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Change in the clinical antifungal sensitivity profile of *Aspergillus flavus* induced by azole and a benzimidazole fungicide exposure

Leandra Martins Meireles, Mariceli Lamas de Araujo, Denise Coutinho Endringer, Marcio Fronza, Rodrigo Scherer *

Pharmaceutical Sciences Graduate Program, University of Vila Velha, Espírito Santo, Brazil

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ABSTRACT

The study evaluated the change in the clinical antifungal sensitivity profile of *A. flavus* strains after exposure to azole and benzimidazole fungicide. Exposure to fungicide altered the sensitivity profile for the antifungal itraconazole, voriconazole and posaconazole. This change was characterized by an increase in the minimum inhibitory concentration (MIC) from 16 to 32 times, evidencing the development of resistance phenotypes. The most significant changes were found after exposure to a pool of the fungicide with MIC of up to 256 times, which is considered, to the best of our knowledge, the first case report of such a high level of resistance induced by azole fungicide exposure. This observation probably indicates a synergistic action among azole compounds that potentiates the development of resistance phenotypes. In addition, exposure to fungicide changed the pigmentation of the colonies from green to white. The development of resistance to fungicides represents risks to human health, since azole fungicides are used widely in the agriculture, and a single agricultural fungicide spray often includes more than one azole compound.

1. Introduction

Aspergillus flavus has a wide environmental distribution favored by the formation of conidia that are highly tolerant to adverse conditions and is also considered an important pathogen that is responsible for several diseases in humans (Hedayati et al., 2007; Natesan et al., 2013). The primary route of infection is characterized by the inhalation of conidia, which, due to their small size, easily reach the pulmonary alveoli and cause local and invasive infections (Hedayati et al., 2007; Paulussen et al., 2017). It is responsible for a broad spectrum of diseases in humans (from hypersensitivity reactions to invasive infections), the type of infection depends on the individual's immune status (Garcia-Rubio et al., 2017). In immunocompromised patients, invasive aspergillosis is associated with high morbidity and mortality, with *A. flavus* the most common non-fumigatus species (accounting for 10% to 20% of the cases) (Amare and Keller, 2014; Krishnan et al., 2009), and responsible for refractory and severe infections that involve several organs (Hedayati et al., 2007; Krishnan-Natesan et al., 2008; Natesan et al., 2013). *A. flavus* also an important crop associated pathogen (such as corn and peanut). The control of this fungus is traditionally carried out with fungicides, since the contamination of food by this fungus poses a great risk to human health by being a producer of mycotoxins (Lv et al., 2018).

Aspergillosis therapy is comprised since the latest generation of azole antifungals, such as voriconazole and posaconazole, has lost efficacy (Garcia-Rubio et al., 2017; Hagiwara et al., 2016; Parker et al., 2014; Patterson et al., 2016). The overuse of azole antifungals in prophylaxis and infection control has led to the emergence of resistance (Liu et al., 2012; Natesan et al., 2013; Paul et al., 2015). However, the recovery of resistant strains in azole-naïve patients and the isolation of resistant strains in the environment indicated a new route of resistance development, possibly of environmental origin. This new route of resistance has been associated with the extensive use of azole fungicides in agricultural crops to treat and prevent damage caused by plant pathogens (Chowdhary et al., 2012; Parker et al., 2014; Pham and Lockhart, 2012), since azoles are a class of compounds used in both agriculture and clinical medicine (Garcia-Rubio et al., 2017). Fungicides azoles are widely used in several countries, in this context, species of *Aspergillus*, which are ubiquitous in nature, undergo strong selective pressure, which reduces the population of sensitive strains and selects for resistant genotypes (Garcia-Rubio et al., 2017; Niels Kleinkauf et al., 2013).

Azole compounds inhibit ergosterol biosynthesis by binding to the enzyme lanosterol 14α-demethylase and inhibiting the production of the sterol (Garcia-Rubio et al., 2017). As a consequence, the cell membrane loses stability, which impairs the growth and replication of the fungus. The occurrence of azole-resistant *Aspergillus* strains has been reported in several parts of the world to be increasing (Liu et al., 2012; Natesan et al., 2013; Pfaller et al., 2011; Pham and Lockhart, 2012). However, studies to evaluate azole resistance in *A. flavus* are sparse

* Corresponding author. Tel.: +55-27-3421-2198; fax: +55-27-3421-2049.
E-mail address: rodrigo.scherer@uvv.br (R. Scherer).

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(Krishnan-Natesan et al., 2008; Liu et al., 2012; Natesan et al., 2013). Aiming to identify strains with reduced sensitivity, epidemiological cutoff values (ECVs) are established based on minimum inhibitory concentration (MIC) assays (Meletiadis et al., 2012). It is important to emphasize that the occurrence of resistance severely limits treatment options and generates great concern in the medical/scientific community, as well as devastating consequences for the world's population health (Fairlamb et al., 2016).

Considering the relevance of infections caused by *A. flavus* and the importance of azole compounds in the treatment of these fungal infections in humans, along with the use of azole fungicides in agriculture, the objective of the present study was to evaluate whether the exposure of strains of *A. flavus* to azole fungicides change the sensitivity profile of these strains to clinical antifungals. The results of the present study revealed that the exposure of these strains to azole fungicides resulted in altered the sensitivity profile to the clinical antifungals. The exposure to a combination of fungicides had even more evident effects reducing the azol sensitivity.

2. Materials and methods

2.1. Microorganisms

Two of the strains of *A. flavus* used in this study were isolated from coffee (green beans) samples, CCDCA 1043 and CCDCA 1049, and donated by the Federal University of Lavras (MG, Brazil), and another one, ATCC 204304 (INCQS 40182), was provided by FIOCRUZ-INCQS (RJ, Brazil).

2.2. Determination of the minimum inhibitory concentration

The sensitivity test was performed according to the CLSI (Clinical and Laboratory Standards Institute) reference protocol M38-A2 (CLSI). The antifungals itraconazole (ITZ), voriconazole (VCZ) and posaconazole (POS) and the fungicides thiabendazole (THI, benzimidazole/nuclear division inhibitors, was chosen to evaluate the cross-interaction between fungicides of different groups in the sensitivity profile), tebuconazole (TEB, azole/demethylation inhibitors) and metconazole (MET, azole/demethylation inhibitors) (Sigma-Aldrich, St. Louis, USA) were used.

The antifungal concentrations ranged from 0.03 to 16 μg/mL. The inoculum was prepared by the spectrophotometric method (530 nm and OD 0.09 to 0.13) to obtain a final concentration between 0.4×10^4 CFU/mL and 5×10^4 CFU/mL. The MIC values were determined after 48 h of incubation at 35 °C by visual reading with the aid of a reading mirror. The lowest concentration that resulted in complete inhibition of the growth of the microorganism compared to the control (microorganism without the drug) was considered the MIC value. The results were validated with the strain *A. flavus* ATCC 204304, which is sensitive. The experiments were performed in duplicate.

2.3. In vitro fungicide exposure experiments

The assay was adapted from the study by Faria-Ramos (2014) for the THI, TEB and MET fungicides active ingredients. One milliliter of the fungal inoculum was added to Erlenmeyer flasks containing 99 mL of YPD broth. Sub-inhibitory concentrations of the fungicides were added separately to the culture medium. In an additional treatment, the fungus was exposed to the fungicides pool (mixture of the three active ingredients). The exposure of the fungal strains to the individual fungicides and fungicide mixture was conducted for 28 days. The initial concentration of fungicides and fungicides pool was 25% of the MIC value described in Table 1 at days zero to 14; the concentration was increased to 35% at days 14 to 21; and to 45% of the MIC value at days 21 to 28. The Erlenmeyers flasks were incubated in an orbital shaker at 35 °C and 180 rpm. After the exposure period, the microorganism was cultivated in YPD broth for 28 days in the absence of fungicides (washout) under the same conditions of temperature and agitation.

Table 1
MIC for clinical antifungals and agricultural fungicides prior to the induction period.

	MIC (μg/mL)					
	Antifungals			Agricultural fungicides		
Strain	ITZ	VCZ	POS	THI	TEB	MET
ATCC 204304	0.25	0.5	0.0625	8.0	1.0	0.5
CCDCA 1049	0.0625	0.125	0.0625	4.0	2.0	1.0
CCDCA 1043	0.125	0.5	0.0625	8.0	2.0	1.0

The culture medium was renewed every 7 days to avoid overloading the concentration of azole fungicide added to the broth, which could influence the results (the same occurred in the washout period). Furthermore, during the period of exposure to fungicides and in the washout period, the strains were collected every 72 h to ensure the viability of the fungus.

The determination of the MIC values for the antifungals was carried after 7, 14, 21, and 28 days of exposure to evaluate the change in the sensitivity profile. For this assay, a sample of the cultured microorganism in YPD broth was transferred to potato agar and the sensitivity test were also performed according to the CLSI reference protocol M38-A2 (CLSI). After the washout period, the sensitivity test was performed to evaluate the stability of the changes. The sensitivity test was performed in duplicate.

2.4. Macromorphological characteristics

The analysis was performed after 28 days of exposure to the azole fungicides to identify the occurrence of possible changes. After the exposure period, 100 μL of inoculum was spread onto potato agar plates and incubated at 35 °C for 7 days to evaluate possible changes in the color, shape and texture.

3. Results

Currently, few studies report the epidemiological cutoff values (ECVs) for *Aspergillus*. In addition, the ECVs have not been established by the CLSI for new azoles such as VCZ and POS. Therefore, the values reported by Meletiadis (2012) were used for the identification of resistant strains of *A. flavus*: 1.0 μg/mL for ITZ and VCZ and 0.125 μg/mL for POS.

3.1. Thiabendazole exposure

All strains studied showed a change in the sensitivity profile for ITZ, VCZ and POS after initial 7th day exposure to thiabendazole, with the exception of the ATCC strain for which the alteration in sensitivity only occurred for ITZ and POS after the 14th day of exposure (Fig. 1). The MIC value for the ATCC strain increased 4-fold, exceeding the ECV (MIC ≫1.0 μg/mL), characterizing the development of resistance phenotype to VCZ (Fig. 1B). In addition, this MIC increase remained in the washout period, indicating that the resistance phenotype may be irreversible. In addition, all strains showed a resistance phenotype for POS (MIC ≫0.125 μg/mL) during exposure time and in the washout period, with an increase in the MIC values of up to 16-fold (Fig. 1C). These results suggest the development of cross-resistance in the ATCC strain for VCZ and in all isolates for POS. Although there was no change in the resistance phenotype of any strain for ITZ, an increase in the MIC values of up to 8 times during exposure and in the washout period was observed (Fig. 1A).

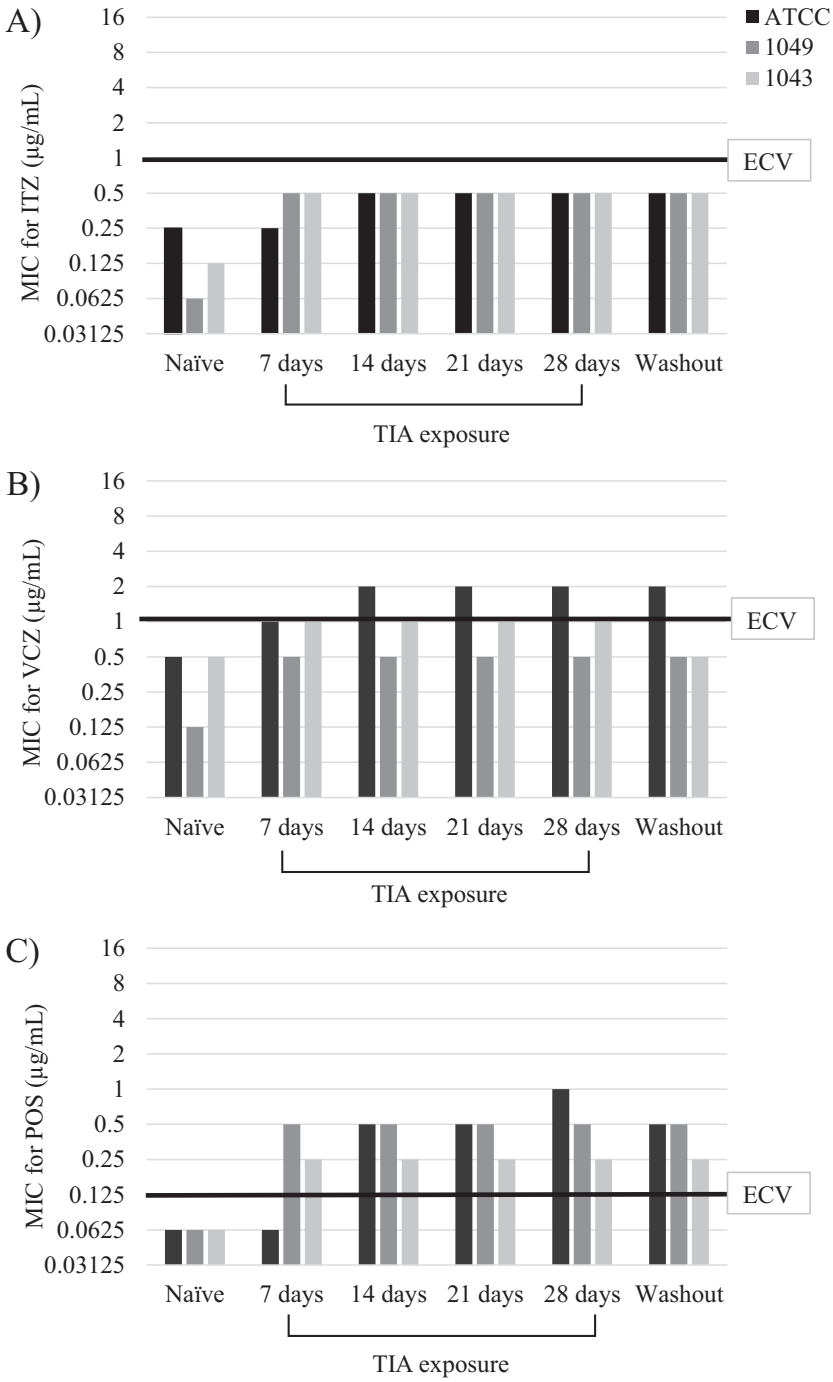

Fig. 1. Changes in the *A. flavus* antifungal sensitivity profile induced by exposure to the fungicide thiabendazole (THI, a benzimidazole). Minimum inhibitory concentration (MIC) for (A) itraconazole (ITZ); (B) voriconazole (VCZ) and (C) posaconazole (POS) under three conditions: naïve (before exposure), after 7–28 days-exposure to increasing concentrations of THI and in the washout treatment.

3.2. Tebuconazole exposure

All isolates showed a reduction in sensitivity to ITZ, VCZ and POS from the 7th day of exposure to TEB. However, the ATCC strain showed a reduction in sensitivity to ITZ only from day 14th (Fig. 2). The MIC value for the CCDCA 1043 strain increased 4-fold and corresponded to a resistance phenotype (MIC ≫1 µg/mL) for VCZ during exposure to the fungicide (Fig. 2B), but this phenotype was not maintained in the washout period. For POS, all the isolates showed an evident reduction in sensitivity (MIC ≫0.125 µg/mL) (Fig. 2C). The MIC values increased up to 32-fold, characterizing a resistance phenotype. All strains maintained a resistance phenotype in the washout period, suggesting the development of cross-resistance between the fungicide TEB and the antifungal POS (Fig. 2C). Although there was no change in the phenotype of any tested strain for ITZ (Fig. 2A), an increase in the MIC values of up to 8 times during exposure and in the washout period was observed.

3.3. Metconazole exposure

All isolates showed a reduction in sensitivity to ITZ, VCZ and POS, but again the ATCC strain showed a change in sensitivity to ITZ from

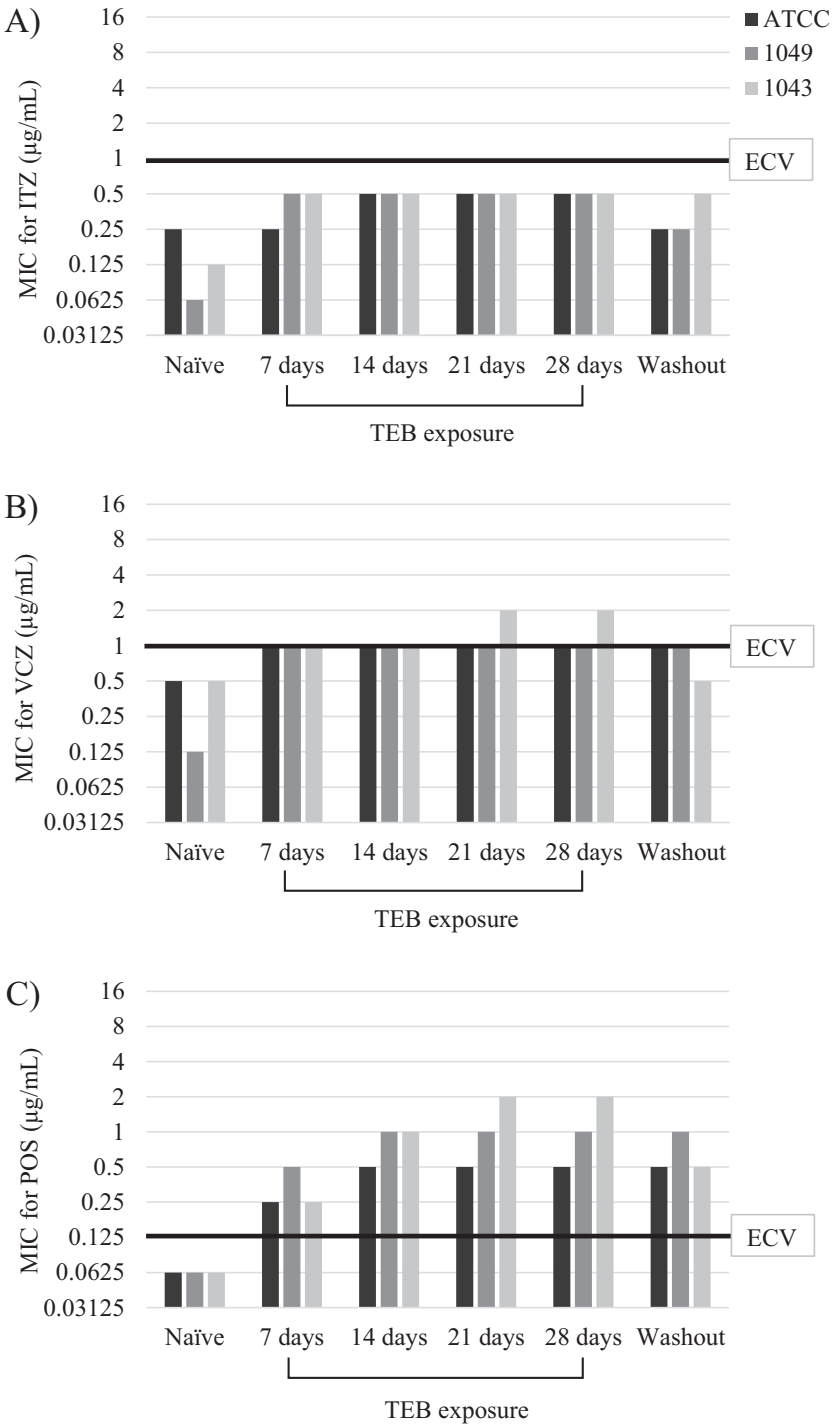

Fig. 2. Change in the *A. flavus* antifungal sensitivity profile induced by exposure to the fungicide tebuconazole (TEB, an azole). Minimum inhibitory concentration (MIC) for (A) itraconazole (ITZ), (B) voriconazole (VCZ) and (C) posaconazole (POS) under three conditions: naïve (before exposure), after exposure to increasing concentrations of TEB and in the washout treatment.

the 14th day (Fig. 3A). For the ATCC and CCDCA 1043 strains, the MIC value (MIC ≫1 µg/mL) for VCZ increased up to 4-fold, which corresponded to a resistance phenotype. However, the phenotype was not maintained in the washout period (Fig. 3B). For POS, all strains showed an increase in the MIC value (MIC≫0.125 µg/mL) of up to 32 times, characterizing a resistance phenotype during exposure and in the washout period and suggesting the development of cross-resistance between MET and POS (Fig. 3C). For ITZ, the isolates showed no change in the sensitivity phenotype during exposure. However, an increase of up to 16-fold in the MIC value was observed (Fig. 3A).

3.4. Exposure to the fungicide pool (thiabendazole + tebuconazole + metconazole)

In addition, the exposure of the strains to the combination of all the tested fungicides was carried out to evaluate the occurrence of synergism in their interactions. All strains showed changes in their sensitivity

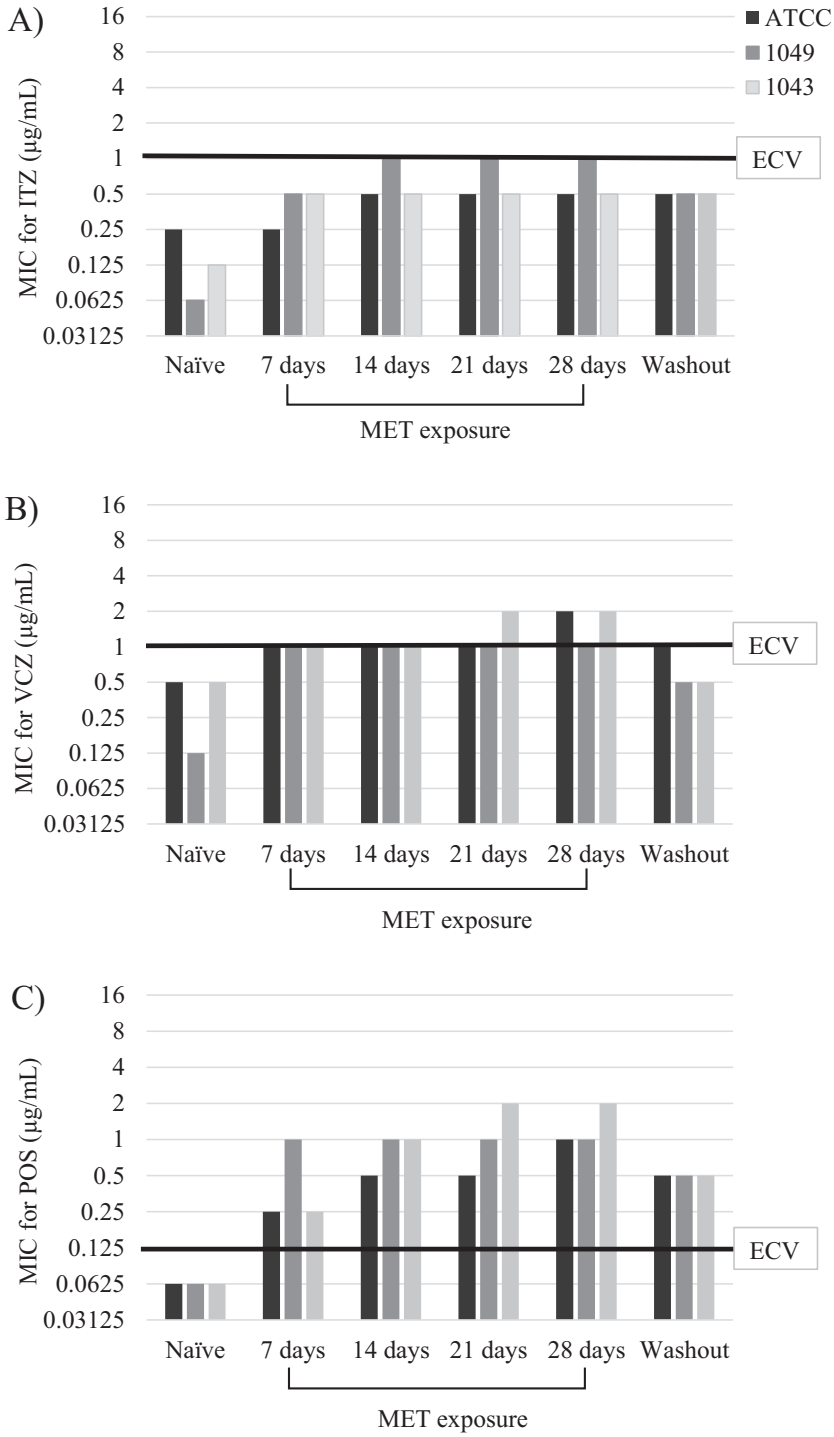

Fig. 3. Change in the *A. flavus* antifungal sensitivity profile induced by exposure to the fungicide metconazole (MET, an azole). Minimum inhibitory concentration (MIC) for (A) itraconazole (ITZ); (B) voriconazole (VCZ) and (C) posaconazole (POS) under three conditions: naïve (before exposure), after exposure to increasing concentrations of MET and in the washout treatment.

profile. For ATCC strain, the change occurred from the 14th day for ITZ (Fig. 4). For VCZ, MIC values of up to 32 times higher for the ATCC strain were observed during exposure to the fungicides pool. However, in the washout period, all strains showed MIC values below the ECV, and the changes in the sensitivity profile were reversible (Fig. 4B). For POS, MIC values of up to 256 times higher than those of the naïve strains (MIC = 16 µg/mL) were observed. Despite a small reduction in the MIC value, all strains-maintained MIC values for POS above the ECV after the washout period. This indicated a synergistic action that may potentiate the change in the sensitivity profile (Fig. 4B and C**).** For ITZ, less conspicuous effects were observed (Fig. 4A**).**

3.5. Macromorphological changes

After the fungicide exposure period, in addition to the change in the sensitivity profile, a change in colony color was observed as the pigmentation changed from green to white (Fig. 5). The colonies remained flat and grainy.

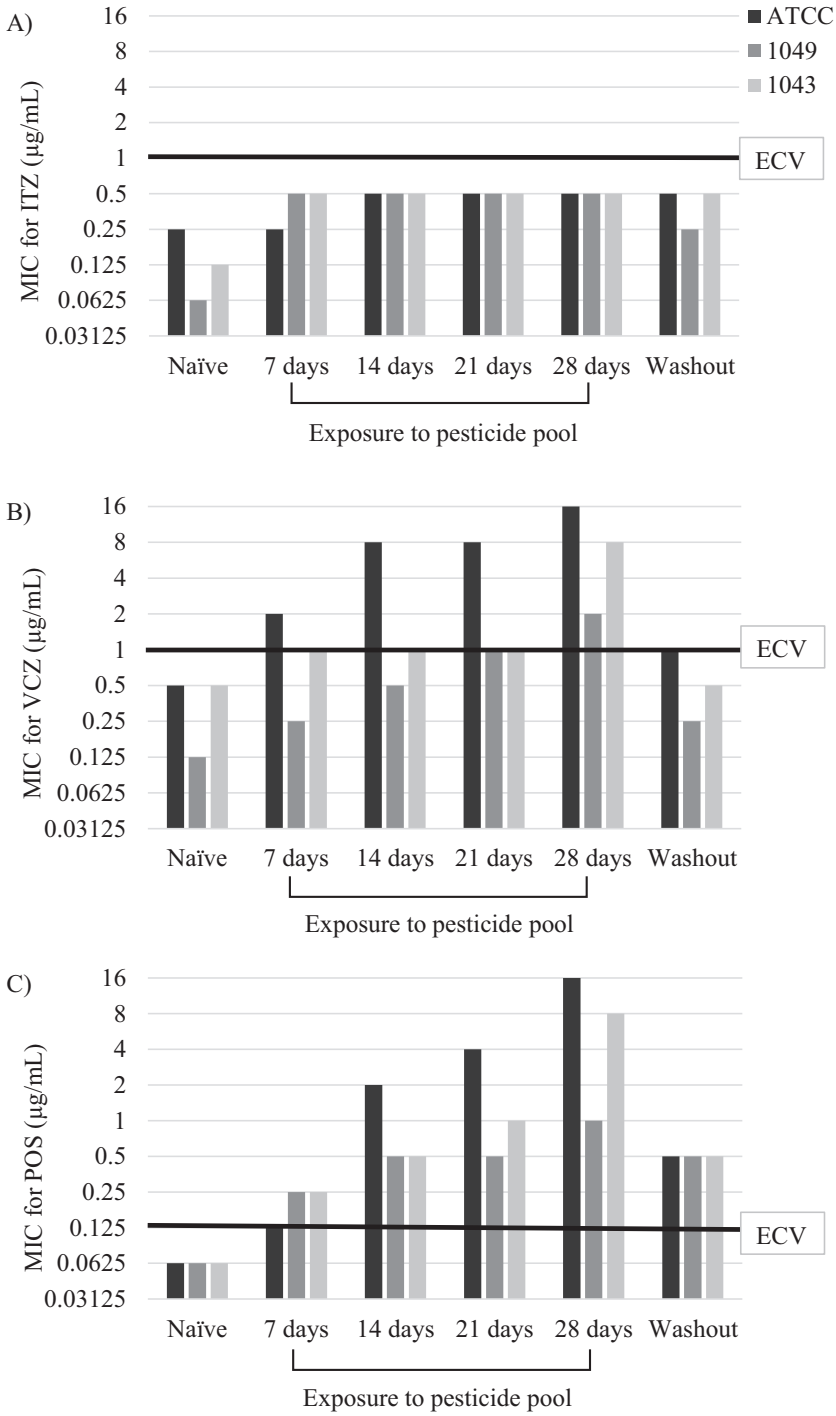

Fig. 4. Change in the *A. flavus* antifungal sensitivity profile induced by exposure to the fungicides pool. Minimum inhibitory concentration (MIC) for (A) itraconazole (ITZ); (B) voriconazole (VCZ) and (C) posaconazole (POS) under three conditions: naïve (before exposure), after exposure to increasing concentrations of the fungicides pool and in the washout treatment.

4. Discussion

The origins of clinical antifungal resistance is thought to be associated with the use of azole fungicides in the environment (Brent and Hollomon, 2007; Parker et al., 2014; Pham and Lockhart, 2012). Studies suggest that this route of antifungal resistance origin has emerged as the main cause of azole resistance in *A. fumigatus*. Additionally, *A. flavus*, which is the most common non-fumigatus species in invasive aspergillosis, is also an important mycotoxin-producer plant pathogen, posing a high risk for food safety (Alastruey-Izquierdo et al., 2013; Farmakiotis and Kontoyiannis, 2017; Pappas et al., 2010; Parker et al., 2014; Taccone et al., 2015). However, studies to evaluate the emergence of azole resistance in *A. flavus* are sparse, especially in Brazil (Krishnan-Natesan et al., 2008; Liu et al., 2012; Natesan et al., 2013).

It is known that azole fungicides account for approximately a third of fungicides sales and that 99% of these fungicides are used in agriculture. Azole fungicides present high efficiency, broad spectrum and are an important example of drugs capable of selecting resistant strains because of their unique mechanism of action and the good chemical stability of these molecules in the environment (Chen et al., 2014; Chowdhary et al., 2013; Parker et al., 2014).

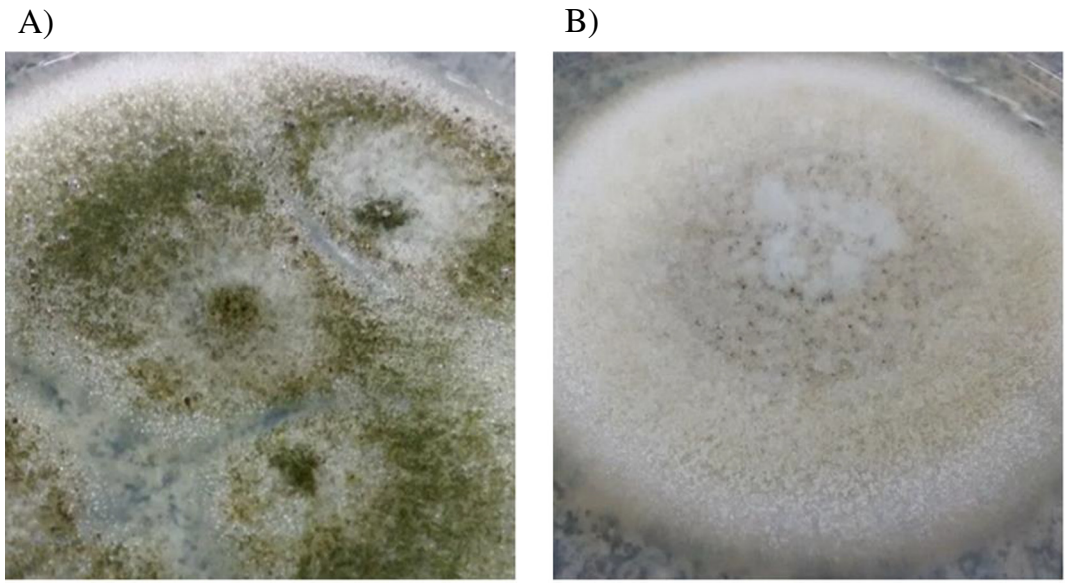

Fig. 5. Potato agar plates with *A. flavus* (ATCC 204304) growth showing macromorphological changes in the colonies after exposure to the fungicides pool. **A.** *A. flavus* (ATCC 204304) control colonies. **B.** *A. flavus* (ATCC 204304) colonies after 28 days of exposure.

It is also important to consider the structural similarity between azole fungicides and clinical antifungals. Previous study (Chowdhary et al., 2013) confirms the structural similarity between clinical antifungals (ITZ, VCZ and POS) and azole fungicides, besides the conformation and docking to the target enzyme in *Aspergillus*. The structural similarity among these molecules is related to the presence of a halogenated aromatic ring near the azole ring (Snelders et al., 2012). This structural similarity is probably the cause of the alterations that were found in the MIC values for clinical antifungals tested here and the resulted in changes in the sensitivity profile of *A. flavus* after exposure to TEB and MET fungicides. The halogenated radical of these compounds is located near the azole ring for these fungicides. The distance between the halogenated radical and the azole ring could justify the differences found in MIC value when the fungus was exposed to TEB and MET. Additionally, the halogen bound to the aromatic ring could influence the binding affinity with the enzyme, a fact that could explain why the alterations in the MIC values found for VCZ and POS (fluorine) were different from those observed for ITZ (chlorine). TEB and MET fungicides inhibit sterol biosynthesis by binding to the enzyme CYP51A (Parker et al., 2014), while as the fungicide THI (benzimidazole) inhibits nuclear division through binding to the tubulin protein (Zhou et al., 2016). The difference in the mechanism of action could explain the difference in sensitivity profile. Additionally, the exposure to fungicides pool potentiate these changes.

The exposure of *A. flavus* to azole fungicides and the subsequent changes in the sensitivity profile to clinical antifungal agents are associated with risks to human health. POS is a second-generation clinical antifungal (available since 2006) (Dekkers et al., 2016), and changes in the sensitivity profile was observed for this antifungal when the microorganism was exposed to all the fungicides studied. VCZ is considered the drug of choice for cases of *Aspergillus* infection (Natesan et al., 2013). However, for this clinical antifungal, changes in the sensitivity profile was also observed after exposure to fungicides, with an increase in the MIC value of up to 32 times. The changes in the sensitivity profile for *A. flavus* were time-dependent. The strains CCDCA 1049 and CCDCA 1043 presented changes in less time, possibly already exposed to fungicides in the environment from where they originated (coffee plantation). This occurrence would further limit therapy with azole in cases of clinical important infection.

Among the mechanisms of resistance already studied for *A. flavus*, the literature reports that mutations in the gene encoding the target enzyme (Krishnan-Natesan et al., 2008). However, other mechanisms may be involved as overexpression of enzyme as a consequence of positive regulation of the *cyp51A, cyp*51B and *cyp*51C genes and increased efflux of azole compounds from the cell due to increased expression of membrane transporters (Parker et al., 2014; Paul et al., 2015; Sharma and Chowdhary, 2017).

Krishnan-Natesan (2008) indicated that 77% of VCZ resistance *A. flavus* isolates lacked *cyp*51A alterations. This observation suggested that other mechanisms including resistance efflux pumps may be associated with azole resistance. In this study, while for POS the change in sensitivity was maintained after exposure to the fungicide pool, in contrast the change in the VCZ sensitivity profile was not maintained during the washout period. Such observation indicates that another mechanism may be involved in the resistance perhaps related to the functioning of the efflux pumps during the exposure.

In the present study, macromorphological alterations were also observed due to exposure of the microorganism to fungicides, corroborating previous studies (Faria-Ramos et al., 2014a, 2014b). Previous study (Griffith et al., 1994) reported the involvement of a molecule homologous to phytochromes involved in the initiation and development of conidiophores in *Aspergillus* species. Azole compounds bind to this molecule and inhibit this process. However, the binding affinity is variable and may explain the difference in inhibition exhibited by the compounds. Macromorphological alterations may be responsible for the incorrect identification of the microorganism, resulting in consequent diagnostic errors in addition to compromising the therapy (Fig. 5).

Considering that a reduction in *in vitro* sensitivity may be associated with therapeutic failure and that more than one resistance mechanism may be involved, it is important to note that further studies should be performed to understand the differences found in the sensitivity profiles and to explain why some fungicides are better inducers of sensitivity changes than others, and to determine whether a longer period of exposure to fungicides can result in definitive changes in sensitivity. Some limitations were present in this study, for example: few strains studied, genotype of resistance and the amphotericin B activity after resistance development was not evaluated. Additionally, a large-scale environmental and human population based assay about the diversity in *A. flavus* clinical antifungals and fungicide sensitivity is also important to be conducted.

5. Conclusion

The results of the present study revealed that the exposure of *A. flavus* strains to agricultural fungicides resulted in change in the sensitivity profile to the antifungal agents VCZ and POS. In addition, exposure to a combination of the three fungicides (the benzimidazole THI, and the azoles TEB and MET) enhanced the effects of the development of resistance (increase in the MIC value of up to 256 times). Further studies should be performed to investigate possible changes in the expression of efflux pumps and genes encoding the target enzyme of azoles. This study serves as a warning for public agencies and regulatory agencies to implement more rigorous control over the use of azole fungicides in the environment and for the medical-scientific community, since therapy for infections caused by *Aspergillus* is limited.

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Declaration of conflict of interest

The authors declare no conflict of interest.

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