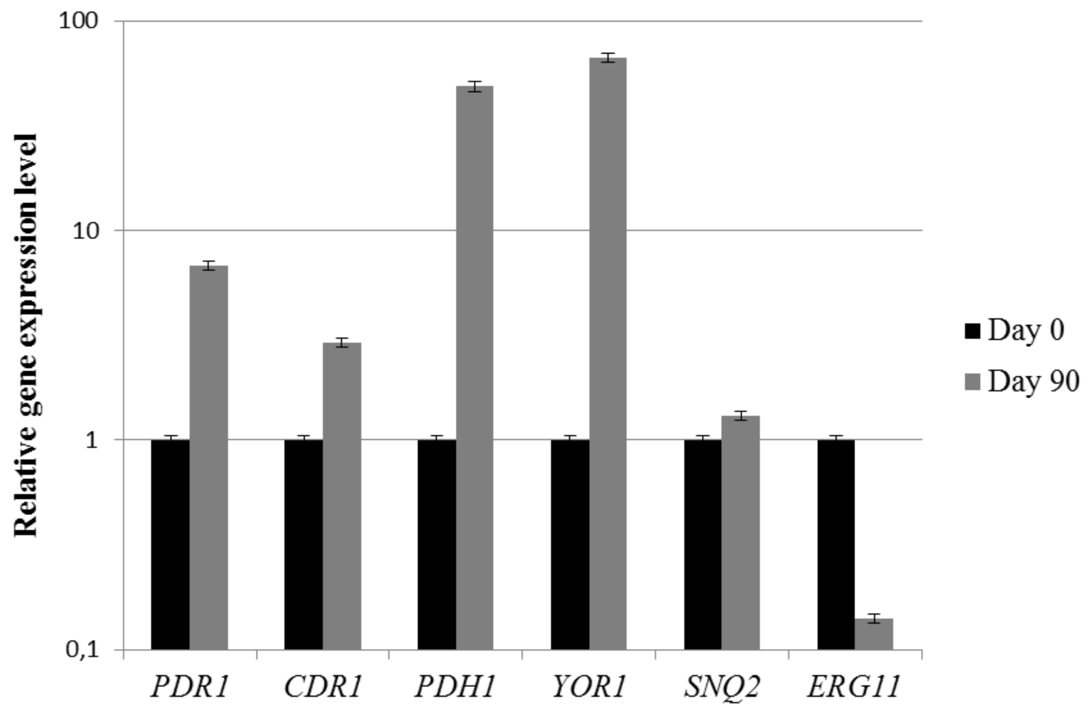

Figure 1. Gene expression alterations triggered by PCZ exposure in *C. glabrata*. Black bars represent the susceptible initial strain – day 0; gray bars represent the same strain after the induction assay, day 90. Comparative gene expression profile between the initial, day 0, susceptible strain and the strain after the induction assay, day 90. Gene expression is expressed as average with standard deviation of three independent experiments. Each mean value was normalized with the *ACT1* gene.

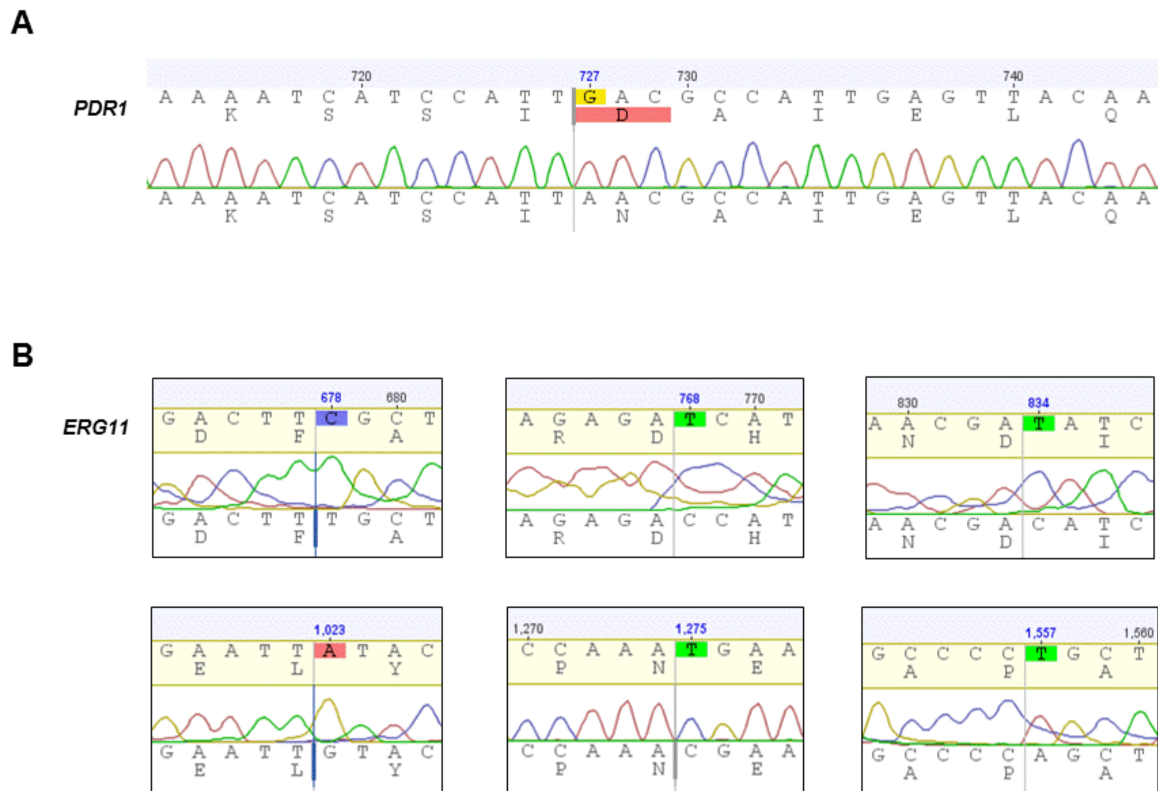

Figure 2. Single Nucleotide Polymorphisms (SNPs) found in *Candida glabrata PDR1* and *ERG11* genes. **(A)** *PDR1* missense mutation G727A results in the amino acid change D243N. **(B)** Several synonymous SNPs throughout the *ERG11* gene.

DISCUSSION

In recent years the concept that the use of azoles in agriculture would not only influence plant pathogenic species but would also inevitably impair susceptible species of opportunistic human pathogens has gained relevance, especially in the case of filamentous fungi like *Aspergillus fumigatus*; such drugs may also leave an impact in saprophytic human flora [113, 125, 158, 159]. In fact, such an imbalance might affect the endogenous population of other medically important pathogens like yeasts, such as *Candida* species. It is generally accepted that a persistent antimicrobial pressure on a complex microbial population will lead to selection of resistant clones, particularly if the antimicrobial effect is not microbiocidal. Systemic infections due to *Candida glabrata* are characterized by a high mortality rate; they are difficult to treat due to the intrinsically low susceptibility of this species to azole drugs, especially to fluconazole [160] . In addition, *C. glabrata* easily develops fluconazole resistance in response to drug exposure during patient treatment. In fact, it is now common to find azole-resistant *Candida* isolates from patients not previously exposed to clinical antifungal agents [161, 162]. In our study, in all the three species PCZ MIC value increased from 32 to 64 fold compared to the initial value. However, neither *C. albicans* nor *C. parapsilosis* developed cross-resistance. Anidulafungin activity was not impaired following the selective pressure of an agricultural azole compound, which is not surprising considering that echinocandins have a different mechanism of action. Our results suggest a different perspective on the way *C. glabrata* species develop stable resistance to medical triazoles. Drug efflux, resulting from the increased expression of ABC transporter proteins is the predominant mechanism by which *C. glabrata* mediates resistance to a wide range of antifungal compounds. Also Pdr1, as the principal regulator of ABC transporter gene expression, has been found to be a key player in such

resistance [63, 157, 163, 164]. These genetic alterations may transform an intrinsically susceptible to a permanently resistant phenotype. In fact, haploid fungal cells - as is the case of *C. glabrata* - might be more prone to such events [165]. We assessed the most common associated genes with azole resistance and found that all ABC transporters were upregulated, as well as their regulatory transcription factor. To our knowledge, this is the first time that *YOR1* was found to have such high expression in a *C. glabrata* azole resistant strain – it was 66.8 fold overexpressed. Also, previous reports addressing genes involved in azole resistance in *C. glabrata* state that the predominant basis for acquired azole resistance is the constitutively upregulated expression of multidrug transporter genes *CDR1* and *PDH1* [63, 157, 166]. Borst and colleagues reported a rapid and stable acquisition of azole resistance by *C. glabrata* after an induction assay with FLC; the same ABC transporters were found overexpressed while no contribution of *ERG11* was verified [154]. As previously described, a single point mutation was found at *PDR1* while *ERG11* only showed the existence of several synonymous SNPs suggesting that this gene was not involved in *C. glabrata* azole resistance in the isolate examined [63, 166]. Certainly, additional studies are necessary to address the involvement of such genes in the development of azole cross-resistance triggered by the selective pressure of an agricultural drug.

In conclusion, apart from very few speculative reports published some years ago, there is still no evidence for a clear correlation between the agricultural use of azoles and the increasing clinical azole resistance [42, 116, 167]. Nevertheless, our results strongly suggest such possibility and have the merit to put in evidence the molecular mechanisms triggered by such an exposure.

4 Development of cross-resistance by *Aspergillus fumigatus* to clinical azoles following exposure to prochloraz, an agricultural azole.

Faria-Ramos I, Farinha S, Neves-Maia J, Tavares PR, Miranda IM, Estevinho LM, Pina-Vaz C, Rodrigues AG.

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ABSTRACT

The purpose of this study is to unveil whether azole antifungals used in agriculture, similar to the clinical azoles used in humans, can evoke resistance among relevant human pathogens like *Aspergillus fumigatus,* an ubiquitous agent in nature. Additionally, cross-resistance with clinical azoles was investigated. Antifungal susceptibility testing of environmental and clinical isolates of *A. fumigatus* was performed according to the CLSI M38-A2 protocol. *In vitro* induction assays were conducted involving daily incubation of susceptible *A. fumigatus* isolates, at 35ºC and 180 rpm, in fresh GYEP broth medium supplemented with PCZ, a potent agricultural antifungal, for a period of 30 days. MIC of PCZ and clinical azoles were monitored every ten days. In order to assess the stability of the developed MIC, the strains were afterwards sub-cultured for an additional 3 0 days in the absence of antifungal. Along the *in vitro* induction process, microscopic and macroscopic cultural observations were registered.

MIC of PCZ increased over 32 times in just ten days after initial PCZ exposure; cross-resistance to all tested clinical azoles was observed. The new MIC value of agricultural and of clinical azoles maintained stable in the absence of the selective PCZ pressure. PCZ exposure was also associated to morphological colony changes: macroscopically the colonies became mostly white, losing the typical pigmentation; microscopic examination revealed the absence of conidiation.

PCZ exposure induced *Aspergillus fumigatus* morphological changes and an evident increase of MIC value to PCZ as well as the development of cross-resistance with posaconazole, itraconazole and voriconazole.

INTRODUCTION

The ubiquitous saprophytic mould *Aspergillus fumigatus* is known to cause a spectrum of diseases in humans, including allergic syndromes, noninvasive infections, as well as invasive aspergillosis, a condition associated with significant morbidity and mortality [168]. *A. fumigatus* is one of the human pathogenic fungi that have a natural habitat in the environment, including soil and plants [169]. Some members of the azole drug class, which includes voriconazole and posaconazole have been shown to be effective in the treatment of invasive aspergillosis [170]. While azole resistance among clinical *A. fumigatus* isolates has for long been considered to be an uncommon finding, recently multiazole resistance has been reported to be emerging and is increasingly recognized as a cause of clinical treatment failure [171, 172]. In agriculture, thousands of tons of azoles are sold annually for the purpose of plant protection, either to control fungal growth that can cause extensive loss of crops or to prevent, or to ease the problem of postharvest spoilage of plants and fruits [87]. All azoles compounds mode of action - irrespectively of their chemical structure and variable biological properties - is based on the interference with the activity of fungal lanosterol 14 alpha-demethylase; such enzyme is responsible for the transformation of lanosterol in ergosterol, an essential component of the fungal cytoplasmatic membrane which is encoded by *Cyp51A* gene in *A. fumigatus*. The inhibition of ergosterol formation results in cell wall disorganization and, finally, the impairment of fungal growth. The mode of action of azoles is, therefore, fungistatic rather than fungicidal. It is well known that a strong and persistent antimicrobial pressure can lead to the selection of resistant clones within a microbial population, particularly if the drug effect is static rather than microbiocidal [159]. Since azoles are the mainstay treatment for both human and agricultural fungal diseases, a major concern is the predictable emergence of cross-resistance to clinical *A.*

fumigatus isolates that is already observed in several countries, driven by the massive use of azole fungicides in agriculture, which have the same mechanism of action as those used in humans [113, 125, 126, 158, 159]. The aim of our study was to investigate whether PCZ, a fungicide extensively use in agriculture, could be associated with the emergence of cross-resistance with clinical azoles among *A. fumigatus*.

MATERIALS AND METHODS

Organisms Two clinical isolates of *A. fumigatus,* LMF05 and LMF11, and one environmental *A. fumigatus* isolate (LMN60, recovered from a garden nearby the hospital), were used in this study. The isolates were identified as belonging to *A. fumigatus* species by macroscopic and microscopic morphology, the ability to grow at 48ºC and by using MALDI-TOF MS to accurately discriminate *A. fumigatus* from a new sibling species *A. lentulus*, which cannot be distinguished by morphological characteristics or growth peculiarities [173]. Long-term preservation of conidial suspensions of the isolates was made in a GYEP medium (2% glucose, 0.3% yeast extract, 1% peptone) broth supplemented with 10% glycerol and stored at -80◦C. Working cultures were subsequently maintained during 2 weeks on Sabouraud dextrose agar slants and plates at 4˚C.

Antifungal agents and susceptibility profile Prochloraz (PCZ) is a non-systemic imidazole fungicide – an ergosterol biosynthesis inhibitor - it was selected and used as a representative of agricultural azoles after a previous MIC screening where it showed to be the less active drug upon the selected strains, i.e., it had the lower MIC values which was a prerequisite for this induction experiment; Fluconazole (FLC), Voriconazole (VCZ), Posaconazole (POS) and Itraconazole (ITZ) as clinical azoles. Prochloraz was ressuspended in 80% acetone solution at a final concentration of 5 mg/L. Clinical azoles were dissolved in dimethysulphoxide (DMSO) to obtain stock solutions of 10 mg/L. All drugs were stored at -20ºC. Broth microdilution susceptibility assay was performed according to the Clinical and Laboratory Standards Institute M38-A2 protocol in order to evaluate the initial minimal inhibitory concentrations (MIC) of

PCZ and of all the clinical azoles [51]. Drug concentration ranged from 0.125 to 64 mg/L of FLC and PCZ; and 0.0313 to 16 mg/L of POS, VCZ and ITZ. *A. fumigatus* ATCC 46645 was included for quality control of susceptibility testing. Also, FLC was used as control, since *A. fumigatus* shows a non-susceptible phenotype and MIC is most often above 64 mg/L for this species. MIC of azoles was defined as the lowest concentration of the drug that produced no visible growth following 48 hours of incubation. MIC determination was repeated at least twice.

***In vitro* induction experiments** Induction experiments were performed with the agricultural azole PCZ. *A. fumigatus* isolates were grown on Saboraud dextrose agar at 37ºC for 72h; conidia were harvested by flooding the surface of the slants with phosphate-buffered saline (PBS) containing 0.025% (vol/vol) tween 80 while gently rocking. The conidial suspensions were then adjusted using specific spectrophotometric readings at 550 nm to a final concentration of $5x10^4$ conidia per militer [174]; one militer of each distinct isolate suspension was transferred to 9 ml of GYEP broth supplemented with sub-inhibitory concentrations of PCZ (0.06 mg/L for both LMF05 and LMF11; 0.125 mg/L for LMN60) and incubated overnight at 37ºC with agitation (180rpm). Daily, after vigorous vortexing for 60 seconds, one militer from each culture was transferred to fresh GYEP medium supplemented with PCZ and in parallel, 1 ml of culture was added with 10% glycerol and frozen at -80ºC. This procedure was repeated along thirty consecutive days.

Susceptibility testing/ Stability of *in vitro* developed resistance phenotype MICs of PCZ were determined every five days along the thirty days of induction assay. No official breakpoints are yet defined for PCZ; therefore, whenever a marked MIC

increase was observed (four fold the initial PCZ MIC), the MIC values of clinical antifungals were determined. In order to assess the stability of the developed MIC increment to PCZ and of the developed cross-resistance to clinical azoles, the induced strains were afterwards sub-cultured for an additional thirty days in the absence of the drug and MIC values re-determined, as previously described.

Culture macro and micro morphology Along the induction process, every two days, a loopful was inoculated in Saboraud Agar slants to check for viability and purity of culture. Macro and microscopical growth characteristics were registered. Colony morphology and pigmentation were recorded photographically using a Reflex Nikon D3200 Camera and images were processed by Adobe Photo Deluxe Image Processing Program (San Jose, CA, USA). Microscopical images of hyphae changes from the original *A. fumigatus* strain and the resistant induced one were capture with a Zeiss-Axioplan-2 microscope equipped with Axio Cam and by using the AxioVision 3.0 digital imaging software.

RESULTS AND DISCUSSION

The 3 isolates developed a progressive increment of PCZ MIC value comparatively to the initially determined value. In addition, a concomitant increase of the MIC of VCZ, POS and ITZ, was observed (**Table 1**). From day 0 of evaluation until day 30 during the induction assay, MIC of PCZ increased 256 times. Concerning the clinical azoles, cross-resistance was developed for all days; all isolates changed from a susceptible to a resistant phenotype, according to Meletiadis and colleagues [175].

Table 1. Susceptibility pattern of tested *A. fumigatus* isolates to PCZ and clinical azoles.

A. fumigatus isolate	Time of exposure (days)	MIC (mg/L)				
		PCZ	**VRC**	**POS**	**ITZ**	**FLC**
LMF05	**0**	0.125	0.125	0.25	2	>64
	10	0.25	0.25	0.5	2	>64
	20	8	2	1	4	>64
	30	32	8	2	8	>64
	Ø30	32	2	2	2	>64
LMF11	**0**	0.125	0.25	0.125	0.5	>64
	10	0.125	2	0.25	1	>64
	20	8	8	1	2	>64
	30	32	>16	4	4	>64
	Ø30	32	2	1	0.5	>64
LMN60	**0**	0.25	0.25	0.125	0.25	>64
	10	4	8	0.25	1	>64
	20	8	8	0.5	2	>64
	30	64	>16	4	4	>64
	Ø30	64	2	1	0.25	>64

PCZ= Prochloraz; VCZ= Voriconazole; POS= Posaconazole; ITZ= Itraconazole; FLU= Fluconazole; Ø= MIC after 30 days of culture in the absence of PCZ.

In fact, there are several studies that have characterized azole resistance in *A. fumigatus* and most recently quite a few addressed the possible cross-resistance between environmental and medical azoles [113, 125, 126, 158]. Our study demonstrated the time frame between the introduction of a widely used agricultural fungicide and the emergence of cross-resistance to medical triazoles. The exposure of other clinical relevant moulds to agricultural azoles might therefore be associated with the emergence of cross-resistance to clinical antifungals. Besides the emergence of cross-resistance, we found that PCZ exposure caused marked morphological colony changes, both at macroscopical level (the colonies turned white losing the characteristic pigmentation) and microscopic examination revealed the progressive nonappearance of conidiation during the induction assay. Initially it was noticeable a macroscopic modification of the pigmentation of *A. fumigatus* colonies, changing from the original green colour to white (**Figure 1** A, B and C).

Figure 1. Photographs of Sabouraud dextrose agar plates showing macroscopic morphological changes of colonies of *A. fumigatus* following exposure to subinhibitory concentration of PCZ. **A.** Initial morphological aspect (control). **B.** After fifteen days. **C.** After thirty days.

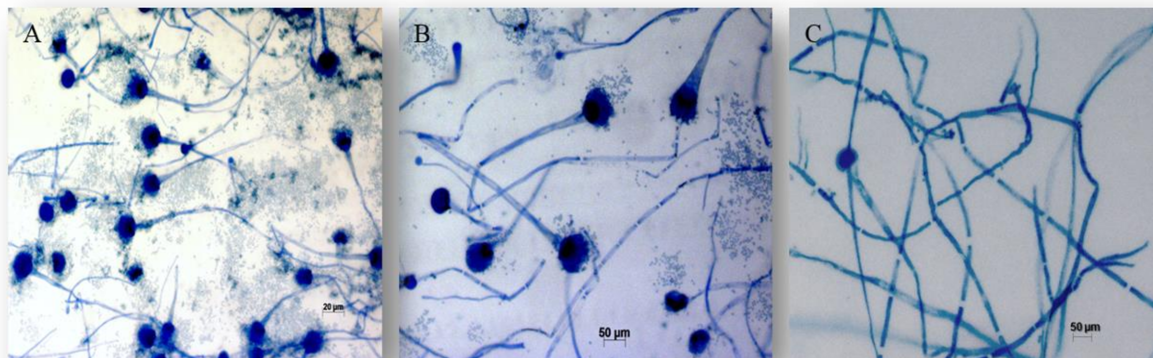

Figure 2. Photomicrographs of *A. fumigatus* colonies using the cellotape flag technique preparation with lactophenol cotton blue staining. Microscopic morphological changes in the development of conidiation of A. fumigatus following exposure to subinhibitory concentration of PCZ. **A.** Initial morphological aspect (control). **B.** After fifteen days. **C.** After thirty days.

With the increase of MIC values of PCZ, colonies of *A. fumigatus* became totally white (**Figure1C**) and also less and smaller. Microscopic examination of such macroscopic pigmentation change showed a progressive absence of conidiation. The original strain (**Figure 1A**) showed normal microscopic features regarding conidiation (**Figure 2A**) while almost white colonies (**Figure 1B**) showed almost complete absence of conidiation (**Figure 2B**), the totally white mycelia (**Figure 1C**) corresponded solely to hyphae and very little conidiophore structures but yet immature, ie, without any conidia (**Figure 2C**). Changes in the pigmentation of *A. fumigatus* colonies and the absence of conidia as consequence of azoles' effect have already been reported. Varanasi and colleagues speculate that azoles may bind to a phytochrome-like regulatory molecule inhibiting the initiation and subsequent development of conidiophores in *Aspergillus* species and such mode of action could also explain the different levels of inhibition displayed by other tested azoles and why echinocandins

and polyenes did not shown this effect [176]. Notably, such morphological changes may be responsible for laboratorial diagnostic misidentification of the fungal genus/species, following its initial isolation, with the inherent consequences [177].

The *in vitro* developed high MIC values of PCZ maintained stable following removal of the selective pressure of the drug. For VRC, the MIC value decreased only after 30 days of incubation without the selective pressure, changing the susceptibility phenotype from resistant to intermediate. For POS, the developed MIC value also decreased but not enough to change the phenotype of resistance. Regarding ITZ, for both LMF11 and LMN60, it was observed the complete reversibility of the resistant phenotype in the absence of PCZ, ie, the MIC reverted to the initial value and that value is categorized as susceptible. However, strain LMF05 has, since day zero, ITZ MIC of 2 mg/L which falls in resistant category. Following removal of the selective pressure of PCZ, conidiation reappeared together with the green typical colour of mature colonies, in all isolates. Since PCZ was responsible for the emergence of stable resistance to itself and to very important medical triazoles in *A. fumigatus*, a resistance mechanism may have been developed. Previous reports describe cyp51A mutation, efflux pump overexpression and/or target upregulation as the main mechanisms responsible for such resistance [28, 59, 111]. A clonal expansion of isolates harbouring the TR34/L98H mutation has been reported across several countries [28, 59, 111, 178]. Interestingly, besides the fact that these resistant isolates are less genetically variable than susceptible ones, no impact on fitness was observed [178]. The phenotypic results (**Figures 1** and **2**) and the stability of the developed resistance (**Table 1**) herein reported suggest the same. Future studies aiming to assess the underlying molecular resistance mechanisms, not only from these induced resistant strains but also from isolates with naturally high MIC values to PCZ and

resistant to medical azoles without previous in vitro induction, will certainly be our next step. Meanwhile, our study suggests that the abuse of azole antifungals in nature may cause serious human health problems since azole resistance and cross-resistance has the potential to further compromise the efficacy of clinical azoles in the future [28, 167, 171, 178, 179]. Furthermore, we can speculate that the exposure of clinically relevant moulds other than *A. fumigatus* to agricultural azoles may also be associated with the emergence of cross-resistance to clinical azoles. Several compounds are being tested in order to find new antifungal alternatives, anticipating the possible loss of efficacy of clinical azoles [180]. On the other hand, efforts should be made to find safer compounds to use in agriculture.

CONCLUSIONS

In order to assess the real dimension of *Aspergillus* resistance, a susceptibility test should be performed in all isolates from patients with *Aspergillus* infection. Moreover, for patients with severe infection initial combination therapy may be considered in geographical areas with high prevalence of environmental azole resistant isolates. Ultimately, surveillance studies in both clinical and in environment settings should be conducted in order to provide updated local data regarding susceptibility profiles.

5 Genesis of Azole Antifungal Resistance from Agriculture to Clinical Settings

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ABSTRACT

Azole fungal resistance is becoming a major public health problem in medicine in recent years. However, it was known in agriculture since several decades. The extensive use of these compounds results in contamination of air, plants and soil.

Indeed, the increasing frequency of life-threatening fungal infections and the increase of prophylactical use of azoles in high-risk patients, taken together both with the evolutionary biology evidence that drug selection pressure is an important factor for the emergence and spread of drug resistance can result in a dramatic scenario.

This study reviews the azole use in agricultural and in medical context and discusses the hypothetical link between its extensive use and the progressive increase of azole resistance among human fungal pathogens.

INTRODUCTION

The use of plant protection products (PPP) may have considerable negative effects on the environment [90]. The use of azole fungicides in agriculture started around mid-1960s; since then, such compounds have been used extensively during the last 30 years [181]. In the European Union half of the total land of cereals and grapevine is treated with azole fungicides. Notably, much smaller quantities of azoles are used in the United States for plant protection [182].

The azoles used in agriculture are generally sprayed every year over the cultured area in order to control mildews, rust and other diseases in cereals, ornamental plants, vegetables, fruits and vineyards. According to Matthews, the widespread use of these compounds results in contamination of air, plants and soil with a lot of small particles of fungicide [183].

The hypothetical risk that the massive use of azoles in agriculture could induce antifungal resistance among human pathogens started to be discussed several years ago. Agricultural azoles could stress fungal species either members of the endogenous saprophytic flora or exogenous to the human body. The imbalance of the endogenous fungal flora ecology could thus upset the population of medically important fungi. Human pathogenic fungi may therefore persist and thrive [87], and in this situation, health risks for human beings increase.

In order to address this problem, in particular regarding the Portuguese situation relatively to azole use in agriculture, this review was organized according the following topics: 1. Use of azole fungicides in agriculture; 2. Azole fungicides in Portugal; 3. Mode of action of the azoles; 4. Hypothetical link between azole use in agriculture and human health; 5. Portuguese National Strategie plan for a sustainable use of plant protection products.

Use of azole fungicides in agriculture The growing request for food in quantity/quality has placed a drastic pressure on the productive sector, often at the expense of the natural elements, with progressive reduction or elimination of pristine landscape. The use of PPP in this context may have strong negative effects on the environment, especially with regard to the possible contamination of surface water and groundwater. Azole compounds are PPP which are also used for preservation of materials, such as paints, coatings, wall paper pastes, and are routinely applied to mattresses in order to prevent fungal growth. The most commonly compounds use in the European Union are imidazoles (**Figure 1A -** [184]), triazoles (**Figure 1B -** [185]), fluconazole (Figure **1C -** [186]) and tebuconazole **(Figure 1D** - [187]).

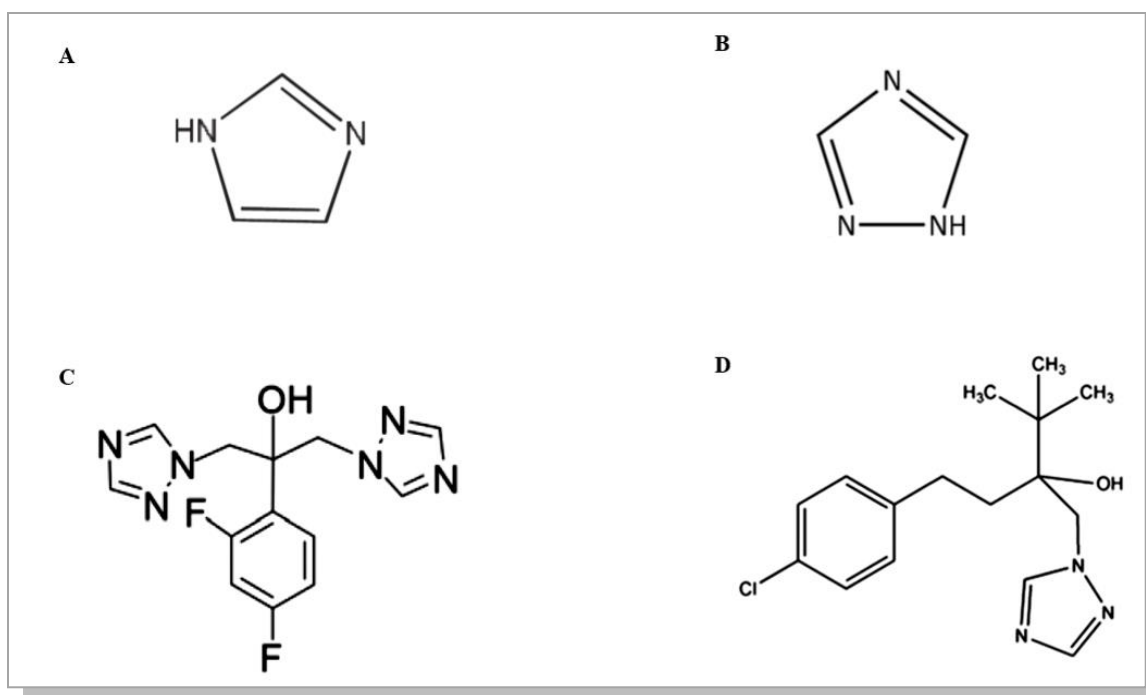

Figure1. Chemical structure of (A) imidazole, (B) 1,2,4 triazole, (C) fluconazole, and (D) tebuconazole.

They are widely used in preharvest phase, in grain-growing and grass-growing environments, as well as at postharvest phase to prevent fungal spoilage [167] by yeasts like *Candida* spp., *Cryptococcus spp, Rhodotorula* spp., *Trichosporon penicillatum*, and *Saccharomyces cerevisiae* or filamentous fungi/moulds like *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., *Geotrichum* spp [95, 96]. In addition, they are also applied to treat plant infection when it becomes apparent.

According to the manufacturer's instructions, doses of 100g/ha should be used, corresponding approximately to 10 mg of azoles applied to 1 m^2 of plant surface [87]. Data from the literature reveals that annually nearly 50% of the total acreage under cereal and grapevine production in Europe is treated with azole fungicides [188]. Comparatively, in the United States, less than 5% of the total crop area is treated with these compounds. In the European Union, the situation of the Netherlands must be stressed in particular, since the utilization of azole fungicides has almost doubled since the mid-1990s [113]. Several reasons are usually forwarded to justify the use of azoles in agriculture, in particular, the fact that these compounds are not expensive and that they exhibit broad spectrum activity. However, since azoles are very stable molecules, they can persist active in the soil and water during several months [91] and several fruits and vegetables [99, 100, 189]. Although the azole residues detected in many samples do not reach health hazards toxic levels, the amount could vary considerably. Data from the literature shows that considerable quantities of azole residues could persist in some food products during long time [87].

Farmers have been strongly encouraged to follow guidelines aiming to reduce the probability of developing resistance to agricultural antifungals and/or to minimise its extent and/or to increase the chance that any developed resistance would still be reversible. Such practices include the rotating use of antifungal products with different

modes of action, as well as the preferential practice of a limited number of interventions using higher dosages as opposed to more frequent applications with lower dosages. This does not, however, imply that the use of antifungal substances in agriculture should not be closely monitored, considering the risk of accumulation of azoles in the soils (the half-life time of azoles is usually longer than 1 year), and because the newer compounds may be also at the origin of resistance development.

Azole fungicides in Portugal In the particular case of Portugal, there is a need to promote agriculture and invest in training farmers. According to the results of the Agricultural Census report [190], the social characterization of the Portuguese family farm population consists of 793 000 individuals representing, 7% of the resident population. The rural population has aged considerably between 1999 and 2009; the average age increased from 46 to 52 years old. The educational level is rather low; 40 % of subjects only attended the 1st cycle and 22% have no schooling. However, despite these social indicators, significant improvements were registered during the ten year period under study; illiteracy rate decreased by 7% and the frequency of senior secondary cycle and higher education increased by 3%. The typical profile of the Portuguese farmer nowadays is a 63 year old male, having just completed the 1st cycle of basic education, only having practical agricultural training and working in agricultural activities about 22 hours per week.

By December 31, 2012, 907 PPP, based on 248 active substances were available in Portugal [191]. The largest volume of these substances available at the national market belong to the fungicides group, followed by herbicides and insecticides, representing the remaining products about 15% of the total PPP marketed. According to the Agricultural Statistics report the ratio between sales of PPP/agricultural area used was in 2008, 2009,

2010 of 4.6, 3.8 and 3.8, respectively. In case the total sales volume is subtracted with the value of sulfur and derivates, this ratio corresponds to values of 1.9, 2.0, and 1.9, respectively [191].

Sales figures of PPP at national level constitute an indicator that can provide an estimation about the use of such products in Portugal. The amount of PPP sold in Portugal during 2012 corresponded to a total of 12 462237 kg, expressed as active substances. The fungicides contributed with about 68.3% of the total of active substances sold, whereas sulphur represented 49.8 % of the total sales and 71.4% of all the fungicides sold. **Figure 2** represents an overview of fungicides sales by chemical group in Portugal between 2002 and 2012. Overall, a decrease in fungicides sales was registered from 2008 until 2012 [192]. In respect to azoles, a consecutive decrease in sales was registered between 2002 and 2005; however, after 2006 the values started to increase again, reaching a peak in 2012 (Figure 3) [192].

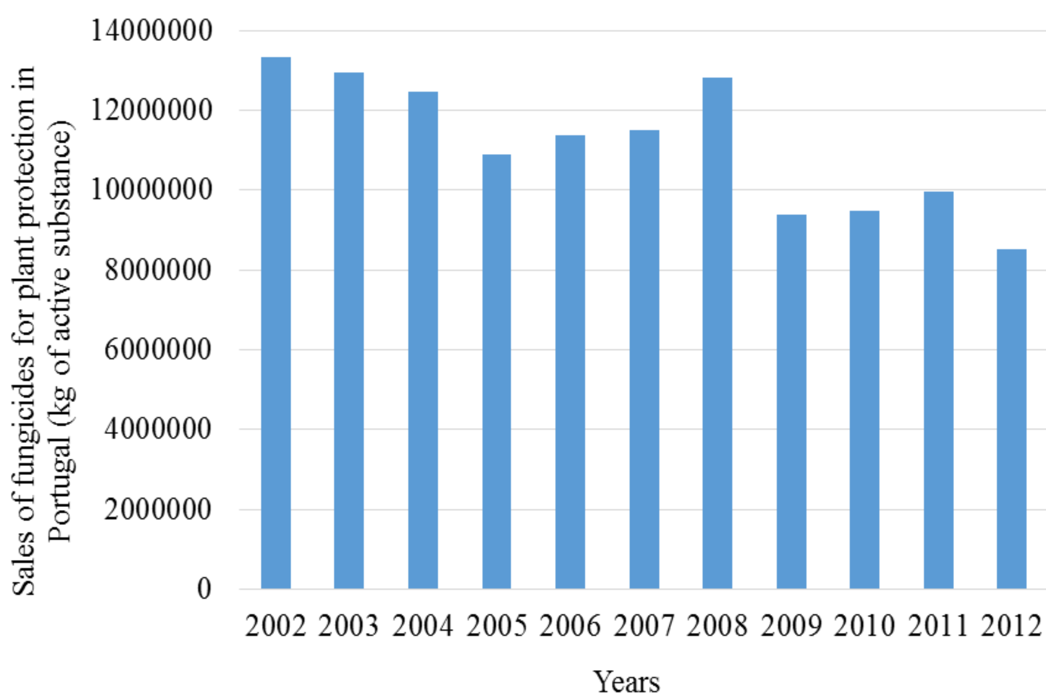

Figure 2. Sales of fungicides in Portugal between 2002 and 2012 [192].

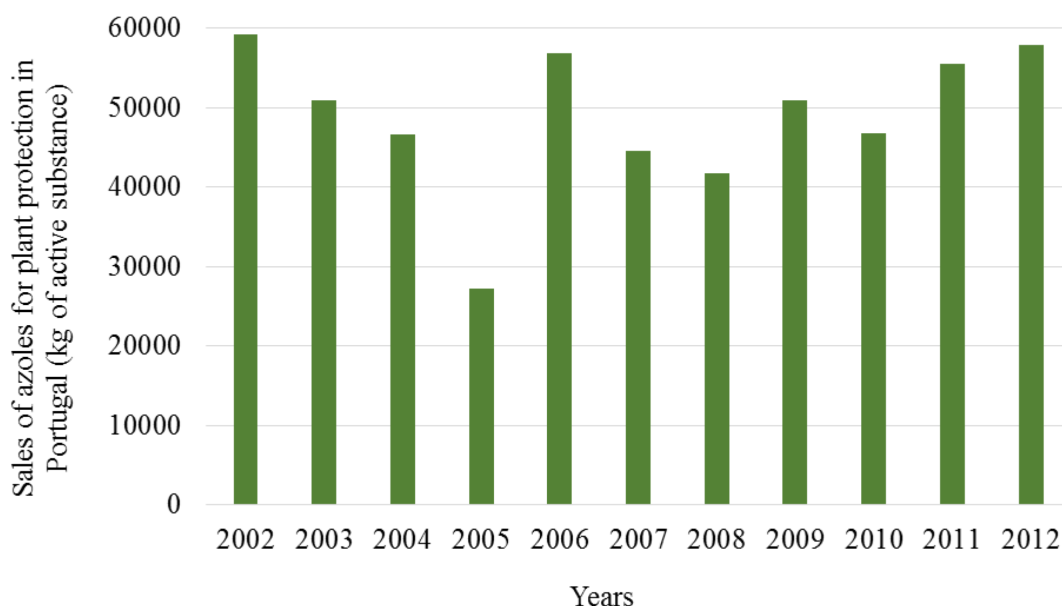

Figure 3. Sales of agricultural azole in Portugal between 2002 and 2012 [192].

Mode of action of the azoles Concerning the mode of action, all the azoles compounds interact and target the same active site in fungal enzyme, so agricultural azole fungicides share the mode of action with medical azoles [125]. This results in a fungistatic rather than fungicidal effect; in this sense the terminology often used in agriculture “fungicides” is ambiguous. This class of compounds are sterol demethylation-inhibitors and impair the synthesis of ergosterol [87, 125]. Sterol biosynthesis inhibitors (SBIs) are major compounds used as medical and agriculture agents for the control of fungal diseases. In many fungi the preponderant sterol is ergosterol. Azoles compounds interfere with the activity of fungal lanosterol 14 alpha-demethylase, an enzyme responsible for the transformation of lanosterol in ergosterol. Ergosterol is an important compound of the fungal cytoplasmatic membrane; the impairment of its metabolism induces cell membrane disorganization and decrease of

fungal growth. Moreover, fungal growth arrest can also result from the accumulation of toxic ergosterol precursors [193].

The available SBIs-fungicides can be classified into four classes according to their respective target sites, which are membrane bound enzymes, namely: thiocarbamates or allylamines (squalene epoxidase), inhibithors of C14-demethylation or DMIs, Amines (Δ^{14} reduction or Δ^{8}-> Δ^{7} – isomerization) and hydroxyanilides (C4-demethylation). However, several resistance mechanisms to SBIs have been described. The two major involve either alterations of the target enzyme, which result in a reduced affinity to fungicides or a decrease of the intracellular drug accumulation, that is linked to an over expression of membrane transporter proteins.

Hypothetical link between azole use in agriculture and human health Many authors equate antifungal resistance only in terms of clinical settings. Indeed, agriculture has been blamed for it, in particular due to the use of concentrated pest control compounds that outflow fungicides into nearby watercourses. Several reports claim that antibiotic resistance is also a natural occurrence in the soil. In fact, microorganisms inhabiting soil have to strive for energy sources; it is known that, some bacteria and fungi developed the ability to produce antibiotic compounds in order to destroy their competitors. Such microorganisms are thus naturally immune to the effects of their own excretions [194].

The development of resistance to azoles in plant fungal pathogens is complex and generally progressing in small steps. First descriptions date from long time ago and claim that some plant pathogens acquired azole resistance following agricultural exposure [195]. Data from the literature describes resistance or tolerance to triazole fungicides for important crop pathogens such as wheat, barley and strawberry [116].

Although in Portugal resistance to triazoles in vineyard powdery mildew has been detected in the past [196], still recent studies described this situation [117].

Over the last two decades, clinically important fungal infections have become more prevalent because medical progress allows the survival of an increasing number of immunocompromised patients. However, resistance to antifungal drugs also became a critical medical problem. Antifungals of a classes similar to those used in clinical practice are widely used in agriculture where resistance to antifungals also became a problem. Improvement the methods of application and treatment control have been devised in order to limit the consequences of this emerging resistance, allowing the continued use of such drugs.

However, it has been proposed, that there may be a direct relation between the development of resistance to azole fungicides used in agricultural practice and the development of resistance to azole antifungals observed in clinical settings [7]. Exposure of a microorganism to any antibiotic involves a risk of resistance developing through the process of selection. The knowledge about the rate and extent of the emergence of resistant organisms [197] and whether this resistance is reversible or not, is a very relevant issue to clinical medicine. Resistance depends upon a variety of factors and conditions, including the mechanisms of action of the compound, the target site(s) in the organism and their number, the risk of resistance transference between individual organisms and species, and the mechanism of such resistance.

Resistance in fungi and the mechanisms involved in its development and transmission differ in some extent from those seen in bacteria. There is no evidence that the genes that confer resistance to fungi can be transferred horizontally, a key difference with bacteria. There are two forms of resistance, primary (or intrinsic) and secondary. In primary resistance, the fungal organism cannot be inhibited even by high concentrations

of an antifungal drug. For example, *Aspergillus fumigatus* is intrinsically resistant to fluconazole and does not respond at all to this drug. Similarly, *Candida krusei* and *C. glabrata* isolates are often intrinsically resistant to fluconazole. Conversely, fluconazole resistance in *Candida albicans* can emerge *de novo* during prolonged treatment; which is an example of secondary resistance [198].

There are at least three different mechanisms by which antifungal drug resistance might arise. They can involve increased efflux of drug, altered target demethylase sites and the availability of alternative pathways for the synthesis of cell membrane sterols. *Candida krusei* shows intrinsic resistance to fluconazole largely due to the last mentioned mechanism. There is also evidence that in clinical settings the adoption of some therapeutic strategies to treat patients over long periods or to prevent infections through antifungal prophylaxis can result in selection of resistant organisms, either of the same or of different species. Several studies have shown that a strong and persistent antimicrobial pressure upon a microbial population will undoubtedly lead to the selection of resistant clones, particularly whenever the antimicrobial agent induces a static effect instead of a biocidal effect [194]. This situation can happen in fungi submitted to azole action. Since azoles represent first line clinical antifungals, the topic of azole resistance is of great medical relevance. Azole resistance can thus hypothetically result from exposure of the fungi to azole compounds, which can happen *in vivo* (in azole treated patients) or due to the use of these compounds in the environment, namely agriculture use [158]. The discussion about the risk that the massive use of azoles in agriculture could induce cross-resistance among human fungal pathogens has been debated along the recent years. The massive use of azoles in agriculture would stress susceptible species of the fungal flora, both in nature but also at human endogenous niches. This disturbance in the fungal flora ecology could result in

considerable changes among the population of medically important fungi; human pathogenic fungi may thus persist and increase [87]. Specifically, species that could develop azole resistance would profit from such an environmental repeated and selective pressure. In this situation, health risks for human beings might increase seriously. In addition, new resistance mutations could also developed in the environment. Studies addressing the epidemiology of invasive fungal infections are of high relevance in order to determine the hypothetical impact of such anthropogenic activities upon the susceptibility profile of yeast and filamentous fungi and also to report any emergent species. In Portugal, two studies conducted at the same institution, within a ten year interval, revealed several relevant differences not only in susceptibility patterns but, most importantly, regarding species distribution [67, 144].

In 2009, Snelders and co-workers provided for the first time evidence that patients with invasive aspergillosis due to azole resistant *A. fumigatus* might have acquired the organism from the environment [158]. Invasive aspergillosis is a very dangerous opportunistic fungal infection. The conidia of *Aspergillus spp*. become airborne from soil or decaying organic matter and patients become infected usually by inhalation from such a reservoir [113]. According to this study, resistant strains of *A. fumigatus* to medical azoles were found in the soil and compost from areas nearby to the admission hospital [158]. The dominance of a single resistance mechanism and the genetic homology between clinical and environmental isolates suggested that the acquisition of azole-resistant isolates from the environment was the most important infection route.

Azole resistance among *A. fumigatus* isolates was not only found in Dutch hospitals [67]; resistant isolates were also detected in several European countries such as in Spain [199], Belgium [200], Denmark [201], Sweden [202], France [203].

Human and animal fungal pathogens such as *Coccidioides*, *Histoplasma*, *Aspergillus* and *Cryptococcus* do also thrive in the environment, including plants and food products. It should also be stressed that only sporadic yeast species are endogenous to the flora of healthy human beings. Thus, in most occasions, the fungal pathogens are taken up from the environment. Thus, environmental resistant isolates could have developed antifungal resistance following a prolonged selective azole pressure. Therefore, the probability that an individual might be exposed to an environmental resistant fungal organisms is real and high. Nevertheless, the effect of residual azoles present in vegetables and other food products upon the endogenous native fungal flora of humans should not be neglected and may also contribute to the emergence of antifungal resistance at endogenous niches.

In the case of *A. fumigatus*, it was concluded that two distinct models of resistance might have developed. One of them occurs following long time-exposure to azole treatment, as might occur in clinical settings which is supported by several possible genetic mutations. The other model is facilitated by a single mechanism, TR/L98H mutation, that occurs in environmental isolates under the selective pressure of azole fungicides used in agriculture. This kind of mutation started spreading worldwide and has nowadays also been described outside Europe [204]. A very recent study conducted in France describes a clinical case of a French farmer who developed invasive aspergillosis caused by an azole-resistant *A. fumigatus* with the TR34/L98H mutation following a hematopoietic stem cell transplantation [205]. This farmer had worked in fungicide-sprayed fields from where *A. fumigatus* TR34/L98H isolates were collected. This study supports the assumption that immunosuppressed patients might be infected with environmental azole resistant strains.

The adoption of measures in order to control this problem should be undertaken, in particular in the Netherlands, where the TR/L98H mutation is endemic [87]. Interestingly, in Portugal, back in 2007 Araújo *et al*. [132] compared the antifungal susceptibility profile of different *Aspergillus* species recovered from environmental and clinical sources and found high MIC levels of clinical antifungals among environmental strains.

Verweij and co-workers reported cross-resistance between azoles, which constitutes a very relevant clinical issue, with relatively few therapeutic options available in the case of invasive *Aspergillus* infections [206]. More recently, several studies demonstrated cross resistance between agricultural and medical azoles [113, 125]. Faria-Ramos and co-workers, clearly demonstrated that exposure of clinically relevant moulds and yeast organisms to azoles used in agriculture does result in the emergence of cross-resistance to clinical azoles, which may not be reversible [118, 119].

Additional research efforts aiming to reduce the emergence of fungicide resistance are urgently needed, both in agriculture and in medical settings. Since the mechanisms providing the basis for fungicide resistance are likely to be conserved between plant and human pathogens, plant pathologists and medical microbiologists should develop effective strategies counteracting the development of antifungal resistance, in a concerted action.

Portuguese National Strategie Plan for a sustainable use of plant protection products The Directive No. 2009/128/EC is an innovation in the context of the Community legislation about PPP. For the first time legislates about the use of PPP in order to protect human health and environment from the possible risks inherent to its use.

In order to operationalize the implementation of this Directive at national level, the law nº. 26/2013 was published. This regulates the distribution, sales and application of PPP by professional users as well as adjuvants for PPP; it also sets the monitoring procedures for the use of PPP. Together with the Law nº. 86/2010 constitutes (which establishes a mandatory inspection scheme of PPP and its application using equipment authorized for professional use) the transposition into national legal order of that Community law.

The article 51º of this law, envisaged the creation of National Action Plans. These plans aimed at reducing the risks and effects of the use of PPP on human health and upon the environment, also promote the development of integrated protection technical alternatives to reduce dependence of the use of conventional PPP.

The Portuguese National Plan has been prepared with the collaboration of various agents, either from public or private sector, without which would not have been possible to achieve the intended goals. It must also be pointed out that the implementation of this plan will only be effective with the committed collaboration of all the agents involved in manufacture, storage, sale and use of PPP, as well as those responsible for managing the effects and risks associated with the use of PPP. The correct application of PPP can be encouraged by the training of professionals, appropriate counselling at the point of sale, and monitoring/supervision of compliance by professional users. In the act of sale, adequate information about the use of PPP and the risks and safety instructions regarding human health, should be provided to buyers, in order to enable the adequate management of the risks inherent to the products concerned.

The protection of professionals configures primarily a matter of safety and health regarding to the professional handling field use and application of pesticides.

Risks may include not only acute intoxication, but also chronic and sub-chronic risks, resulting from prolonged exposure. The lines of action chosen for this intervention area are: i) Protection of professional users (commercial and storage settings); ii) Reduction of exposure; iii) Limitation of the use of certain PPP [207].

The main measure of protection for non-professional users is restricting its access to a limited category of PPP. In any case, such buyers should be provided awareness of the risks associated with PPP use and about promotion of good general practices regarding its use. On the other hand, it is intended that at points of sale buyers are properly advised, as recommended by the legislation.

In order to assess the progress made in reducing the risks and adverse effects of the use of PPP, both to human health and the environment, monitoring plans should be in place so that, based on a sample of professional users and predefined criteria, it will be possible to assess the progresses made in the implementation of the general principles of integrated protection.

In addition, is also very important to emphasize the importance of the contribution of scientific research and field application of the most recently available scientific and technological information in improving resources and instruments available to the professional users, including complementary or alternative means of use of PPP.

Concluding, fungicide-based plant protection is at present indispensable for efficient and large-scale crop production. Notably, modern fungicides should exhibit negligible acute toxicity, and the regulations for fungicide application should ensure the availability of safe food. As long time periods and enormous financial investments are required to develop new fungicides, particular care must be taken in order to prevent the emergence of fungicide resistance. This is particularly important, since fungicides with

novel modes of action are rarely found, and resistance to single-target fungicides may occur within the first few years of its field use.

The key to prevent or at least delay the onset of development of fungicide resistance in agriculture is the preparation of efficient fungicide mixtures as well as the rotation between fungicides with different modes of action during a crop campaign. The recommendations to maintain highly efficient fungicides in the market preclude the use of monofungicides, in order to keep the use of fungicides applied above a threshold allowing quantitative resistance to develop. The strategy developed during the last decades was to apply single-target fungicides, which resulted in a strong selective pressure for mutants with a modified fungicide-binding site. As fungicides with more than one target are not easy to be overcome by mutations, the focus in fungicide screening should also be directed towards identifying multi-site inhibitors. After having such fungicides available, the risk of rapid occurrence of qualitative resistance might be strongly and effectively reduced. Particular attention should be devoted to the assessment of development of cross-resistance to clinical antifungals while a strict monitoring of the emergence of fungal strains with resistance mechanisms, as well as variations in species distribution regarding both plant and human pathogens should be implemented.

6 Overall Discussion and Conclusions

Overall discussion

Over the past three decades, the world has witnessed the rise of fungi as a major cause of human disease, especially among debilitated and immunocompromised patients, whose number continues to increase [5]. Despite its growing importance, many fungal infections have been neglected over the years. However, there are two well-known responsible fungal pathogens: *Aspergillus fumigatus* and yeasts belonging to the *Candida* genus. More recently, the emergence of antifungal resistance among such pathogens along with a somewhat narrow antifungal armamentarium have stressed the relevance of the topic. Charles Darwin´s evolutionary concept of "natural selection" tells us that it was inevitable for this to happen in result either from antifungal prophylactic (perhaps abusive) use or also from prolonged regimen therapies that are also very common in clinical practice; sooner or later resistance would emerge [208]. The question is: where natural resistant strains came from? Where did the antifungal resistance of isolates that where never before in contact with clinical antifungals came from? Can this resistance develop by cross-resistance with agricultural antifungals? This question has been raised a little all over the world by now. We tried to address it with two original studies using *Candida* and *Aspergillus*, as described in **chapters 3** and **4**, respectively.

The prevalence of IFIs is changing greatly across different geographic areas and it is also dependent of many factors, starting on the host condition itself, passing through the antifungal therapy modalities used and, finally, to the pathogen virulence attributes [70, 84, 85]. In order to understand our national reality a multicentric survey on yeast species distribution and its *in vitro* susceptibility profile to clinical antifungals was carried out, as described in **chapter 2**. From this study we could obtain important

information not only about clinical and demographic data but also about cryptic species such as *C. parapsilosis* and *C. glabrata* complex, along with the resistance rate to the most frequently used clinical antifungals. From the 240 fungemia episodes reported in this study we have calculated an incidence of 2.4 per 1.000 admissions, decreasing to 2.1/1.000 when defined as nosocomial fungemia, corresponding to 86% of all the fungemia episodes. In fact, in most surveillance studies, healthcare-associated fungemia in hospitalized patients remains the main source of infection, frequently representing more than 80% of the cases [43, 145]. *C. albicans* remained the leading agent of fungemia; however, in agreement with findings in Europe the proportion of non-*C. albicans* is rising [133]. Our data confirmed other Spanish and Brazilian studies where the second position was occupied by *C. parapsilosis*, being *C. glabrata* the third [142, 148]. The latter was also the deadliest species with more than a half of the patients dying within 30 days after fungemia detection which represents the highest death burden in Europe [37, 43, 141, 145]. We did not find any isolate of the sibling species of *C. glabrata*, *C. nicavariensis* or *C. bracarensis*; only *C. glabrata* sensu stricto was found in this study. The same occurred in similar studies from Mediterranean countries and also in reports from Brazil [142, 148-150]. Interestingly, from 55 *C. parapsilosis*, 49 were sensu stricto while 4 were *C. orthopsilosis* and 2 *C. metapsilosis*. Most yeasts were isolated from patients admitted in intensive care units and surgical wards, being 57% males and 32% aged between 41-60 years old. The overall mortality rate was 25% and it was associated with more than thirty days of hospitalization and patients with one of the following condition: 1) female gender; 2) more than sixty years old; 3) under dialysis and 4) infected by *C. glabrata*. The susceptibility testing results were interpreted by two distinct protocols, CLSI and EUCAST, and the overall susceptibility rates ranged from 74 to 97% for echinocandins and from 84% to 98% for azoles. It is

noteworthy that resistance rates discrepancies between protocols were found with *C. albicans* and *C. glabrata* for echinocandins and with *C. parapsilosis* and *C. tropicalis* for azoles. Besides the obvious importance of establishing an accurate and actual portuguese scenario detailing species distribution and susceptibility profiles, this study enabled us to obtain a considerable number of yeast isolates from several species and with different susceptibilities to the most commonly used clinical antifungals. From this newly assembled collection, our next step was also to determine its corresponding MIC values to the most frequently used fungicides in crop fields. Such results helped us to selected one isolate each of *C. albicans*, *C. parapsilosis* and *C. glabrata* that were susceptible to clinical and agricultural antifungals. Fulfilled this premise, we carried on with *in vitro* induction assays with one of the most frequently fungicide used in Portugal, PCZ – a study detailed in **chapter 3**. During the 90 days of induction, the MIC values were determined every 10 days and, at the end, we found that cross-resistance did develop. In fact, the three species under investigation revealed a corresponding progressive increase of MIC value of PCZ comparatively with the respective parental strain. A concomitant increase was achieved for each clinical azole tested – FLC, VCZ and POS – but only for *C. glabrata*; in fact, cross-resistance emerged following the selective pressure of a fungicide. No cross-resistance was observed regarding echinocandin for any of the tested species. We were expecting such a result since echinocandins belong to a class of antifungals that act very distinctly from azoles or DMIs fungicides. Interestingly, the obtained resistance and cross-resistance was stable, i.e., the MIC values did not revert to the initial value in the absence of the inducer fungicide, in any of the tested species. Since this cross-resistance phenomena only happened with *C. glabrata* we considered that a specific genetic event should be responsible for such cross-resistance, not only because it was a permanent susceptible-

to-resistant phenotype alteration but also because *C. glabrata* is a haploid cell and for that reason cells might be more prone to genetic modifications. We screened the most common azole resistance associated genes, namely: *CDR1*, *PDH1*, *YOR1*, *SNQ2*, *PDR1* and *ERG11*. In contrast to several other studies reporting genetic alterations responsible for azole resistance, to the best of our knowledge, this was the first time that genes like *YOR1* and *PDH1* have such an upregulation, 66.8 and 45.5 fold overexpressed, respectively. All the other genes were also upregulated but to a smaller extent while no ERG11 contribution was verified. Can this particular genetic context of resistance only arise from a fungicide azole selective pressure? It would certainly needs additional studies to address whether the involvement of these genes is specifically triggered after a fungicide induction of cross-resistance. Besides a few speculative reports published years ago [42, 116, 167], the only work done with yeasts that can allow to clearly trace a correlation between agricultural use of fungicides and azole clinical resistance was latter herein discussed.

In contrast, the evidence that *A. fumigatus* species are extremely stressed by the use of agricultural fungicides is now fully documented around the globe. In fact, there is a strong indication that *A. fumigatus* clinical azole resistance arose from the environment, namely from fungicides use in crop fields. So, we decided to recreate the same methodologic approach described in chapter 3 but this time with *A. fumigatus* species – as detailed in **chapter 4**. Following the induction assays we found that besides the emergence of cross-resistance, PCZ exposure also caused marked morphological colony changes, both macro and microscopically. The macroscopic colony changed in pigmentation from the original greenish color to a totally white mycelia, corresponding to hyphae with immature little conidiophore structures without conidia. Although these pigmentation changes had already been described as a consequence of azole exposure,

yet no clear explanation is given of such phenomena besides one report by Varansi and colleagues suggesting that it possible may be due to the fact that azoles bind to a phytochrome-like regulatory molecule, inhibiting the initiation and development of conidiophores in *Aspergillus* species [176]. The high MIC values achieved for PCZ maintained stable. On the other hand, in the absence of PCZ, we observed different repercussions either within the isolates tested and also regarding the cross-resistance achieved for the three clinical azoles. For all the isolates tested, the MIC value of VRC and POS changed but not enough to revert to a susceptible phenotype, while for ITZ we observed a complete reversion to the initial MIC value, i.e., LMF11 and LMN60 became susceptible again and although LMF05 reverted to the original MIC value, 2mg/ml, this was already falling into the resistant category since the beginning of the induction assay. The morphological typical aspect of all isolates reappeared after PCZ removal.

It should be stressed that cross-resistance developed soon after 30 days of exposure to an agricultural antifungal. Certainly, we cannot deny the fact that an *in vitro* assay lacks the influence of innumerous factors happening either *in vivo* or in the field; the poor relationship between *in vitro* and *in vivo* is known in science [48]**.** Yet, the clear suggestion of our studies was that, indeed, the use of fungicides in nature may cause serious problems in the medical field – the potential of saprophyte fungi to suffer the selective pressure of fungicides with very similar mode of action to those antifungals used to treat humans and develop cross-resistance and then compromise clinical efficacy of medical drugs.

Finally, in **chapter 5**, we discussed about the origin/emergence of azole antifungal resistance and its hypothetical link to fungicide use in agriculture, addressing in particular the Portuguese situation. We report the national strategy plan for the use of

Plant Protection Products (PPP) upon several directives and law implemented since 2009. It regulates the distribution, sales and application of PPP. The Portuguese plan aims to reduce the risks and effects of the use of PPP on human health and the environment but most importantly to promote the development of alternatives to reduce the dependence of the use of conventional PPP. In Portugal the largest volume of these substance available at the national market belongs to the fungicides group, followed by herbicides and insecticides. More than 12. 000 tons of PPP have been sold during 2012; fungicides represent about 68% and, from these, more than 5 000 000 kg were azoles [191]. Indeed, several reasons are given to justify the use of azoles in agriculture, in particular, the fact of being inexpensive and having a broad spectrum of activity. However, they can persist active in soils and water for several months [91] as well as in fruits and vegetables [99, 100, 189]. Therefore, farmers are being instructed to follow the most recent guidelines in order to minimize the collateral damage of such use. All the working plans upon agriculture activities must be monitored on site in order to prevent or at least delay the onset of azole fungicide resistance. The most important factor to prevent it must be the preparation of efficient fungicide mixtures, i.e., to join fungicides with multiple modes of action and exclude the use of monofungicides [207]. It is known that the use of these single-target fungicides (monofungicides) results in a strong selective pressure for genetic events overcoming its target site. Also, it is of extremely importance to insure surveillance studies addressing the susceptibility profile of both environmental and clinical isolates either for yeast or for filamentous fungi. The last and perhaps the biggest challenge is to develop new antifungal drugs – part of the challenge is related to the difficulty in finding suitable mammalian-excluding biochemical targets in eukaryotic pathogens and to the pharmaceutical economic interests and priorities. Nonetheless, new antifungals are indeed urgently needed mainly

because, in the setting of azole resistance, treatment failure is closely associated with resistance and alternative therapeutic options are very scarce.

Soon after the first article on this topic in 2007, many studies addressing the same question have been published and all suggest the existence of an environmental route of resistance driven by fungicides [64, 101, 113, 115, 117, 127, 167, 209-212]. The repercussions and impact of such cross-resistance in human health is also being discussed but still no major measures have been taken in agriculture. In fact, simply prohibiting use of the fungicides is not feasible, as this might result in the return of crop disease epidemics and consequent food shortages and incalculable economic losses. Two extreme scenarios can be hypothetically raised: 1) on the medical field what could happen if the efficacy of the current and limited antifungals is lost? What if clinicians have no antifungal available antifungal to treat patients with an invasive fungal infection by a resistant isolate? This is already a real concern among the medical/clinical scientific community [71, 115]; 2) With an estimated global population of more than 7.5 billion people, agriculture cannot afford to exclude the use of fungicides. What would happen if cereals, vegetables and fruits crops production was tremendously reduced? Agricultural industry has to use fungicides to optimize food production; otherwise, how could so many people be fed? Also, several reports from agricultural scientific area debate such hypothetical scenario [213-216]. The most recent effort is called Integrated Pest Management (IPM) and tries to provide feasible alternatives to fungicide application, maintaining a high agricultural production pattern [217, 218].

The concern is real and translational within these two scientific communities,: clinical and agricultural [127, 209]. It must be stressed that ITC was introduced in early 1980s and the first isolation of a resistant *A. fumigatus* from a patient with invasive aspergillosis and receiving long-term azole therapy occurred in 1989 [106]. On the other

hand, azole fungicides were introduced in the 70s but just very recently (2007) resistant isolates were recovered from patients with invasive aspergillosis with no prior or actual azole therapy [67]. Therefore, it was suggested that selection for resistance could occurred in the environment since *A. fumigatus*, as a saprophyte, inhabit soils and decaying matter. The hypothesis of agricultural fungicides being the main responsible cause for antifungal cross-resistance, is still controversial. Firstly, from an ecological point of view, *A. fumigatus* is not commonly isolated from residue surface in cropping areas [219] but it can be found at farms sewage [220], compost [220] and flower fields [128]. Also, it is estimated that azole fungicides applied in crop fields have a half-time that varies from 20 to 200 days depending on soil type but more than two years are needed before soils are expected to be considered "azole free" [221]. However, azoles are moderately lipophilic so they are strongly adsorbed to soil organic matter and may become quickly unavailable to soil microflora [222]. Secondly, laboratories involving selection for resistance are scarce but on the other hand surveys analyzing genotypic changes in isolates from both clinical and environmental sources are considerable [54, 114]. Interestingly, such surveys always suggest that evolutionary changes involving different resistance targets are far greater in the clinic than in nature, which probably reflects higher selective pressure in patients receiving azole therapy than what might occurs in field crops. Another feature that certainly biases this situation is the existence of sibling species of *A. fumigatus*, that are known to be less susceptible or even resistant to medical azoles and are difficult to identify in a routine hospital laboratories [16, 17, 48]. Several authors have acknowledged that the studies herein described are the ones confirming the selection, convey by agricultural fungicides exposure, for resistance and promotion of cross-resistance to clinical antifungals [15, 127, 210, 223-230].

As a conclusion of this thesis, altogether, it seems evident that both medical relevant yeasts and filamentous fungi can develop cross-resistance to medical antifungals following its exposure to agricultural fungicides.

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8 Publications

Publication I

(Chapter 2)

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ARTICLE

Species distribution and in vitro antifungal susceptibility profiles of yeast isolates from invasive infections during a Portuguese multicenter survey

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Abstract This is the first Portuguese multicenter observational and descriptive study that provides insights on the species distribution and susceptibility profiles of yeast isolates from fungemia episodes. Ten district hospitals across Portugal contributed by collecting yeast isolates from blood cultures and answering questionnaires concerning patients' data during a 12-month period. Molecular identification of cryptic species of *Candida parapsilosis* and *C. glabrata* complex was performed. The susceptibility profile of each isolate, considering eight of the most often used antifungals, was determined. Both Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocols were applied. The incidence of 240 episodes of fungemia was 0.88/1,000 admissions. Fifteen different species were found, with *C. albicans* (40 %) being the most prevalent, followed by *C. parapsilosis* (23 %) and *C. glabrata* (13 %). Most isolates were recovered from patients admitted to surgical wards or intensive care units, with 57 % being males and 32 % aged between 41 and 60 years. For both the CLSI and EUCAST protocols, the overall susceptibility rates ranged from 74 to 97 % for echinocandins and from 84 to 98 % for azoles. Important resistance rate discrepancies between protocols were observed in *C. albicans* and *C. glabrata* for echinocandins and in *C. parapsilosis* and *C. tropicalis* for azoles. Death associated with fungemia occurred in 25 % of the cases, with more than half of *C. glabrata* infections being fatal. The great number of *Candida* non-*albicans* is noteworthy despite a relatively low antifungal resistance rate. Studies like this are essential in order to improve empirical treatment guidelines.

Introduction

The increased incidence of fungemia represents a clinical problem and its impact has significantly risen during the last

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20 years [1]. Fungemia is an important cause of morbidity and mortality, being related to longer hospital stays and high economic costs. *Candida albicans* still remains the leading cause of fungemia worldwide [2]; *Candida parapsilosis*, *Candida glabrata*, and *Candida tropicalis* occupy places two to four, varying according to region. Due to the medical relevance of fungemia and its strong association with unfavorable outcomes, epidemiological surveillance studies are urgently needed in order to evaluate species' geographic distribution and potential changes in susceptibility profiles. In addition, there is a limited number of therapeutic options, with the main class of drugs (azoles) being controversially used for prophylaxis, possibly leading to decreased susceptibility and the selection of non-*albicans* species [3–5]. Regarding the susceptibility testing, there are currently two independent organizations suggesting standard protocols: the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [6–8]. Their protocols differ not only on the laboratory execution and interpretation, but also with respect to the breakpoints proposed for each species/drug combination, leading to discrepancies in the categorization of the isolates as susceptible or resistant [9]. The aim of this study was to provide an overview of blood-borne yeast infections in Portugal and to evaluate the susceptibility profiles provided by both protocols from the CLSI and the EUCAST.

Materials and methods

Study design

Ten hospitals from northern (4), central (2), and southern (4) regions of Portugal accepted to participate in this study, providing isolates collected from patients with fungemia from September 1st 2011 to August 31st 2012. All hospitals enrolled in this study were able to admit adults, children, and neonates. Of those hospitals, two had more than 1,000 beds, three had between 600 and 1,000 beds, and five had less than 600 beds. All were asked to collect and send the strains to the Microbiology Department of the Faculty of Medicine of the University of Porto, where the study was conducted. In addition, a questionnaire regarding patients' clinical and demographic data was also sent.

Definitions

The start of an episode of fungemia was defined as the first isolation from a blood culture of a yeast strain from a patient with related signs and symptoms. Nosocomial fungemia was defined whenever the yeast isolate was obtained more than 48 h after hospital admission. Patients were grouped into five age categories: less than 19 years, 19–40 years, 41–60 years, 61–70 years, and more than 70 years old. For each individual patient, the outcome of the fungemia episode was evaluated at 30 days after the first yeast isolation. Death associated with fungemia was defined as death within 30 days after the recovery of the first yeast isolate, without any other concomitant cause of death, such as intracerebral or gastrointestinal bleeding or pulmonary embolism.

Identification and in vitro antifungal susceptibility testing of yeast isolates

Yeasts were identified using VITEK 2 YST cards from bioMérieux (Marcy l'Etoile, France). The characterization of *Candida glabrata* sensu stricto, *Candida bracarensis*, and *Candida nivariensis* was confirmed as previously described by Romeo et al. [10]. *Candida parapsilosis* sensu stricto, *Candida orthopsilosis*, and *Candida metapsilosis* isolates were identified as previously described by Tavanti et al. [11]. Furthermore, the identities of these species were confirmed by DNA amplification and further sequencing of the *ITS1* and *ITS4* regions of rRNA operons. The minimum inhibitory concentration (MIC) was considered as the lowest concentration showing a 50 % reduction on growth compared with the control after 24 h of incubation for azoles and echinocandins. For AMB and 5FC, the MIC was the lowest concentration showing a complete absence of growth compared to the control and 50 % reduction of growth compared to the control, respectively, both after 48 h of incubation. MICs were determined for fluconazole (FLC), voriconazole (VOR), posaconazole (POS), anidulafungin (AND), caspofungin (CAS), micafungin (MCF), amphotericin B (AMB), and 5-flucytosine (5FC) by broth microdilution, following two different protocols: CLSI M27-A3 (M27-S4) and EUCAST EDef 7.2 document [6–8]. Also, being aware of the interlaboratory variation of CAS MIC values [12], we tried to minimize it by using a single drug powder lot and stock solution solvent. In order to compare both protocols, we assigned the categorical agreement (CA), expressing the level of discrepancy on the number of resistant isolates when categorized by one protocol or another. Reference powders for each antifungal agent were obtained from their respective manufacturers. FLC, CAS, and MCF were dissolved in water and the other drugs in DMSO. Antifungal resistance rates were calculated regarding both protocols, although for CLSI, several times, the epidemiological cutoff values (ECVs) were used; for EUCAST, many breakpoints are still undefined. For AMB, yeasts inhibited by $\leq$1 mg/L were considered to be susceptible according to suggestions by Pfaller et al. [13]. The quality control (QC) strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included in all assays [6–8].

Eur J Clin Microbiol Infect Dis (2014) 33:2241–2247

Statistical analysis

A descriptive revision of the collected data was performed using SPSS v21.0 (SPSS Inc., Chicago, IL, USA). Chi-squared tests were used to compare proportions and analyze differences in the species distribution and antifungal susceptibility profiles. A *p*-value below 0.05 was considered significant.

Results

Patient data

A total of 240 fungemia episodes were reported in the current survey (corresponding to 240 different patients) and included in the study. The mean incidence of fungemia was 0.88 per 1,000 admissions, ranging from 0.15 to 2.4, depending on the hospital. The mean incidence of nosocomial fungemia was 0.74 per 1,000 admissions, ranging from 0.14 to 2.1. This corresponds to approximately 86 % of all episodes of fungemia. In Portugal, pediatrics departments admit neonates and children aged 18 years and under. However, often, these patients—especially adolescents—are admitted by other departments due to numerous and unspecified issues (Table 1). Fifty-seven percent of the patients with fungemia were males and the most affected age group was 41–60 years (32 %), closely followed by the group of patients over 70 years of age, together accounting for 62 % of cases (Table 1). Most patients enrolled in this study were admitted to the intensive care unit (ICU; 39 %) and surgical wards (30 %). The mortality rate due to fungemia was 25 %. The deadliest species was *C. glabrata*, with more than 50 % of patients dying within 30 days of fungemia detection (Table 1).

Species distribution and identification

Table 1 shows the most important characteristics regarding the top five recovered species, the total of the others species, and an overall perspective. Fifteen different yeast species were identified and nearly 60 % of fungemias were caused by non-*albicans* species. The five most prevalent species were *C. albicans* (40.4 %), *C. parapsilosis* (22.9 %), *C. glabrata* (13.3 %), *C. tropicalis* (6.3 %), and *C. krusei* (5 %). *Cryptococcus neoformans* was responsible for eight (3.3 %) episodes of fungemia, followed by *C. lusitaniae* (2.5 %), *Candida guilliermondii* (1.7 %), *Candida dubliniensis* (1.3 %), and *Candida famata* (1.3 %). Single cases due to *Candida sake*, *Candida inconspicua*, *Candida haemulonii*, *Candida kefyr*, and *Trichosporon mucoides* were also found, as a whole accounting for 1.7 % of all cases. Regarding the molecular identification of the cryptic species, from 55 isolates of *C. parapsilosis* sensu lato, 49 corresponded to *C. parapsilosis* sensu stricto, four to *C. orthopsilosis* (three isolated from medicine departments and one from an ICU), and two to *C. metapsilosis* (both from medicine departments). The 32 isolates primarily identified as belonging to the *C. glabrata*

Table 1 Distribution and characteristics of the isolated yeasts

	No. of isolates (%)						
	C. albicans	*C. parapsilosis*	*C. glabrata*	*C. tropicalis*	*C. krusei*	Other species	Overall
Gender							
Male	59 (61)	26 (47)	21 (66)	11 (73)	4 (33)	16 (55)	137 (57)
Female	38 (39)	29 (53)	11 (34)	4 (27)	8 (67)	13 (45)	103 (43)
Total	97 (40)	55 (23)	32 (13)	15 (6)	12 (5)	29 (12)	240 (100)
Age group (years)							
<19	8 (8)	13 (23)	1 (3)	–	–	12 (42)	34 (14)
19–40	10 (10)	4 (7)	3 (9)	2 (13)	2 (17)	6 (21)	27 (11)
41–60	35 (35)	13 (24)	9 (29)	7 (47)	6 (50)	7 (24)	77 (32)
61–70	15 (16)	7 (13)	3 (9)	3 (20)	1 (8)	2 (7)	31 (13)
>70	29 (30)	18 (32)	16 (50)	3 (20)	3 (25)	2 (10)	71 (30)
Hospital department							
ICU	42 (43)	21 (38)	12 (38)	4 (27)	5 (42)	10 (35)	94 (39)
Surgery	22 (23)	18 (33)	8 (25)	8 (53)	3 (25)	11 (38)	70 (30)
Medicine	25 (26)	13 (24)	11 (34)	3 (20)	4 (33)	6 (21)	62 (26)
Pediatrics	8 (8)	3 (6)	1 (3)	–	–	2 (7)	14 (6)
Outcome							
Death within 30 days	23 (24)	12 (22)	17 (53)	3 (20)	–	4 (14)	59 (25)

group all corresponded to *C. glabrata* sensu stricto. Two cases of fungemia in neonates were observed: one infection caused by *C. parapsilosis* and the other by *C. glabrata*.

We report three cases of fungemia in children under 2 years old, all caused by *C. albicans*.

Very distinct geographic areas were covered by these hospitals, although no significant differences in species distributions were found. *C. glabrata* appears in more than half of the cases in ICUs and Medicine Departments, while *C. tropicalis* was mostly found in surgery wards.

Species susceptibility profiles

Table 2 details all the data for susceptibility testing using the CLSI and EUCAST protocols. Readings were made after 24 h of incubation for azoles and echinocandins. For AMB and 5FC, 48-h readings were registered. Similar MIC results were obtained despite differences arising from both protocols (one dilution discrepancy above or below for each protocol has not been taken into account). Generally, the CLSI breakpoints categorized fewer isolates as resistant (R) than EUCAST. Among the group of azoles, the highest percentage of resistance (9 %) coupled *C. glabrata* to FLC, according to both protocols. Concerning the echinocandins, several differences were observed with the application of specific breakpoints for each protocol. Resistance to AMB and 5FC was rare. Regarding simultaneous resistance to antifungals of different classes, applying both protocols and excluding strains with intrinsic resistances such as *C. krusei* for FLC and *C. neoformans* for the echinocandins, we detected one isolate of *T. mucoides* (resistant to FLC, AND, CAS, and MCF), one of *C. krusei* (resistant to AMB and CAS), and one of *C. parapsilosis* (resistant to FLC and CAS) (data not shown). Resistance to drugs within the same class was more frequent, with eight strains of *C. albicans* and one strain of *C. tropicalis* showing resistance to the three azoles and six strains of *C. parapsilosis* being resistant to the three echinocandins. With the exception of three isolates (one each of *C. glabrata*, *C. krusei*, and *C. kefyr*), which corresponded to a high MIC value (2 mg/L), all the other strains were susceptible to AMB with low MIC values, ranging from 0.03 to 0.125 mg/L. The four *C. orthopsilosis* and two *C. metapsilosis* strains were found to be susceptible to all antifungals tested. Regarding the CA, half of the possible comparisons between the CLSI and EUCAST protocols had a CA of 100 %, while the other half ranged from 42 %, the lowest CA obtained relating to both protocols in the case of *C. krusei* isolates to 5FC, to 97 % in the case of *C. albicans* to VOR (see Table 2).

Discussion

Our study reports a mean incidence of fungemia of 0.88/1,000 admissions (ranging from 0.15 to 2.4), which is comparable to recent data from other European countries [14, 15], but being somewhat lower than in Brazil [16]. A previous study in Portugal, based on a single university hospital, found higher a incidence of 2.7/1,000 admissions [17]. Healthcare-associated fungemia remains as comprising the vast majority, representing more than 80 % in most studies [18, 19]. Thus, it is not surprising that most of the isolates were recovered from ICU, surgery, and internal medicine departments, since most of the patients admitted to these wards are in poor health conditions and are submitted to invasive procedures and aggressive antibiotic and immunosuppressive drug regimens, both of which are associated with increased rates of fungal infections [20, 21]. We report a 30-day mortality rate of 25 %, with more than half (53 %) of *C. glabrata* fungemias being lethal. Costa-de-Oliveira et al. reported even higher values 5 years ago, with a 78 % mortality rate for *C. glabrata* fungemia and 46 %, 30 %, and 53 % for *C. albicans*, *C. parapsilosis*, and *C. tropicalis*, respectively [17]. The reasons behind this decrease can only speculated. In other European studies, rates of fungemia ranged from around 30 % [14, 18] to 40 % [19, 22], but *C. glabrata* did not carry such a death burden.

C. albicans appears as the leading causative agent of fungemia in our study (40 %), as reported in other European studies [14, 18, 22–24]. However, the second position is not occupied by *C. parapsilosis* in most European countries; instead, *C. glabrata* appears repeatedly as the most common non-*albicans* species isolated from blood cultures [2], comprising almost a third of species [19, 23]. The causes of the difference in incidence are unknown but, curiously, our data parallel those of Spanish and Brazilian studies, where *C. parapsilosis* is responsible for 27 % and 37 % of cases of fungemia, respectively [15, 25]. *C. orthopsilosis* and *C. metapsilosis* represented 7.3 % and 3.6 %, respectively, of the *C. parapsilosis* sensu lato isolates, while *C. nivariensis* and *C. bracarensis* were not identified, coinciding with the results obtained in other Mediterranean countries [15, 26, 27] and Brazil [25].

Concerning our susceptibility results, comparing the CLSI and EUCAST protocols implies limitations because several breakpoints are undefined and some are discordant, with the EUCAST protocol being predisposed to lower breakpoint values. However, in recent years, great efforts have been made to overcome those differences and to harmonize both protocols [9, 28, 29]. The similar MIC values obtained by both protocols, despite the differences in practical details and test execution, are in agreement with those obtained by Pfaller and colleagues [28, 29]. This study represents another step towards a unified categorization of resistant isolates,

Eur J Clin Microbiol Infect Dis (2014) 33:2241–2247

Table 2 Distribution of minimum inhibitory concentration (MIC) values comparing the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocols

Species (no. of isolates)	Drug	R breakpoint or ECV		No. of isolates with MIC (mg/L)														No. (%) of R		CA (%)
		CLSI	EUCAST	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	CLSI	EUCAST	
C. albicans (97)	AND	>0.5	>0.03	88	2	3				1	2		1	–	–	–	–	4 (4)	8 (8)	96
	CAS	>0.5	[a]		75	1	1	5	10	3			2	–	–	–	–	5 (5)	–	–
	MCF	>0.5	>0.016	82	1	3	2	3	3	1	1		1	–	–	–	–	3 (3)	15 (15)	88
	FLC	>4	>4	–	–	–	27	53	6	7	1	1	1			1		2 (2)	2 (2)	100
	VOR	>0.5	>0.125	–	75	7	11	1	2					1	–	–	–	1 (1)	4 (4)	97
	POS	>0.06	>0.06	–	81	5	5	5						1	–	–	–	11 (11)	11 (11)	100
	AMB	>1	>1	11	21	54	1	2	4	4				–	–	–	–	0	0	100
	5FC	>0.5	> 0.5	–	–	–	61	18	10	5	2					1	–	8 (8)	–	–
C. parapsilosis (55)	AND	>4	>4	17			1	1	4	1	6	25			–	–	–	0	0	100
	CAS	>4	[a]	20	1	2	1	1	3	12		3	10	2	–	–	–	12 (22)	–	–
	MCF	>4	>2	16	4		8	2	4	9	3	9				–	–	0	9 (16)	84
	FLC	>4	>4	–	–	–	10	16	17	4	2	4	2					2 (4)	2 (4)	100
	VOR	>0.5	>0.125		17		23	8	7						–	–	–	0	13 (24)	76
	POS	>0.25	>0.06		11	20	12	3	9						–	–	–	9 (16)	24 (44)	73
	AMB	>1	>1		42	8	4	1							–	–	–	0	0	100
	5FC	>0.5	>0.5	–	–	–	49	3	2	1								1 (2)	–	–
C. glabrata (32)	AND	>0.25	>0.06		19	7	3	2	1					–	–	–	–	1 (3)	6 (19)	84
	CAS	>0.25	[a]		8	6	3	4	10	1				–	–	–	–	11 (34)	–	–
	MCF	>0.12	>0.03	6	21		5							–	–	–	–	0	5 (16)	84
	FLC	>32	>32	–	–	–			2	1	8	7	11			3		3 (9)	3 (9)	100
	VOR	>0.5	IE		5	10	3	9	3	1	1				–	–	–	2 (6)	–	–
	POS	>2	IE		4	4	7	5	3	9					–	–	–	0	–	–
	AMB	>1	>1		4	9	7	6	3	2	1							1 (3)	1 (3)	100
	5FC	>0.5	>0.5	–	–	–	22	2	5	3								3 (9.4)	–	–
C. tropicalis (15)	AND	>0.5	>0.06		7	3	4	1						–	–	–	–	0	5 (33)	67
	CAS	>0.5	[a]		5		2	4	3	1				–	–	–	–	1 (7)	–	–
	MCF	>0.5	IE		3		5	3	4					–	–	–	–	0	–	–
	FLC	>4	>4	–	–	–	5	3	3			4						0	0	100
	VOR	>0.5	>0.125		1	3	1	5	3	1	1				–	–	–	2 (13)	10 (67)	47
	POS	>0.12	>0.06		2	1	3	4	2	3					–	–	–	9 (60)	12 (80)	80
	AMB	>1	>1		12	3												0	0	100
	5FC	>0.5	>0.5				6	4	4				1					1 (7)	–	–
C. krusei (12)	AND	>0.5	>0.06		8		2	2						–	–	–	–	0	4 (33)	67
	CAS	>0.5	[a]				3	2	2	3	1	1		–	–	–	–	5 (42)	–	–
	MCF	>0.5	IE				3	6	3					–	–	–	–	0	–	–
	FLC	>64	[b]											2	7	3		–	–	–
	VOR	>1	IE		6	5	1								–	–	–	0	–	–
	POS	>0.5	IE		3	1	1	7							–	–	–	0	–	–
	AMB	>1	>1		9	1		1			1							1 (8)	1 (8)	100
	5FC	>32	>8									2	3	6	1			0	7 (58)	42
Other spp.(20)[c]	AND	>2	IE	2	3	1	7	1	1	3	1	1						1 (3)	–	–
	CAS	>2	IE	4	1	3	2	1	5	1	2	1						1 (3)	–	–
	MCF	>2	IE	2	1	3	2	3	2		4	2	1					3 (10)	–	–
	FLC	>64	>4			1	4	3	4	3		1		3		1		1 (3)	4 (14)	90
	VOR	>4	IE		10	2	3	4	1									0	–	–

Table 2 (continued)

Species (no. of isolates)	Drug	R breakpoint or ECV		No. of isolates with MIC (mg/L)														No. (%) of R		CA (%)
		CLSI	EUCAST	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	CLSI	EUCAST	
	POS	>4	IE		10	3	3	2	1			1			–			0	–	–
	AMB	>1	IE		4	2	1	7	4	1	1							1 (3)	–	–
	5FC	>32	IE			3	6	3	6	1	2				1			1 (3)	–	–
Overall (231)	AND	NA	NA	107	39	14	13	7	6	5	9	26	1					6 (3)	23 (10)	93
	CAS	NA	NA	24	90	12	12	17	33	21	3	5	12	2				35 (15)	–	–
	MCF	NA	NA	106	30	6	25	17	16	10	8	11	2					6 (3)	29 (13)	90
	FLC	NA	NA			1	46	75	32	15	11	17	14	5	7	8		8 (4)	11 (5)	99
	VOR	NA	NA		114	27	42	27	16	13	2			1				3 (1)	27 (12)	89
	POS	NA	NA		111	34	31	26	15	12		1	1					29 (13)	47 (20)	93
	AMB	NA	NA	11	92	77	13	17	11	7	3							3 (1)	2 (1)	100
	5FC	NA	NA			3	144	30	27	10	4	2	4	6	2	1		14 (6)	7 (3)	97

ECV epidemiological cutoff value; *IE* insufficient evidence; *NA* not applicable

[a] Due to significant interlaboratory variation in MIC ranges for caspofungin, EUCAST breakpoints have not yet been established

[b] Isolates of *Candida krusei* are assumed to be intrinsically resistant to fluconazole (FLC)

[c] Except *Cryptococcus neoformans* and *Trichosporon mucoides*

strengthened by the concordant results. Overall, it is important to highlight the relatively small size of our sample and point out that more isolates (either through greater participation or by extending the period of study) would boost our results.

This is the first multicenter study carried out in Portugal on this topic. These studies provide valuable data on the species distribution and antifungal susceptibility. This information is important since it can guide clinicians in the treatment of fungal infections and, ultimately, reduce the mortality due to fungemia, which still remains at unacceptably high values. .

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Conflict of interest No conflicts to declare.

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Publication II

(Chapter 3)

FEMS YEAST RESEARCH

SHORT COMMUNICATION

Environmental azole fungicide, prochloraz, can induce cross-resistance to medical triazoles in *Candida glabrata*

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agriculture; *Candida glabrata*; clinical resistance.

Abstract

Acquisition of azole resistance by clinically relevant yeasts in nature may result in a significant, yet undetermined, impact in human health. The main goal of this study was to assess the development of cross-resistance between agricultural and clinical azoles by *Candida* spp. An *in vitro* induction assay was performed, for a period of 90 days, with prochloraz (PCZ) – an agricultural antifungal. Afterward, the induced molecular resistance mechanisms were unveiled. MIC value of PCZ increased significantly in all *Candida* spp. isolates. However, only *C. glabrata* developed cross-resistance to fluconazole and posaconazole. The increased MIC values were stable. *Candida glabrata* azole resistance acquisition triggered by PCZ exposure involved the upregulation of the ATP binding cassette multidrug transporter genes and the transcription factor, *PDR1*. Single mutation previously implicated in azole resistance was found in *PDR1* while *ERG11* showed several synonymous single nucleotide polymorphisms. These results might explain why *C. glabrata* is so commonly less susceptible to clinical azoles, suggesting that its exposure to agricultural azole antifungals may be associated to the emergence of cross-resistance. Such studies forward potential explanations for the worldwide increasing clinical prevalence of *C. glabrata* and the associated worse prognosis of an infection by this species.

Although *Candida albicans* is still the most frequently isolated *Candida* species, *C. glabrata* and *C. parapsilosis* have emerged as the second or third most common agent of invasive candidosis, depending on the region (Pfaller & Diekema, 2007; Costa-de-Oliveira *et al.*, 2008; Tortorano *et al.*, 2013).

In an attempt to understand the growing clinical relevance of *Candida* species, several facts were considered. *Candida* species are human commensals, but they are also ubiquitous in the environment (Odds, 1988); antifungal agents used for crop protection of the azole class, such as prochloraz (PCZ), very similar to those used in human therapy and are extensively used in agriculture within the EU (Hof, 2001). Such long antimicrobial pressure is recognized to lead to drug resistance. Fungal diseases are problematic for both human health and agriculture, and azole drugs represent the core therapy for both; such circumstance may represent an initial step in the emergence of clinically resistant fungal isolates. Therefore, the purpose of this study was to evaluate the potential development of cross-resistance by the extensive use of azole fungicides in agriculture, similar to azoles used in humans.

A preliminary broth microdilution susceptibility assay was performed in order to evaluate the initial MIC of the isolates to PCZ and to confirm results for all the clinical azoles, according to the Clinical and Laboratory Standards Institute M27-S4 protocol (Clinical & Laboratory

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Standards Institute, 2012). Within the fungal collection of the Microbiology Department of Faculty of Medicine – University of Porto, we found that isolates, irrespectively from their species, resistant to clinical azoles showed also high MIC value to PCZ. Therefore, three clinical isolates each of *C. albicans*, *C. parapsilosis*, and *C. glabrata* were selected based upon their susceptibility profile: susceptible to clinical azoles and low MIC value to PCZ. Strains were kept in a YPD medium broth supplemented with 10% glycerol, stored at −80 °C. Prochloraz (PCZ) was used as a representative of agriculture azoles because its initial minimal inhibitory concentration (MIC) value was the lowest encountered. Among clinical antifungals, we used fluconazole (FLC), voriconazole (VRC), and posaconazole (POS) – clinical azoles – and anidulafungin (AND) as representative of echinocandins. Prochloraz was resuspended in 80% acetone solution at a final concentration of 5 mg L^{-1}. Clinical azoles were dissolved in dimethyl sulphoxide to obtain a stock solution of 10 mg L^{-1}. All drugs were stored at −20 °C until use. Drug concentration ranged from 0.125 to 64 mg L^{-1} of FLC and PCZ and from 0.0313 to 16 mg L^{-1} of POS, VRC, and AND; MIC determination was repeated at least twice.

In vitro induction experiments were performed as described by Borst *et al.* (2005) and Pinto e Silva *et al.* (2009). It was carried out daily for 90 days keeping a constant subinhibitory concentration of PCZ. MIC of PCZ was determined every ten days throughout the 90 days of assay. Whenever a marked MIC increase was observed (fourfold the initial PCZ MIC), the MIC values of clinical antifungals were determined as well.

To assess the stability of the developed elevated MIC, the induced strains were afterward subcultured for an additional ninety days in the absence of antifungal and MIC values re-determined, as previously described in the induction assay.

RNA was extracted as described by Köhrer & Domdey (1991). For each real-time quantitative PCR (RT-qPCR) analysis, three replicates for each species, of the initial and final strain, were included: for *C. albicans CDR1*, *CDR2*, *MDR1*, and *ERG11*; for *C. parapsilosis MRR1*, *MDR1*, *UPC2*, *NDT80*, *ERG6*, and *ERG11*; for *C. glabrata PDR1*, *CDR1*, *PDH1*, *YOR1*, *SNQ2*, and *ERG11* (Kanafani & Perfect, 2008). The signal obtained for each gene was normalized with the *ACT1* for *C. albicans* and *C. glabrata* and with *TUB4* for *C. parapsilosis*.

In vitro induction assays were performed with three isolates of each species with similar results within the same species – one isolate representative of each species was shown in the results. The three species developed a progressive increment of PCZ MIC value in comparison to the initially determined value. After 10 days of induction, all *Candida* species developed a 32–64 times higher PCZ MIC value. In addition, a concomitant increase of the MIC of FLC, VRC, and POS was observed, but only for *C. glabrata*; regarding POS, cross-resistance was well established after 60 days of induction. No cross-resistance was registered regarding AND (Table 1).

Table 1. Susceptibility profile of *Candida* species tested to PCZ, triazoles, and anidulafungin

Species	Time of exposure to PCZ (days)	MIC (mg L^{-1})				
		PCZ	FLC	VRC	POS	AND
C. albicans	0	0.5	0.25	0.03	0.03	0.015
	10	16	0.25	0.06	0.5	0.03
	30	16	0.5	0.06	0.5	0.03
	60	16	0.5	0.06	0.5	0.03
	90	16	1	0.06	0.5	0.03
	Ø90	16	0.5	0.06	0.5	0.03
C. parapsilosis	0	2	0.5	0.03	0.03	1
	10	64	1	0.06	1	1
	30	64	1	0.06	1	1
	60	64	1	0.06	2	2
	90	64	1	0.06	2	2
	Ø90	64	1	0.06	2	2
C. glabrata	0	1	1	0.25	0.03	0.06
	10	64	16	0.5	16	0.125
	30	64	16	0.5	16	0.125
	60	64	32	1	16	0.125
	90	64	64	1	16	0.125
	Ø90	64	64	0.25	16	0.125

PCZ, prochloraz; FLC, fluconazole; VRC, voriconazole; POS, posaconazole; AND, anidulafungin; Ø, MIC after 90 days of culture in the absence of PCZ.

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The *in vitro* developed high MIC values of PCZ for all species were stable in the absence of the inducing antifungal PCZ as well as the cross-resistance observed in *C. glabrata*.

No significant difference was obtained between the initial and final strain, regarding gene expression in *C. albicans* or *C. parapsilosis*. *C. glabrata* was the only species that consistently overexpressed genes previously associated with azole resistance due to upregulation of efflux pumps (Bennett *et al.*, 2004). Meanwhile, *ERG11* was found to be downregulated (0.141-fold and $P = 0.775$). PCZ exposure triggered overexpression of ATP Binding Cassette (ABC) multidrug transporters *PDH1*, *YOR1*, *CDR1*, and *SNQ2*. The first two genes were found to be 48.5-fold ($P < 0.001$) and 66.8-fold ($P < 0.001$) overexpressed, respectively; *CDR1* and *SNQ2* showed an expression level of 2.9-fold ($P = 0.008$) and 1.3-fold ($P = 0.193$), respectively. These multidrug transporters are regulated by *PDR1* encoded transcription factor, which was also found to be overexpressed – 6.8-fold ($P < 0.001$) (Fig. 1). Therefore, both *C. glabrata* transcriptional factor and related efflux genes were upregulated following the *in vitro* induction assay. To determine whether resistance was associated with mutations: *ERG 11* and *PDR1* genes were sequenced for a single *C. glabrata* isolate. DNA products were sequenced in an ABI Prism 3130 genetic analyzer (Applied Biosystems). A G727A point mutation in *PDR1* gene was found, leading to an aspartic acid to asparagine amino acid substitution at codon 243. *ERG11* analysis revealed several synonymous single nucleotide polymorphisms (SNPs) (Supporting information, Fig. S1).

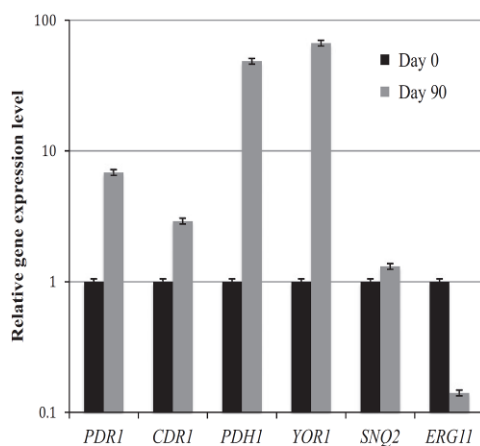

Fig. 1. Gene expression alterations triggered by PCZ exposure in *C. glabrata*. Black bars represent the susceptible initial strain – day 0; gray bars represent the same strain after the induction assay, day 90. Comparative gene expression profile between the initial, day 0, susceptible strain and the strain after the induction assay, day 90. Gene expression is expressed as average with standard deviation of three independent experiments. Each mean value was normalized with the *ACT1* gene.

The concept that the use of azoles in agriculture would not only influence plant pathogenic species but also impair susceptible species of opportunistic human pathogens has gained relevance; such drugs may also have an impact in saprophytic fungal species found in human microbial communities (Snelders *et al.*, 2009, 2012; Verweij *et al.*, 2009; Bowyer & Denning, 2014). In fact, such an imbalance might affect the endogenous population and medically important pathogens. It is generally accepted that a persistent antimicrobial pressure on a complex microbial population will lead to selection of resistant clones. Systemic infections due to *C. glabrata* are characterized by a high mortality rate; they are difficult to treat due to the intrinsically low susceptibility of this species to azole drugs (Pfaller *et al.*, 2003). In addition, *C. glabrata* easily develops fluconazole resistance during patient treatment. In fact, it is now common to find azole-resistant *Candida* isolates from patients not previously exposed to clinical antifungal agents (Pfaller & Diekema, 2004; Pfaller *et al.*, 2004). In our study, in all the three species PCZ MIC value increased from 32- to 64-fold compared to the initial value. However, neither *C. albicans* nor *C. parapsilosis* developed cross-resistance. Anidulafungin activity was not impaired following the selective pressure of an agricultural azole compound, which is not surprising considering that echinocandins have a different mechanism of action. Our results suggest a different perspective on the way *C. glabrata* species develop stable resistance to medical triazoles. Drug efflux, resulting from the increased expression of ABC transporter proteins, is the predominant mechanism by which *C. glabrata* mediates resistance to a wide range of antifungal compounds. Also Pdr1, as the principal regulator of ABC transporter gene expression, has been found to be a key player in such resistance (Bennett *et al.*, 2004; Tsai *et al.*, 2006; Vermitsky *et al.*, 2006; Ferrari *et al.*, 2009). These genetic alterations may transform an intrinsically susceptible to a permanently resistant phenotype. In fact, haploid fungal cells – as is the case of *C. glabrata* – might be more prone to such events (Brockert *et al.*, 2003). We assessed the most common associated genes with azole resistance and found that all ABC transporters were upregulated, as well as their regulatory transcription factor. To our knowledge, this is the first time that *YOR1* was found to have such high expression in a *C. glabrata* azole-resistant strain – it was 66.8-fold overexpressed. Also, previous reports addressing genes involved in azole resistance in *C. glabrata* state that the predominant basis for acquired azole resistance is the constitutively upregulated expression of multidrug transporter genes *CDR1*

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and *PDH1* (Bennett *et al.*, 2004; Sanguinetti *et al.*, 2005; Ferrari *et al.*, 2009). Borst *et al.* (2005) reported a rapid and stable acquisition of azole resistance by *C. glabrata* after an induction assay with FLC; the same ABC transporters were found overexpressed while no contribution of *ERG11* was verified. As previously described, a single-point mutation was found at *PDR1* while *ERG11* only showed the existence of several synonymous SNPs suggesting that this gene was not involved in *C. glabrata* azole resistance in the isolate examined (Sanguinetti *et al.*, 2005; Ferrari *et al.*, 2009) Certainly, additional studies are necessary to address the involvement of such genes in the development of azole cross-resistance triggered by the selective pressure of an agricultural drug.

In conclusion, apart from very few speculative reports published some years ago, there is still no evidence for a clear correlation between the agricultural use of azoles and the increasing clinical azole resistance (Müller *et al.*, 2007; Serfling *et al.*, 2007; Hof, 2008). Nevertheless, our results strongly suggest such possibility and have the merit to put in evidence the molecular mechanisms triggered by such an exposure.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Single Nucleotide Polymorphisms (SNPs) found in *Candida glabrata PDR1* and *ERG11* genes.

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Publication III

(Chapter 4)

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BMC Microbiology

RESEARCH ARTICLE Open Access

Development of cross-resistance by *Aspergillus fumigatus* to clinical azoles following exposure to prochloraz, an agricultural azole

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Abstract

Background: The purpose of this study was to unveil whether azole antifungals used in agriculture, similar to the clinical azoles used in humans, can evoke resistance among relevant human pathogens like *Aspergillus fumigatus,* an ubiquitous agent in nature. Additionally, cross-resistance with clinical azoles was investigated. Antifungal susceptibility testing of environmental and clinical isolates of *A. fumigatus* was performed according to the CLSI M38-A2 protocol. *In vitro* induction assays were conducted involving daily incubation of susceptible *A. fumigatus* isolates, at 35°C and 180 rpm, in fresh GYEP broth medium supplemented with Prochloraz (PCZ), a potent agricultural antifungal, for a period of 30 days. Minimal inhibitory concentrations (MIC) of PCZ and clinical azoles were monitored every ten days. In order to assess the stability of the developed MIC, the strains were afterwards sub-cultured for an additional 30 days in the absence of antifungal. Along the *in vitro* induction process, microscopic and macroscopic cultural observations were registered.

Results: MIC of PCZ increased 256 times after the initial exposure; cross-resistance to all tested clinical azoles was observed. The new MIC value of agricultural and of clinical azoles maintained stable in the absence of the selective PCZ pressure. PCZ exposure was also associated to morphological colony changes: macroscopically the colonies became mostly white, losing the typical pigmentation; microscopic examination revealed the absence of conidiation.

Conclusions: PCZ exposure induced *Aspergillus fumigatus* morphological changes and an evident increase of MIC value to PCZ as well as the development of cross-resistance with posaconazole, itraconazole and voriconazole.

Keywords: Aspergillus fumigatus, Cross-resistance, Clinical and agricultural azoles

Background

The ubiquitous saprophytic mould *Aspergillus fumigatus* is known to cause a spectrum of diseases in humans, including allergic syndromes, noninvasive infections, and invasive aspergillosis, a condition associated with significant morbidity and mortality [1]. *A. fumigatus* is one of the human pathogenic fungi that have a natural habitat in the environment, including soil and plants [2]. Some members of the azole drug class, which includes voriconazole (VRC) and posaconazole (POS), have been shown to be effective in the treatment of invasive aspergillosis [3], and for a long time, azole resistance among clinical *A. fumigatus* isolates was considered to be an uncommon finding. However, multiazole resistance is emerging and is increasingly recognized as a cause of treatment failure [4,5]. In agriculture, thousands of tons of azoles are sold annually for the purpose of plant protection, either to prevent or to control fungal growth that can cause extensive loss of crops or to ease the problem of postharvest spoilage of plants and fruits [6]. The mechanism of action of all azoles - irrespectively of their chemical structure and variable biological properties - is based on its interference with the activity of fungal lanosterol 14 alpha-demethylase, an enzyme encoded by *Cyp51A* gene in *A. fumigatus* that is responsible for the transformation

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of lanosterol in ergosterol, an essential component of the fungal cytoplasmatic membrane. The inhibition of ergosterol formation results in cell membrane disorganization and impairment of fungal growth. Therefore, azoles are considered fungistatic rather than fungicidal, and it is well known that a strong and persistent antimicrobial pressure can lead to the selection of resistant clones, particularly if the drug effect is static rather than microbicidal [7]. Since azoles are the mainstay treatment for both human and agricultural fungal diseases, a major concern is the predictable emergence of cross-resistance to clinical *A. fumigatus* isolates that is already observed in several countries, driven by the massive use of azole fungicides in agriculture, which have the same mechanism of action as those used in humans [7-11]. The aim of our study was to investigate whether Prochloraz (PCZ), an azole extensively used in agriculture, could be associated with the development of cross-resistance to clinical azoles among *A. fumigatus*.

Results and discussion

The three isolates developed a progressive increment of PCZ minimal inhibitory concentrations (MIC) value. In addition, a concomitant increase of the MIC of VRC, POS and Itraconazole (ITZ) was also observed (Table 1). During the induction assay, MIC of PCZ increased 256 times from day 0 until day 30. Concerning the clinical azoles, cross-resistance was developed since all isolates changed from a susceptible to a resistant phenotype, according to Meletiadis and colleagues [12].

Table 1 Susceptibility pattern of tested *A. fumigatus* isolates to Prochloraz and clinical azoles

A. fumigatus isolate	Time of exposure (days)	MIC (mg/L)				
		PCZ	VRC	POS	ITZ	FLC
LMF05	**0**	0.125	0.125	0.25	2	>64
	10	0.25	0.25	0.5	2	>64
	20	8	2	1	4	>64
	30	32	8	2	8	>64
	Ø30	32	2	2	2	>64
LMF11	**0**	0.125	0.25	0.125	0.5	>64
	10	0.125	2	0.25	1	>64
	20	8	8	1	2	>64
	30	32	>16	4	4	>64
	Ø30	32	2	1	0.5	>64
LMN60	**0**	0.25	0.25	0.125	0.25	>64
	10	4	8	0.25	1	>64
	20	8	8	0.5	2	>64
	30	64	>16	4	4	>64
	Ø30	64	2	1	0.25	>64

PCZ, Prochloraz; VRC, Voriconazole; POS, Posaconazole; ITZ, Itraconazole; FLC, Fluconazole; Ø, MIC after 30 days of culture in the absence of PCZ.

There are several studies that have characterized azole resistance in *A. fumigatus*, and most recently some addressed the possible cross-resistance between environmental and medical azoles [8-11]. Our study demonstrated the time frame between the introduction of a widely used agricultural antifungal and the emergence of cross-resistance to medical triazoles. During the induction assay, we found that besides the emergence of cross-resistance, PCZ exposure caused marked morphological colony changes, both macroscopically and microscopically. Macroscopic modification of the pigmentation of *A. fumigatus* colonies, changing from the original green colour to white (Figure 1A, B and C) was remarkable at the beginning of the assay. With the increase of MIC values of PCZ the colonies became scarcer, smaller and totally white (Figure 1C). Microscopic examination showed a progressive absence of conidiation: the original strain (Figure 1A) showed normal microscopic features regarding conidiation (Figure 2A) while almost white colonies (Figure 1B) showed nearly complete absence of conidiation (Figure 2B). The totally white mycelia (Figure 1C) corresponded solely to hyphae and immature little conidiophore structures without conidia (Figure 2C). These changes in pigmentation and in conidiation as a consequence of exposure to azoles have already been reported. Varanasi and colleagues speculate that azoles may bind to a phytochrome-like regulatory molecule inhibiting the initiation and subsequent development of conidiophores in *Aspergillus* species [13]. Such mechanism of action could also explain the different levels of inhibition displayed by other tested azoles and why echinocandins and polyenes did not show this effect [13]. Notably, such morphological changes may be responsible for laboratorial diagnostic misidentification of the fungal genus/species [14]. The high MIC values for PCZ that were achieved *in vitro* maintained stable following removal of the selective pressure of the drug. For VRC, the MIC value decreased only after 30 days of incubation without the selective pressure, changing the susceptibility phenotype from resistant to intermediate. For POS, the developed MIC value also decreased but not enough to change the phenotype of resistance. Regarding ITZ, for both LMF11 and LMN60, it was observed the complete reversibility of the resistant phenotype in the absence of PCZ, ie, the MIC reverted to the initial value (susceptible). However, strain LMF05 had, since day zero, ITZ MIC of 2 mg/L, which falls in resistant category. In all the isolates conidiation reappeared together with the typical green colour of mature colonies following the removal of PCZ.

Since PCZ was responsible for the emergence of stable resistance to itself and to very important medical triazoles in *A. fumigatus*, a resistance mechanism may have been developed. Previous reports describe cyp51A mutation, efflux pump overexpression and/or target upregulation as the main mechanisms responsible for

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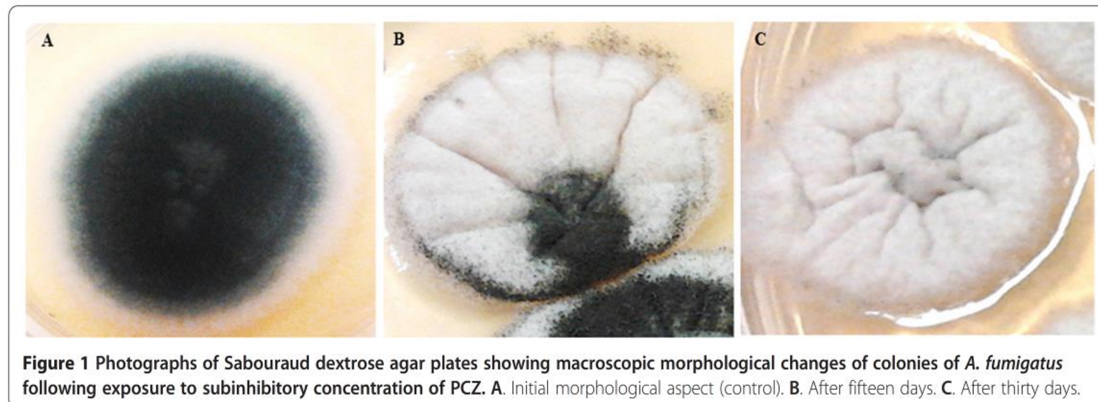

Figure 1 Photographs of Sabouraud dextrose agar plates showing macroscopic morphological changes of colonies of *A. fumigatus* following exposure to subinhibitory concentration of PCZ. A. Initial morphological aspect (control). **B**. After fifteen days. **C**. After thirty days.

such resistance [15-17]. A clonal expansion of isolates harbouring the TR34/L98H mutation has been reported across several countries [15-18]. Interestingly, besides the fact that these resistant isolates are less genetically variable than susceptible ones, no impact on fitness was observed [18]. The phenotypic results (Figures 1 and 2) and the stability of the developed resistance (Table 1) herein reported suggest the same. Future studies aiming to assess the underlying molecular resistance mechanisms, not only from these induced resistant strains but also from isolates with naturally high MIC values to PCZ and resistant to medical azoles without previous *in vitro* induction, will certainly be our next step. Meanwhile, our study suggests that the abuse of azole antifungals in nature may cause serious human health problems since azole-resistance and cross-resistance has the potential to further compromise the efficacy of clinical azoles in the future [4,17-20]. Furthermore, we can speculate that the exposure of clinically relevant moulds other than *A. fumigatus* to agricultural azoles may also be associated with the emergence of cross-resistance to clinical azoles. Several compounds are being tested in order to find new antifungal alternatives, anticipating the possible loss of efficacy of clinical azoles [21]. On the other hand, efforts should be made to find safer compounds to use in agriculture.

Conclusions

In order to assess the real dimension of *Aspergillus* resistance, a susceptibility test should be performed in all isolates from patients with *Aspergillus* infection. Moreover, for patients with severe infection initial combination therapy may be considered in geographical areas with high prevalence of environmental azole resistant isolates. Ultimately, surveillance studies in both clinical and in environment settings should be conducted in order to provide updated local data regarding susceptibility profiles.

Methods

Organisms

Two clinical isolates of *A. fumigatus*, LMF05 and LMF11, and one environmental *A. fumigatus* isolate (LMN60, recovered from a garden nearby the hospital), were used in this study. The isolates were identified as belonging to *A.*

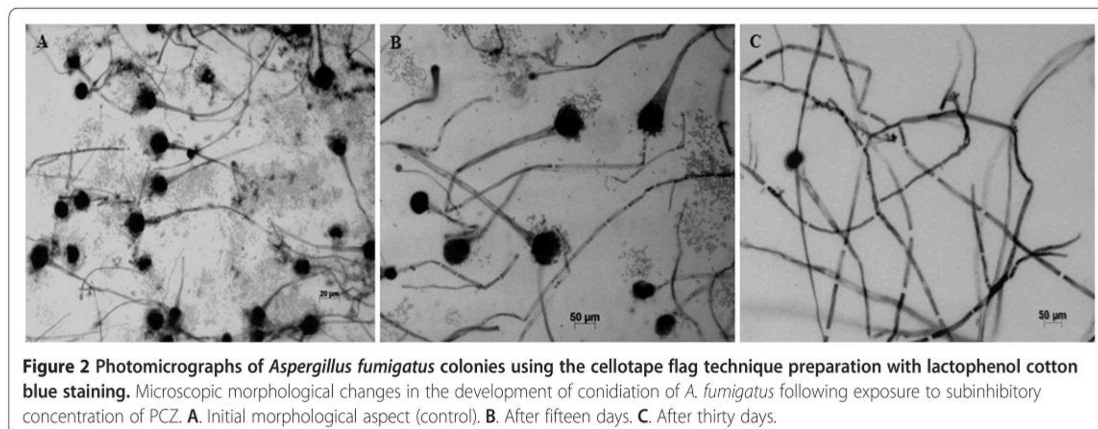

Figure 2 Photomicrographs of *Aspergillus fumigatus* colonies using the cellotape flag technique preparation with lactophenol cotton blue staining. Microscopic morphological changes in the development of conidiation of *A. fumigatus* following exposure to subinhibitory concentration of PCZ. **A**. Initial morphological aspect (control). **B**. After fifteen days. **C**. After thirty days.

fumigatus species by macroscopic and microscopic morphology, the ability to grow at 48°C and by using MALDI-TOF MS to accurately discriminate *A. fumigatus* from a new sibling species *A. lentulus*, which cannot be distinguished by morphological characteristics or growth peculiarities [22]. Long-term preservation of conidial suspensions of the isolates was made in a GYEP medium (2% glucose, 0.3% yeast extract, 1% peptone) broth supplemented with 10% glycerol and stored at −80°C. Working cultures were subsequently maintained during 2 weeks on Sabouraud dextrose agar slants and plates at 4°C.

Antifungal agents and susceptibility profile

PCZ is an imidazole and one of the main drugs used within European Union for crop protection [23]. This ergosterol biosynthesis inhibitor was selected as a representative of agricultural azoles after a previous MIC screening, where it showed to be the less active agricultural drug on the selected strains, ie, it had the lower MIC values, which was a prerequisite for this induction experiment. Fluconazole (FLC), VRC, POS and ITZ were selected as clinical azoles. PCZ was ressuspended in 80% acetone solution at a final concentration of 5 mg/L. Clinical azoles were dissolved in dimethysulphoxide (DMSO) to obtain stock solutions of 10 mg/L. All drugs were stored at -20°C. Broth microdilution susceptibility assay was performed according to the Clinical and Laboratory Standards Institute M38-A2 protocol in order to evaluate the initial MIC of PCZ and of all the clinical azoles [24]. Drug concentration ranged from 0.125 to 64 mg/L of FLC and PCZ; and 0.0313 to 16 mg/L of POS, VRC and ITZ. *A. fumigatus* ATCC 46645 was included for quality control of susceptibility testing. Also, FLC was used as control, since *A. fumigatus* shows a non-susceptible phenotype and MIC is most often above 64 mg/L for this species. MIC of azoles was defined as the lowest concentration of the drug that produced no visible growth following 48 hours of incubation. MIC determination was repeated at least twice.

In vitro induction experiments

Induction experiments were performed with the agricultural azole PCZ. *A. fumigatus* isolates were grown on Saboraud dextrose agar at 35°C for 72 h; conidia were harvested by flooding the surface of the slants with phosphate-buffered saline (PBS) containing 0.025% (vol/vol) tween 80 while gently rocking. The conidial suspensions were then adjusted using specific spectrophotometric readings at 550 nm to a final concentration of $5{\times}10^4$ conidia per militer [25]; one militer of each distinct isolate suspension was transferred to 9 ml of GYEP broth supplemented with sub-inhibitory concentrations of PCZ (0.06 mg/L for both LMF05 and LMF11; 0.125 mg/L for LMN60) and incubated overnight at 35°C with agitation (180 rpm). Daily, after vigorous vortexing for 60 seconds, one militer from each culture was transferred to fresh GYEP medium supplemented with PCZ and in parallel, 1 ml of culture was added with 10% glycerol and frozen at -80°C. This procedure was repeated along thirty consecutive days.

Susceptibility testing/ Stability of *in vitro* developed resistance phenotype

MICs of PCZ were determined every ten days along the thirty days of induction assay. No official breakpoints are yet defined for PCZ; therefore, whenever a marked MIC increase was observed (four fold the initial PCZ MIC), the MIC values of clinical antifungals were determined.

In order to assess the stability of the developed MIC increment to PCZ and of the developed cross-resistance to clinical azoles, the induced strains were afterwards sub-cultured for an additional thirty days in the absence of the drug and MIC values re-determined, as previously described.

Culture macro and micro morphology

Along the induction process, every two days, a loopful was inoculated in Saboraud Agar slants to check for viability and purity of culture. Macro and microscopical growth characteristics were registered. Colony morphology and pigmentation were recorded photographically using a Reflex Nikon D3200 Camera and images were processed by Adobe Photo Deluxe Image Processing Program. Microscopic images of hyphae changes from the original *A. fumigatus* strain and from the resistant induced strain were captured with a Zeiss-Axioplan-2 microscope equipped with Axio Cam. AxioVision 3.0 digital imaging software was used for editing.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IFR, IMM and AGR: conceived the study and designed the experiments; IFR and SF: performed the experiments; IFR, SF, JNM and PRT: analysed the data; IMM, LME, CPV and AGR: Contributed with reagents/material and analysis tools; IFR, SF, IMM and AGR: wrote and revised the manuscript. All authors read and approved the final manuscript.

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Publication IV

(Chapter 5)

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Review

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Genesis of Azole Antifungal Resistance from Agriculture to Clinical Settings

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ABSTRACT: Azole fungal resistance is becoming a major public health problem in medicine in recent years. However, it was known in agriculture since several decades; the extensive use of these compounds results in contamination of air, plants, and soil. The increasing frequency of life-threatening fungal infections and the increase of prophylactical use of azoles in high-risk patients, taken together with the evolutionary biology evidence that drug selection pressure is an important factor for the emergence and spread of drug resistance, can result in a dramatic scenario. This study reviews the azole use in agricultural and medical contexts and discusses the hypothetical link between its extensive use and the emergence of azole resistance among human fungal pathogens.

KEYWORDS: *antifungals, azoles, fungal resistance, Candida, Aspergillus fumigatus, Portuguese agriculture*

■ INTRODUCTION

The use of plant protection products (PPP) may have considerable negative effects on the environment.[1] The use of azole fungicides in agriculture started around mid-1960s; since then, such compounds have been used extensively.[2] In the European Union, half of the total land of cereals and grapevine is treated with azole fungicides. Notably, much smaller quantities of azoles are used in the United States for plant protection.[3]

The azoles used in agriculture are generally sprayed every year over the cultured area to control mildews, rust, and other diseases in cereals, ornamental plants, vegetables, fruits, and vineyards. According to Matthews, the widespread use of these compounds results in contamination of air, plants, and soil with a lot of small particles of fungicide.[4]

The hypothetical risk that the massive use of azoles in agriculture could induce antifungal resistance among human pathogens started to be discussed several years ago. Agricultural azoles could stress fungal species either members of the endogenous saprophytic flora or exogenous to the human body. The imbalance of fungal ecology could thus upset the population of medically important fungi. Human pathogenic fungi may therefore persist and thrive,[5] and in this situation, health risks for humans increase.

To address this problem, in particular regarding the Portuguese situation relative to azole use in agriculture, this review was organized according to the following topics: (1) use of azole fungicides in agriculture, (2) use of agricultural azole fungicides in Portugal, (3) mode of action of azole drugs, (4) hypothetical link between azole use in agriculture and human health, and (5) Portuguese national strategie plan for a sustainable use of PPP.

■ USE OF AZOLE FUNGICIDES IN AGRICULTURE

The growing request for food in quantity/quality has placed a drastic pressure on the productive sector, often at the expense of the natural elements, with progressive reduction or elimination of a pristine landscape. The use of PPP in this context may have strong negative effects on the environment, especially with regard to the possible contamination of surface water and groundwater. Azole compounds are PPP, which are also used for preservation of materials, such as paints, coatings, and wall paper paste, and are routinely applied to mattresses to prevent fungal growth. The most common compounds used in the European Union are imidazoles[6] (Figure 1A),[7] triazoles (Figure 1B),[8] fluconazole Figure 1C),[9] and tebuconazole (Figure 1D).[10] They are widely used in the preharvest phase, grain- and grass-growing environments, and postharvest phase to prevent fungal spoilage[9] by yeasts, such as *Candida* spp., *Cryptococcus* spp, *Rhodotorula* spp., *Trichosporon penicillatum*, and *Saccharomyces cerevisiae*, or filamentous molds, such as *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., and *Geotrichum* spp.[10,11] In addition, they are also applied to treat plant infection when it becomes apparent.

According to the instructions of the manufacturer, doses of 100 g/ha should be used, corresponding approximately to 10 mg of azoles applied to 1 m^2 of the plant surface.[5] Data from the literature reveals that annually nearly 50% of the total acreage under cereal and grapevine production in Europe is treated with azole fungicides.[12] Comparatively, in the United

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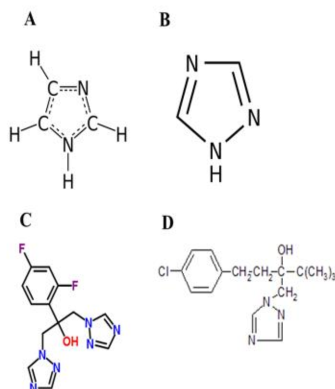

Figure 1. Chemical struture of (A) imidazole, (B) 1,2,4-triazole, (C) fluconazole, and (D) tebuconazole.

States, less than 5% of the total crop area is treated with these compounds. In the European Union, the situation of the Netherlands stands out in particular, because the utilization of azole fungicides has almost doubled since the mid-1990s.[13] Several reasons are usually forwarded to justify the use of azoles in agriculture, in particular, the fact that these compounds are not expensive and that they exhibit broad spectrum activity. However, because azoles are very stable molecules, they can persist active in the soil and water for several months[14] as well as in several fruits and vegetables.[15−17] Although the azole residues detected in many samples do not reach health hazard toxic levels, the amount could vary considerably. Data from the literature show that considerable quantities of azole residues could persist in some food products for a long time.[5]

Farmers have been strongly encouraged to follow guidelines aiming to reduce the probability of developing resistance to agricultural antifungals, to minimize its extent, and/or to increase the chance that any developed resistance would still be reversible. Such practices include the rotating use of antifungal products with different modes of action as well as the preferential practice of a limited number of interventions using higher dosages as opposed to more frequent applications with lower dosages. This does not, however, imply that the use of antifungal substances in agriculture should not be closely monitored, considering the risk of accumulation of azoles in the soils (the half-life time of azoles is usually longer than 1 year) and because the newer compounds may also be at the origin of resistance development.

■ USE OF AGRICULTURAL AZOLE FUNGICIDES IN PORTUGAL

In the particular case of Portugal, there is a real need to promote agriculture and invest in training farmers. According to the results of the Agricultural Census report,[18] the social characterization of the Portuguese family farm population consists of 793 000 individuals representing, 7% of the resident population. The rural population aged considerably between 1999 and 2009; the average age increased from 46 to 52 years. The educational level is rather low; 40% of subjects only attended the first cycle, and 22% have no schooling. However, despite these social indicators, significant improvements were registered during the 10 year period under study; illiteracy rate decreased by 7%, and the frequency of senior secondary cycle and higher education increased by 3%. At the present, the typical profile of the Portuguese farmer nowadays is a 63-year-old male, having just completed the first cycle of basic education, only having practical agricultural training, and working in agricultural activities about 22 h per week.

By December 31, 2012, 907 PPP, based on 248 active substances, were available in Portugal.[19] The largest volume of these substances available at the national market belong to the fungicides group, followed by herbicides and insecticides, representing the remaining products about 15% of the total PPP marketed. According to the Agricultural Statistics report, the ratio between sales of PPP/agricultural area used was in 2008, 2009, and 2010 of 4.6, 3.8, and 3.8, respectively. In the case of the total sales volume being subtracted with the value of sulfur and derivates, this ratio corresponds to values of 1.9, 2.0, and 1.9, respectively.[19]

Sales figures of PPP at the national level constitute an indicator that can provide an estimation about the use of such products in Portugal. The amount of PPP sold in Portugal during 2012 corresponded to a total of 12 462 237 kg, expressed as active substances. The fungicides contributed to about 68.3% of the total of active substances sold, whereas sulfur represented 49.8% of the total sales and 71.4% of all of the fungicides sold. Figure 2 depicts an overview of fungicide sales by chemical group in Portugal between 2002 and 2012. Overall, a decrease in fungicide sales was registered from 2008 until 2012.[20] With respect to azoles, a consecutive decrease in sales was registered between 2002 and 2005; however, after 2006, the values started to increase again, reaching a peak in 2012 (Figure 3).[20]

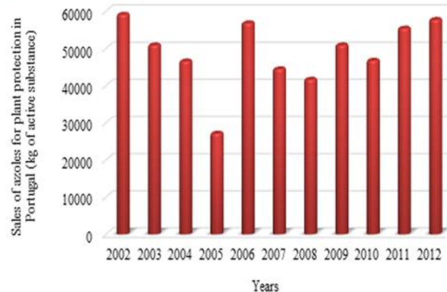

Figure 2. Sales of fungicides in Portugal between 2002 and 2012.[20]

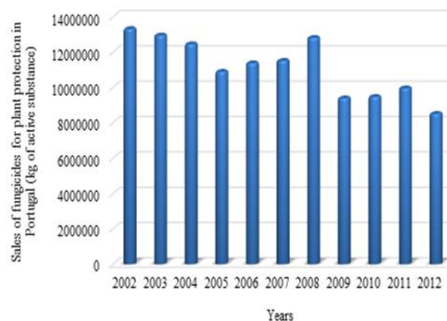

Figure 3. Sales of agricultural azole in Portugal between 2002 and 2012.[20]

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■ MODE OF ACTION OF AZOLE DRUGS

Concerning the mode of action, all of the azole drugs interact and target the same active site in fungal enzyme; thus, agricultural azole fungicides share the mode of action with medical azoles.[21] This results in a fungistatic rather than fungicidal effect; in this sense, the terminology often used in agriculture "fungicides" is ambiguous.

This class of compounds are sterol demethylation inhibitors (SBIs) and impair the synthesis of ergosterol.[5,21] Azole drugs interfere with the activity of fungal lanosterol 14α-demethylase, an enzyme responsible for the transformation of lanosterol in ergosterol. Ergosterol is an important compound of the fungal cytoplasmatic membrane; the impairment of its metabolism induces cell membrane disorganization and decreases fungal growth. Moreover, fungal growth arrest can also result from the accumulation of toxic ergosterol precursors.[22]

The available SBIs–fungicides can be classified into four classes according to their respective target sites, which are membrane-bound enzymes, namely, thiocarbamates or allylamines (squalene epoxidase), inhibithors of C_{14} demethylation or DMIs, amines (Δ^{14} reduction or $\Delta^{8} \rightarrow \Delta^{7}$ isomerization), and hydroxyanilides (C_4 demethylation). However, several resistance mechanisms to SBIs have been described. The two major mechanisms involve either alterations of the target enzyme, which result in a reduced affinity to fungicides, or a decrease of the intracellular drug accumulation, which is linked to an overexpression of membrane transporter proteins, usually, namely, effux pumps.

■ HYPOTHETICAL LINK BETWEEN AZOLE USE IN AGRICULTURE AND HUMAN HEALTH

Several reports claim that antibiotic resistance can be a natural occurrence in the soil. In fact, microorganisms inhabiting soil have to strive for energy sources; it is known that some bacteria and fungi develop the ability to produce antibiotic compounds to destroy their competitors. Such microorganisms are thus naturally immune to the effects of their own excretions.[23]

The development of resistance to azoles among plant fungal pathogens is complex and generally progressing in small steps. First descriptions date from a long time ago and claim that some plant pathogens acquired azole resistance following agricultural exposure.[24] Data from the literature describe resistance or tolerance to triazole fungicides for pathogens of crops, such as wheat, barley, and strawberry.[25] While, in Portugal, resistance to triazoles in vineyard powdery mildew has been detected in the past,[26] still recent studies describe this situation.[27]

Over the last 2 decades, clinically important fungal infections have become more prevalent because medical progress allows for the survival of an increasing number of immunocompromised patients. However, resistance to antifungal drugs also became a critical medical problem.

It has been proposed that there may be a direct relation between the development of resistance to azole fungicides used in agricultural practice and the development of resistance to azole antifungals observed in clinical settings.[28] Exposure of a microorganism to any antibiotic involves a risk of emergence of resistance through the process of selection. The knowledge about the rate and extent of the emergence of resistant organisms[29] and whether this resistance is reversible or not is a very relevant issue to clinical medicine. Resistance depends upon a variety of factors and conditions, including the mechanisms of action of the compound, the target site(s) in the organism and their number, the risk of resistance transference between individual organisms and species, and the mechanism of such resistance.

Resistance in fungi and the mechanisms involved in its development and transmission differ to some extent from those seen in bacteria. There is no evidence that the genes that confer resistance to fungi can be transferred horizontally, a key difference with bacteria. There are two forms of resistance, primary (or intrinsic) and secondary. In primary resistance, the fungal organism cannot be inhibited even by high concentrations of an antifungal drug. For example, *Aspergillus fumigatus* is intrinsically resistant to fluconazole and does not respond at all to this drug. Similarly, *Candida krusei* and *Candida glabrata* isolates are often intrinsically resistant to fluconazole. Conversely, fluconazole resistance in *Candida albicans* can emerge *de novo* during prolonged treatment, which is an example of secondary resistance.[30]

There are at least three different mechanisms by which antifungal drug resistance might arise. They can involve increased efflux of drug, altered target demethylase sites, and availability of alternative pathways for the synthesis of cell membrane sterols. *C. krusei* shows intrinsic resistance to fluconazole largely as a result of the last mentioned mechanism.

There is also evidence that, in clinical settings, the adoption of some therapeutic strategies to treat patients over long periods or to prevent infection through antifungal prophylaxis can result in selection of resistant organisms, of either the same or different species. Several studies have shown that a strong and persistent antimicrobial pressure upon a microbial population will undoubtedly lead to the selection of resistant clones, particularly whenever the antimicrobial agent induces a static effect instead of a biocidal effect.[23] This situation can happen in fungi submitted to azole pressure. Because azoles represent first line clinical antifungals, the topic of azole resistance is of great medical relevance. Azole resistance can thus hypothetically result from exposure to azole compounds, which can happen *in vivo* (azole-treated patients) or as a result of the presence of these compounds in the environment, namely, agriculture use.[31] The massive use of azoles in agriculture could stress susceptible species of the fungal flora, both in nature but also at human endogenous niches. This disturbance in the fungal flora ecology could result in changes among the population of medically important fungi; human fungi pathogens may thus persist and thrive.[5] In this situation, health risks might increase seriously. In addition, new resistance mutations could also develop in the environment. Studies addressing the epidemiology of invasive fungal infections are of high relevance to determine the hipothetical impact of anthropogenic activities upon the susceptibility profile of yeast and filamentous fungi and also to report any emergent species. In Portugal, two studies conducted at the same institution, within a 10 year interval, revealed several relevant differences not only in susceptibility patterns but, most importantly, with regard to species distribution.[29,32]

Invasive aspergillosis is a very dangerous opportunistic fungal infection. The conidia of *Aspergillus* spp. usually become airborne from soil or decaying organic matter, and patients become infected usually by inhalation from such a reservoir.[13] In 2009, Snelders and co-workers provided for the first time evidence that patients with invasive aspergillosis as a result of azole-resistant *A. fumigatus* might have acquired the organism from the environment.[31] According to this study, resistant

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strains of *A. fumigatus* to medical azoles were found in the soil and compost from areas nearby the admission hospital.[31] The dominance of a single resistance mechanism and the genetic homology between the clinical and environmental isolates suggested that the acquisition of azole-resistant isolates from the environment was the most important infection route.

Azole resistance among *A. fumigatus* isolates was not only found in Dutch hospitals;[33] resistant isolates were also detected in several European countries, such as Spain,[34] Belgium,[35] Denmark,[36] Sweden,[37] and France.[38]

Human and animal fungal pathogens, such as *Coccidioides*, *Histoplasma*, *Aspergillus*, and *Cryptococcus*, also thrive in the environment, including plants and food products. It should also be stressed that only a few yeast species are endogenous to the flora of healthy humans; in most occasions, the fungal pathogens are taken up from the environment. Thus, environmental-resistant isolates could have developed antifungal resistance following a prolonged environmental-selective azole pressure. Therefore, the probability that an individual might be exposed to environmental-resistant fungal organisms is real and high. Nevertheless, the stress of residual azoles that might be present in vegetables and other food products upon the endogenous native fungal flora of humans should not be neglected and may also contribute to the emergence of antifungal resistance at endogenous niches.

In the case of *A. fumigatus*, it was concluded that two distinct models of resistance might have developed. One of them occurs following long time exposure to azole treatment, as might occur in clinical settings, supported by several possible genetic mutations, and the other model is facilitated by a single mechanism, TR/L98H mutation, that occurs in environmental isolates under the selective pressure of agricultural azole fungicides. This kind of mutation started spreading worldwide and has nowadays also been described outside Europe.[39] A very recent study conducted in France describes a clinical case of a French farmer who developed invasive aspergillosis caused by an azole-resistant *A. fumigatus* with the TR34/L98H mutation following hematopoietic stem cell transplantation.[40] This farmer had worked in fungicide-sprayed fields from where *A. fumigatus* TR34/L98H isolates were also recovered. This study supports the assumption that humans might indeed be infected with environmental azole-resistant isolates.

Measures to control this problem should be adopted, in particular in the Netherlands, where the TR/L98H mutation became endemic.[5] Interestingly, in Portugal, back in 2007, Araújo et al.[41] compared the antifungal susceptibility profile of different *Aspergillus* species recovered from environmental and clinical sources and found high minimal inhibitory concentration (MIC) levels of clinical antifungals among environmental strains.

Verweij and co-workers reported cross-resistance between azole drugs, which constitutes a very relevant clinical issue, with relatively few therapeutic options available in the case of invasive *Aspergillus* infections.[42] More recently, several studies demonstrated the development of cross-resistance between agricultural and medical azoles.[13,21] Faria-Ramos and co-workers[43] clearly demonstrated that exposure of clinically relevant molds and yeast organisms to azoles used in agriculture does result in the emergence of cross-resistance to clinical azoles, which may not be reversible.

Additional research efforts aiming to reduce the emergence of fungicide resistance are urgently needed, in both agriculture and medical settings. Because the mechanisms providing the basis for fungicide resistance are likely to be conserved between plant and human pathogens, plant pathologists and medical microbiologists should develop effective strategies counteracting the development of antifungal resistance, in a concerted action.

■ PORTUGUESE NATIONAL STRATEGIE PLAN FOR A SUSTAINABLE USE OF PPP

The Directive no. 2009/128/EC is an innovation in the context of the European Community legislation about PPP; for the first time, legislation was provided about the use of PPP, to protect human health and the environment from possible risks inherent to its use.

To operationalize the implementation of this Directive at the national level, the law no. 26/2013 was published; it regulates the distribution, sales, and application of PPP by professional users as well as adjuvants for PPP and also sets the monitoring procedures for the use of PPP. Together with the law no. 86/2010 (which establishes a mandatory inspection scheme of PPP and its application using equipment authorized for professional use), it constitutes the transposition into national legal order of that European Community law.

The article 51 of this law envisaged the creation of National Action Plans. These plans aimed at reducing the risks and effects of the use of PPP on human health and the environment and to promote the development of integrated protection technical alternatives to reduce dependence of the use of conventional PPP.

The Portuguese National Plan has been prepared with the collaboration of various agents, from either public or private sector, without which would not have been possible to achieve the intended goals. It must also be pointed out that the implementation of this plan will only be effective with the committed collaboration of all of the agents involved in manufacture, storage, sale, and use of PPP as well as those responsible for managing the effects and risks associated with the use of PPP. The correct application of PPP can be encouraged by the training of professionals, appropriate counselling at the point of sale, and monitoring/supervision of compliance by professional users. In the act of sale, adequate information about the use of PPP and the risks and safety instructions regarding human health should be provided to buyers, to enable the adequate management of the risks inherent to the products concerned.

The protection of professionals configures primarily a matter of safety and health regarding the professional handling field use and application of pesticides. Risks may include not only acute intoxication but also chronic and subchronic risks resulting from prolonged exposure. The lines of action chosen for this intervention area are (i) protection of professional users (commercial and storage settings), (ii) reduction of exposure, and (iii) limitation of the use of certain PPP.[44]

The main measure of protection regarding non-professional users is restricting its access to a limited category of PPP. In any case, buyers should be provided awareness of the risks associated with PPP use and about promotion of good general practices regarding its use.

To assess the progress made in reducing the risks and adverse effects of the use of PPP, to both human health and the environment, monitoring plans should be in place so that, on the basis of a sample of professional users and predefined criteria, it will be possible to assess the progresses resulting the

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implementation of the general principles of integrated protection.

In addition, it should be emphasized the relevance of the contribution of scientific research and field application of the most recently available scientific and technological information in improving resources and instruments available to the professional users, including complementary or alternative means of use of PPP.

In conclusion, fungicide-based plant protection is at present indispensable for efficient and large-scale crop production. Notably, modern fungicides should exhibit negligible acute toxicity, and the regulations for fungicide application should ensure the availability of safe food. Because long time periods and enormous financial investments are required to develop new fungicides, serious efforts must be taken to prevent emergence of fungicide resistance. This is particularly important because fungicides with novel modes of action are rarely found and resistance to single-target fungicides may occur within the first few years of its field use.

The key to prevent or at least delay the onset of development of fungicide resistance in agriculture is the preparation of efficient fungicide mixtures as well as the rotation between fungicides with different modes of action during a crop campaign. The recommendations are to maintain highly efficient fungicides in the market, precluding the use of monofungicides, to keep its use above a threshold allowing for quantitative resistance to develop. The strategy adopted during the last few decades was to apply single-target fungicides, resulted in a strong selective pressure for mutants with a modified fungicide-binding site. Because fungicides with more than one target are not easy to overcome by mutations, the focus in fungicide screening should also be directed toward identifying multi-site inhibitors. After having such fungicides available, the risk of rapid occurrence of qualitative resistance might be strongly and effectively reduced. Nevertheless, particular attention should be devoted to the assessment of the development of cross-resistance to clinical antifungals, strict monitoring of the emergence of fungal strains with resistance mechanisms, and variations in species distribution regarding both plant and human pathogens, which should be implemented.

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