Exposure to the agricultural fungicide tebuconazole promotes Aspergillus fumigatus cross-resistance to clinical azoles. [Comini S. is the co-first author; Banche G. is the corresponding author; Cuffini A.M. is the co-corresponding author]

Full paper

Exposure to the agricultural fungicide tebuconazole promotes *Aspergillus fumigatus* cross-resistance to clinical azoles

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SUMMARY

Resistance to clinical triazoles in *Aspergillus fumigatus* is a growing concern for individuals at high risk of *Aspergillus* infection. Two triazole resistance selection routes are currently being investigated: one occurring in triazole-treated patients in healthcare settings, and the second taking place in the environment due to the widespread use of agricultural triazoles. This study aimed to assess the ability of agricultural azoles to promote cross-resistance to clinical azoles in *A. fumigatus*. Five *A. fumigatus* isolates susceptible to clinical azoles were exposed to the triazole 14α-demethylase inhibitor, tebuconazole (TBC), and then antifungal susceptibility tests for voriconazole, itraconazole, posaconazole and isavuconazole were performed. Under TBC selection pressure, all *A. fumigatus* isolates exhibited resistance to clinical triazoles. However, only two displayed a multiresistant phenotype to clinical azoles. TBC exposure was also associated with delayed conidia formation and progressive absence of conidiation. Noteworthy, no TBC-exposed clones harbored TR34/L98H mutation, as judged by real-time PCR assays. The observation that TBC exposure promotes cross-resistance to clinical triazoles warrants careful and thorough assessment of the human health risk associated with agricultural azoles. The absence of TR34/L98H mutation in cross-resistant *A. fumigatus* isolates suggests that other *cyp51A* mutations may be involved in clinical azole cross-resistance.

Keywords: Aspergillus fumigatus; tebuconazole; cross-resistance; clinical azole resistance; agricultural triazole.

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INTRODUCTION

Among the pathogenic *Aspergillus* spp., *Aspergillus fumigatus* is a ubiquitous ascomycete fungus commonly found in soil, flora and decaying vegetation (Faria-Ramos et al., 2014; Sewell et al., 2019). The general population is usually exposed to this opportunistic and widely-distributed pathogen, which can cause a number of syndromes, allergic diseases, and non-invasive infections (Faria-Ramos et al., 2014; Pérez-Cantero et al., 2020). In particular, *A. fumigatus* is responsible for invasive aspergillosis (IA), a severe disease mainly affecting immunocompromised individuals, which can lead to a high case fatality rate in this patient group (Jeanvoine et al., 2020; Prigitano et al., 2019; Schoustra et al., 2019; Sewell et al., 2019; Zhang et al., 2019).

Aspergillus spp. infection typically occurs in the alveoli, where conidia germinate and proliferate, eventually resulting in IA (Snelders et al., 2009). However, prompt administration of appropriate antifungal agents is effective in reducing the morbidity and mortality associated with IA (Snelders et al., 2008). There are several commercially available antifungal drugs indicated for the treatment of *Aspergillus* spp. infections in humans or plants. Clinical antifungals include polyenes, azoles, echinocandins, allylamines, and pyrimidines. However, only four triazoles — i.e., itraconazole (ITZ), voriconazole (VRC), posaconazole (POS) and isavuconazole (IZV) — are employed as first-line treatment and prophylaxis against IA (Bedin Denardi et al., 2018; Chowdhary et al., 2012; Prigitano et al., 2019). In contrast, there is a much larger number of antifungals used for agricultural purposes. Among them, propiconazole, tebuconazole (TBC), prochloraz, bromuconazole, difenoconazole, and epoxiconazole have a basic molecular structure similar to that of clinical azoles and, *in vitro*, are active against *A. fumigatus* (Bowyer and Denning, 2014; Prigitano et al., 2019).

Regardless of their chemical structure and different biological characteristics, all azoles are capable of inhibiting the synthesis of the fungal enzyme lanosterol 14 alpha-demethylase. In *A. fumigatus*, this enzyme encoded by the *cyp51A* gene catalyzes the conversion of lanosterol to ergosterol, which is an essential molecule for the correct formation of the cytoplasmic membrane of the fungus. Indeed, inhibition of ergosterol synthesis leads to cell membrane disruption and fungal growth arrest (Faria-Ramos et al., 2014; Jeanvoine et al., 2020; Prigitano et al., 2014).

Even though resistance in *A. fumigatus* isolates was a rare occurrence in past years, it has now become a prominent health concern due to its growing, albeit variable, prevalence across multiple countries (Chowdhary et al., 2013; Dunne et al., 2017; Faria-Ramos et al., 2014). In particular, azole resistance, mostly ascribable to mutations in the *cyp51A* gene, is associated with an increased risk of treatment failure, which can be life-threatening in immunocompromised patients. A 34-bp insertion in the promoter region of *cyp51A* and a leucine to histidine substitution at codon 98 (TR34/L98H) are the most commonly found gene alterations, followed by a double substitution combined with a 46-bp

tandem duplication in the same *cyp51A* promoter (TR46/Y121F/T289A) (Ahangarkani et al., 2020; Bedin Denardi et al., 2018; Snelders et al., 2012).

Remarkably, 64-71% of patients with *Aspergillus spp*., harboring a confirmed resistant *A. fumigatus* strain, were reported to be triazole naïve — that is, with no history of past triazole therapy (Cui et al., 2019). This has led to the hypothesis that exposure of *A. fumigatus* to agricultural fungicides in the environment may play a crucial role in the emergence of cross-resistance to clinical triazoles (Cho et al., 2019; Chowdhary et al., 2013; Faria-Ramos et al., 2014). Thus, here we sought to determine whether exposure of clinical isolates of *A. fumigatus* to TBC, an agricultural azole with a chemical structure similar to that of clinical azoles, would favor the development of clinical azole cross-resistant isolates *in vitro*. Furthermore, we investigated the temporal relationship between TBC exposure and *A. fumigatus* azole resistance.

MATERIALS AND METHODS

Selection of A. fumigatus strains

Four *A. fumigatus* strains were selected from a collection of filamentous fungi isolated from clinical specimens at the Microbiology Unit of "Città della Salute e della Scienza di Torino" (Turin, Italy). The selected strains were isolated from sputum specimens collected from four hospitalized patients, triazole naïve with no history of prior triazole therapy. The fifth *A. fumigatus* isolate was the sputum specimen 3231 from the UK NEQAS External Quality Assessment Services (Distribution No. 3918, Mycology, distribution date: June 6, 2016).

All isolates were identified as belonging to *A. fumigatus* by macroscopic and microscopic examination and colonies were additionally identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis (bioMérieux, Marcy l'Etoile, France) (Bianco et al., 2020), which has been proven useful in the determination of fungi belonging to *A. fumigatus* (Verwer et al., 2014). In detail, the 5 strains were cultured for 48 and 192 h at 30°C. Fungal material was gently removed with a sterile swab and suspended in medium suspension (bioMérieux, Ref. 20150). Ethanol absolute (0.9 mL; Sigma-Aldrich, Lyon, France) was added, samples were mixed and centrifuged for 2 min at 15,000g, and the supernatant was discarded. Next, 40 µl of 70% formic acid (Sigma-Aldrich) and 40 µl acetonitrile (Sigma-Aldrich) were added, the samples were mixed and centrifuged for 2 min at 15,000g. The supernatant was spotted on a single-use target (VITEK-MSDS, bioMérieux) and the samples were allowed to dry. One microliter of α-cyano-4-hydroxycinnamic acid (CHCA; bioMérieux) was added as a matrix and the targets were allowed to dry. Spectra were determined by MALDI-TOF MS in a linear positive ion extraction mode within a mass range from 2,000 to 20,000 Da.

Azole resistance induction assays

Induction tests were performed using the triazole 14α-demethylase inhibitor (DMI) TBC, chosen as a representative agricultural azole. A TBC stock water solution of 40 mg/L was prepared starting from a commercially available agricultural fungicide (MYSTIC® 250 E, Nufarm, Milan, Italy). Subsequently, scalar doubling dilutions ranging from 1:2 (solution A, 20 mg/L) to 1:64 (solution F, 0.625 mg/L) were obtained.

For each *A. fumigatus* isolate, the following procedure was carried out: the conidia were gently collected from the colony top on the Sabouraud Dextrose Agar plates (SAB, Becton–Dickinson, Franklin Lakes, New Jersey, USA) and suspended in 1 ml of sterile distilled water, added with Tween 80, to reach 0.5 McFarland turbidity; 15 µl of this suspension were further diluted in 1 ml of sterile distilled water, and 100 µl of the conidia inoculum (final concentration of about 10^4 conidia per milliliter) were then separately inoculated in 1 ml of the TBC solutions (A-F). After mixing, 1 ml of the suspensions was separately inoculated on SAB agar and gently spread onto the entire agar surface. The seeded plates were incubated at 35°C ambient atmosphere for 5 days. The TBC sub-inhibitory concentration was determined as the highest TBC concentration producing visible development and conidiation on agar plates after 5 days of incubation.

The *A. fumigatus* colonies grown at the subinhibitory concentration of TBC were used for the next induction phase, repeating the aforementioned procedure. The isolates were subjected to 10 consecutive induction phases. In parallel, control assays in the same experimental conditions were carried out in a TBC-free water solution.

Evaluation of medical triazole resistance

Antifungal susceptibility testing to ITZ, VRC, POS, and IZV was performed using *A. fumigatus* strains exposed or not to TBC at time 0 (before the induction assay), time 1 (after two induction phases), time 2 (after five induction phases) and time 3 (after ten induction phases). Minimal inhibitory concentrations (MICs) were measured by Etest® (BioMérieux) and interpreted according to the manufacturer's instructions and compared to EUCAST breakpoints (v 9.0; http://www.eucast.org/astoffungi/ clinicalbreakpointsforantifungals/).

Multiplex Real-time PCR assay

To detect TR34/L98H mutations in TBC exposed *A. fumigatus* strains at time 3, a commercially available multiplex real-time PCR assay (MycoGENIE *Aspergillus fumigatus* real-time PCR Kit, Ademtech, Pessac, France) was performed. DNA was extracted from 200 µl of the 1:100 0.5 McFarland conidia suspension using QIA Symphony instrument (Qiagen GmbH, Hilden, Germany). Real-time PCR amplifications were performed by 7500 Real-Time PCR System (Applied Biosystems, Monza, Italy) (Bianco et al., 2020).

RESULTS

To assess whether triazole resistance in *A. fumigatus* could be triggered by TBC exposure *in vitro*, we exposed five *A. fumigatus* strains, four isolated from the sputum of 4 hospitalized, triazole naïve patients and one obtained from the UK NEQAS collection, to concentrations of TBC ranging from 0 to 20 mg/L for ten consecutive resistance induction experiments. The susceptibility of all clinical isolates to ITZ, VRC, POS, and IZV, before and after each resistance induction experiment, i.e., at passages 2 (~8 days), 5 (~20 days) and 10 (~40 days), was examined according to both manufacturer's instructions and EUCAST antimicrobial susceptibility testing guidelines.

Table 1 shows the high minimum inhibitory concentration (MIC) values obtained for each condition. In line with the clinical resistance breakpoints of different triazoles for *A. fumigatus*, at time zero, all strains were found to be susceptible to all four antifungal agents tested. By contrast, under TBC selection pressure, the resistance level of each strain to the different triazoles increased over time (**Table 1**). Specifically, at the last time point of the experiment (~40 days), all isolates showed remarkably high MIC values of ITZ (MIC ≥ 4 mg/L), three strains were of intermediate susceptibility to POS (MIC = 0.25 mg/L) and two were resistant to POS (MIC ≥ 1 mg/L). Regarding VRC and IZV, two of the five strains displayed a resistance phenotype to both antifungals assayed, with MICs of > 32 and = 3 mg/L, respectively.

Even though all isolates increased their resistance to clinical triazoles over the entire selection period, from a clinical standpoint, not all strains could be regarded as having a multiresistant phenotype. In fact, only two *A. fumigatus* could be clinically considered multiresistant with high MIC values for all azoles tested. One of these two strains was from the four aforementioned sputum specimens, while the second one corresponded to the sputum specimen 3231 from NEQAS. Noteworthy, the *A. fumigatus* isolates grown on control plates (i.e., medium without TBC) maintained the initial susceptible pattern (i.e., ITZ MIC range 0.5-1 mg/L; VRC MIC range 0.125-0.9 mg/L; POS MIC range 0.047-0.094 mg/L; IZV MIC range 0.125-0.9 mg/L), implying that the TBC susceptibility of *A. fumigatus* isolates had not changed during the experiment.

The increased resistance level of *A. fumigatus* to clinical triazoles was probably the result of consecutive mutation fixation events, as suggested by a consistent, stepwise increase in relative MIC values and fitness over time. This was also accompanied by increased TBC subinhibitory concentrations (**Table 1**).

We also evaluated the main phenotypic characteristics of the *A. fumigatus* colonies before and after exposure to TBC. In **Figure 1,** a demonstrative photograph of macroscopic morphological changes in the resistant strains of *A. fumigatus* colonies following exposure to TBC is shown. Upon TBC exposure, the morphology of *A. fumigatus* colonies changed at both the macroscopic and microscopic

levels. Macroscopically, we noticed a consistent and progressive variation in colony pigmentation — the normal green color turned to pale brown and white, with a reduction of fungal growth — leading to macroscopic alterations. Furthermore, microscopic examination revealed delayed conidia formation and progressive reduction in conidiation. On the other hand, with regard to the susceptible *A. fumigatus* strains, no changes in morphology were revealed.

The two resistant strains were cultivated five times on SAB Agar without azoles (neither human nor agricultural ones), and the changes in the macroscopic/ microscopic morphological features were maintained. Following further MIC determination, it was confirmed that the generated resistance was stable.

To gain mechanistic insights into TBC-induced cross-resistance, we investigated *cyp51A* from all strains exposed to TBC for 40 days (time 3) by multiplex real-time PCR but found no TR34/L98H mutation in any of them, suggesting that other mutations conferring drug resistance might have arisen.

DISCUSSION

It is predicted that in the years to come, the widespread use of azoles in agriculture will spur fungicide resistance not just in plant pathogenic fungi but also in susceptible species of human saprophytic flora (Chowdhary et al., 2012). In this regard, two possible routes of emerging azole cross-resistance in *A. fumigatus* have been envisaged: one due to prolonged exposure to azoles in the clinical setting, and the second caused by the selective pressure of agricultural azole fungicides, a resistance typified by the TR34/L98H mutation in the *cyp51A* gene (Azevedo et al., 2015; Snelders et al., 2012). Based on these assumptions, the aim of the current study was to determine whether clinical triazole resistance in *A. fumigatus* isolates could be induced *in vitro* following exposure to TBC, an azole widely used for agricultural purposes. Furthermore, we sought to define the temporal relationship between TBC exposure and *A. fumigatus* resistance.

In agreement with Zhang J. *et al*. (2017) (Zhang et al., 2017), we found that exposure of five *A. fumigatus* clinical isolates to TBC resulted in increased MIC values to clinical azoles. Specifically, these susceptible *A. fumigatus* strains showed resistance to clinical triazoles under the selection pressure of TBC at a maximum concentration of 20 mg/L in solid cultural medium. In particular, while all strains increased their resistance to triazoles, only two of them could be deemed clinically resistant, as they showed high MIC of ITZ (> 32 mg/L), VRC (> 32 mg/L), POS (≥ 1 mg/L) and IZV (= 3 mg/L). As expected, no resistance was observed in strains grown in the absence of TBC. Furthermore, the novelty of our results pertains to the observation of the cross-resistance to IZV, a new azole molecule used in IA therapy (Pagano et al., 2020; Pérez-Cantero et al., 2020), further confirming a key role of agricultural azoles in determining resistance to recent antifungal drugs that have been commercialized.

Our results are also in line with very recent findings by Cui and co-workers (Cui et al., 2019), indicating that TBC exposure results in cross-resistance of *A. fumigatus* to clinical triazoles. In comparison to Cui et al., the originality of our approach lies in the fact that we exposed *A. fumigatus* strains to TBC while growing on a solid and organic medium, a condition mirroring that occurring in the natural environment, where agricultural antifungals are disperse on the soil or plants in the presence of fungal conidia.

Consistent with Zhang *et al.* (2017) (Zhang et al., 2017) and Faria-Ramos *et al.* (2014) (Faria-Ramos et al., 2014), we observed similar phenotypic features for all resistant strains, which was characterized by changes in colony pigmentation — from green to white — and loss in conidia production. These morphological changes are of particular relevance in terms of laboratory diagnostics, as they may lead to errors in the identification and enumeration of fungal genus/species.

Extensive stepwise cross-resistance to clinical triazoles occurred in all five *A. fumigatus* strains assayed within about 40 days of TBC exposure. Thus, we can speculate a temporal relationship, indicating that exposure time is a major determinant of resistance to agricultural azoles, probably due to the manifestation of sequential mutation-fixation events.

The association between nonhuman use of antimicrobials and cross-resistance to clinically used medications has long been recognized in bacteria. Even though the role of fungicides in azole cross-resistance is still heavily debated (Chowdhary et al., 2013), our results in *A. fumigatus* suggest that a similar mechanism may also occur in fungi.

It has previously been demonstrated that alterations in *cyp51A*, a gene involved in ergosterol synthesis, are associated with the emergence of a resistance phenotype to both agricultural and clinical azoles, possibly due to similarities between the chemical structures of these antifungals (**Figure 2**) (Pagano et al., 2020). In this regard, the TR34/L98H mutation appears to be associated mainly with environmental resistance and is responsible for resistance to ITZ and a variable susceptibility pattern to VRC, IZV and POS (i.e., susceptible, intermediate or resistant, respectively) (Prigitano et al., 2019). However, despite being resistant to clinical triazoles, none of the *A. fumigatus* strains subjected to multiplex real-time PCR analysis revealed a TR34/L98H mutation. In this regard, the emergence of two fully-resistant isolates, as judged by high MIC values for all azoles tested, suggests the existence of other resistance mechanisms. In support of this hypothesis, several azole-resistant *A. fumigatus* strains without mutations in the *cyp51A* gene have been reported. Specifically, Hagiwara *et al.* (2018) (Hagiwara et al., 2018) found that several of his azole-resistant *A. fumigatus* isolates harbored mutations in *erg6* and *hmg1*, two genes involved in ergosterol biosynthesis, thereby proposing that these mutations may play a role in azole resistance. Furthermore, TR46/Y121F/T289A *cyp51A* mutations, overexpression of multidrug resistance efflux transporters (i.e., ATP-binding

cassette (ABC) transporter superfamily and major facilitator superfamily (MFS)), and/or target upregulation have all been involved in azole resistance (Ahangarkani et al., 2020; Chen et al., 2020; Cui et al., 2019; Faria-Ramos et al., 2014; Jeanvoine et al., 2020; Lavergne et al., 2015; Parker et al., 2014).

Overall, the isolation of triazole-resistant *A. fumigatus* strains from triazole naïve patients raises the possibility that exposure to agricultural triazole fungicides may promote resistance of *A. fumigatus* to clinical triazoles, as previously suggested (Ren et al., 2017). Consequently, this factor should be carefully taken into account when assessing the human health risk associated with agricultural azoles (Snelders et al., 2012). This is particularly important in light of the fact that there is a limited number of antifungal agents available for treating noninvasive and invasive fungal diseases (e.g., IA), with triazoles being the only group that can be administered orally (Snelders et al., 2012).

The present study is limited by the low number of *A. fumigatus* strains analyzed and by the incomplete elucidation of the harbored mutation that led to the azole resistance phenotype of the two resistant isolates. However, our results, obtained through an *in vitro* approach that closely recapitulates the natural environment, further substantiate the notion that chronic exposure to agricultural fungicides from the surrounding environment may result in cross-resistance to clinical triazoles. Additional studies based on whole genome sequencing are warranted to investigate all resistance mechanisms involved in clinical triazole resistance following TBC exposure. Further work on a larger sample size is also required to obtain conclusive evidence of the connection between clinically sourced strains, their environmental counterparts, and the role of agricultural azoles in determining cross-resistance.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Figure 1: Representative photographs of macroscopic morphological changes in *A. fumigatus* colonies following exposure to TBC: before (a) and after (b) induction experiments. Photomicrographs of *A. fumigatus* colonies underlying microscopic morphological changes in the development of conidiation of *A. fumigatus* following exposure to TBC: initial (c) and final (d) morphological aspects.

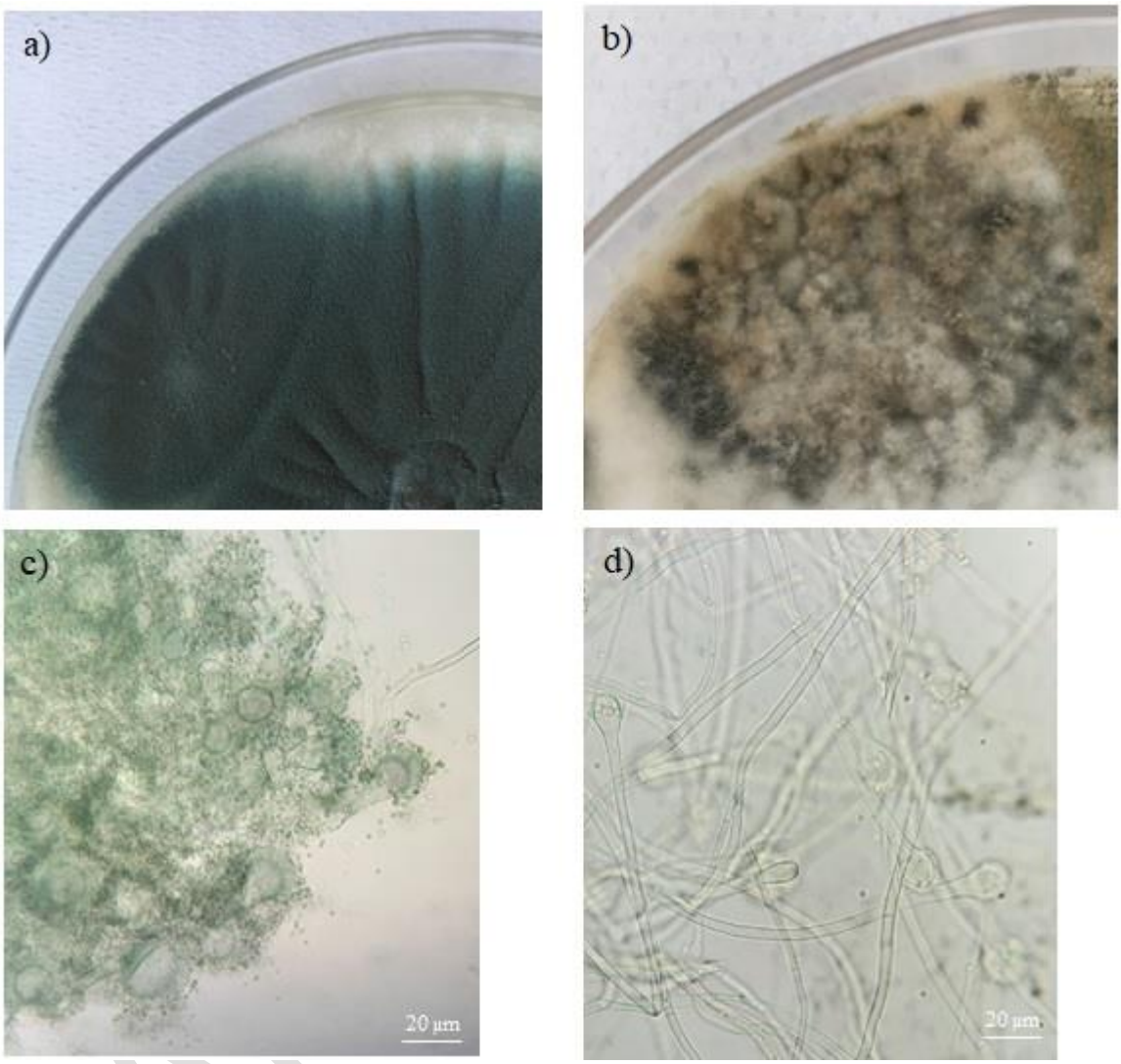

Figure 2: Structures of important triazoles in human (voriconazole, VOR; isavuconazole, IVZ; itraconazole, ITRA; posaconazole, POS) and agricultural use (propiconazole, tebuconazole and prochloraz).