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Research Article

RESEARCH ARTICLE

Nectar yeasts of the *Metschnikowia* clade are highly susceptible to azole antifungals widely used in medicine and agriculture

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One sentence summary: Yeast isolates from the *Metschnikowia* clade retrieved from the floral nectar of diverse wild plants and their insect pollinators were found to be highly susceptible to azole antifungals widely used in agriculture and medicine.
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ABSTRACT

The widespread use of azole antifungals in medicine and agriculture and the resulting long-persistent residues could potentially affect beneficial fungi. However, there is very little information on the tolerance of non-target environmental fungi to azoles. In this study, we assessed the susceptibility of diverse plant- and insect-associated yeasts from the *Metschnikowia* clade, including several ecologically important species, to widely used medical and agricultural azoles (epoxiconazole, imazalil, ketoconazole and voriconazole). A total of 120 strains from six species were tested. Minimum inhibitory concentrations (MICs) were determined by the EUCAST broth microdilution procedure after some necessary modifications were made. The majority of species tested were highly susceptible to epoxiconazole, ketoconazole and voriconazole (>95% of strains showed MICs $\leq$ 0.125 mg l^{-1}). Most strains were also very susceptible to imazalil, although MIC values were generally higher than for the other azoles. Furthermore, certain *Metschnikowia reukaufii* strains displayed a 'trailing' phenotype (i.e. showed reduced but persistent growth at antifungal concentrations above the MIC), but this characteristic was dependent on test conditions. It was concluded that exposure to azoles may pose a risk for ecologically relevant yeasts from the *Metschnikowia* clade, and thus could potentially impinge on the tripartite interaction linking these fungi with plants and their insect pollinators.

Keywords: azoles; broth microdilution; EUCAST; floral nectar; insect pollinators; *Metschnikowia* clade

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INTRODUCTION

Azoles are currently the largest and most widely used class of antifungal agents in clinical medicine (Sheehan, Hitchcock and Sibley 1999; Pierce *et al.* 2013; Allen *et al.* 2015), and also represent a mainstay for crop protection and material preservation (Hof 2001; Groenier and Lebow 2006; Price *et al.* 2015). Their mechanism of action is mainly based on the alteration of cell membrane structure and function through interference with the biosynthesis of ergosterol, but alterations in nutrient transport and other pleiotropic effects not yet fully understood have also been described (Ghannoum and Rice 1999; Price *et al.* 2015; Sanguinetti, Posteraro and Lass-Flörl 2015).

Recently, azole resistance has been increasingly reported in many fungal pathogens of animals and plants, which is a cause of great public concern (Serfling, Wohlrab and Deising 2007; Chakrabarti 2011; ECDC 2013; Vermeulen, Lagrou and Verweij 2013; Price *et al.* 2015). For some of such pathogens, it has been suggested that antifungal resistance can arise during prolonged treatment or, alternatively, through exposure of the microorganism to sub-lethal concentrations of the compounds in the environment (ECDC 2013). Moreover, azole residues can disperse and persist in the environment (Kahle *et al.* 2008; Battaglin *et al.* 2011; Bollmann *et al.* 2014) and potentially affect non-pathogenic or even beneficial fungi, and may therefore have a considerable impact on ecosystem health and functionality. However, there is very little information on the tolerance of non-target fungal species to medical and agricultural azoles, and risk assessments for antifungal use do not take into account their effects on entire fungal communities (Dijksterhuis *et al.* 2011; Dimitrov *et al.* 2014).

The *Metschnikowia* clade (Saccharomycetales) consists of an ancient and diverse group of ascomycetous yeasts harboring around 50 *Metschnikowia* species; the three species of genus *Clavispora* and several asexual forms currently assigned to the genus *Candida* (Lachance 2011; Guzmán, Lachance and Herrera 2013). Members of this clade are adapted to a wide variety of habitats, including flowers and their pollinators (e.g. *Metschnikowia gruessii*, *M. proteae* and *M. reukaufii*), plant surfaces, fruits and agricultural soils (e.g. *M. pulcherrima*) and aquatic environments (e.g. *M. bicuspidata*) (Lachance 2011; Guzmán, Lachance and Herrera 2013). Also, some *Metschnikowia* species are being used in agriculture since they are highly effective in the control of plant pathogens (Piano *et al.* 1997; Kurtzman and Droby 2001; Sipiczki 2006). The widespread presence of *Metschnikowia* species in natural and agricultural plant communities makes them a potential accidental target for azole antifungals. However, except for some reference strains from culture collections and a few clinical and agricultural isolates (Jawich *et al.* 2006; Desnos-Ollivier *et al.* 2012; Savini *et al.* 2013; Cordero-Bueso, Arroyo and Valero 2014), little is known about the possible effects that azoles might have on this ecologically important fungal group.

Floral nectar is a sugary solution essential for the attraction of pollinators that provide a key ecosystem service (Kearns, Inouye and Waser 1998; Vanbergen and the Insect Pollinators Initiative 2013). But nectar is also a crucial habitat for nectarivorous members of the *Metschnikowia* clade (Brysch-Herzberg 2004; Herrera *et al.* 2010), and this group of yeasts seems to play important ecological functions, including the attraction of pollinators (Herrera, Pozo and Medrano 2013; Schaeffer and Irwin 2014; Schaeffer *et al.* 2014; Pozo, Lievens and Jacquemyn 2015). It is well known that pesticides, chemical residues and azoles can accumulate in floral nectar and may have sublethal effects on plant pollinators (Rortais *et al.* 2005; Desneux, Decourtye and Delpuech 2007; Wallner 2009; Blacquière *et al.* 2012; Bernauer, Gaines-Day and Steffan 2015). However, the impact of such anthropogenic contaminants on nectar yeast communities has not been investigated to date.

In this study, we determined the susceptibility of a large collection of strains from the *Metschnikowia* clade isolated from the floral nectar of diverse wild plants and their insect pollinators to a number of azole antifungals that are widely used in agriculture (epoxiconazole and imazalil) and medicine (ketoconazole and voriconazole). Imazalil and ketoconazole are two imidazoles, i.e. compounds containing two nitrogen atoms in the azole ring, whereas epoxiconazole and voriconazole belong to the triazoles and have three nitrogen atoms in the azole ring (Sheehan, Hitchcock and Sibley 1999). Minimum inhibitory concentrations (MICs) were determined by a modification of the reference European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution (BMD) method. In addition, as antifungal susceptibility testing of yeasts from the *Metschnikowia* clade is still rarely performed, we explored the effect of some specific parameters (incubation time, MIC end point and culture medium) on the performance of the EUCAST method for this yeasts group.

MATERIALS AND METHODS

Isolates

A total of 120 strains from the *Metschnikowia* clade, most of which were obtained and identified to the species level by molecular methods in the course of previous studies (Pozo, Herrera and Bazaga 2011; de Vega *et al.* 2012, 2014; Pozo, Lachance and Herrera 2012; Jacquemyn *et al.* 2013; Lenaerts *et al.* 2015), were tested (Table S1, Supporting Information). Studied strains belonged to the following six species: *M. reukaufii* ($n = 46$); *M. proteae* ($n = 23$); *M. gruessii* ($n = 22$); *M. koreensis* ($n = 11$); *M. caudata* ($n = 7$); and *Candida rancensis* ($n = 11$). Most strains originated from the floral nectar of wild plants (87.7% of total, excluding type strains) and insect floral visitors (12.3%) from South Africa (44.7%), Spain (36.0%), Morocco (10.5%) and Belgium (8.8%). In addition, the type strains of the six tested species were also included in the experiments (Table S1). All strains were stored at −80°C as cell suspensions in 25% glycerol stocks.

Antifungal susceptibility testing

In vitro antifungal susceptibility of strains was determined by the reference EUCAST BMD procedure guidelines for yeasts (Arendrup *et al.* 2012), with some modifications in incubation conditions that had to be made for testing the strains investigated in this study (see below). RPMI 1640 (Sigma-Aldrich, Diegem, Belgium) supplemented with glucose (Sigma-Aldrich) to a final concentration of 20 g l^{-1} and buffered with 3-(N-morpholino) propanesulfonic acid (Sigma-Aldrich) to a pH of 7.0 (hereafter referred to as RPMI + 2%G) was used as test medium (Arendrup *et al.* 2012). The antifungal agents tested (all purchased from Sigma-Aldrich) were the imidazoles imazalil and ketoconazole, and the triazoles epoxiconazole and voriconazole. Final concentrations of the antifungal agents were in the range of 0.016–8 mg l^{-1}, and a positive control (i.e. drug-free medium)

was included in each test. Assay plates (96 wells, flat-bottom; Thermo Fisher Scientific/Nunc, Roskilde, Denmark) were prepared in batches according to the EUCAST guidelines and stored until used (but always for less than 3 months) at −80°C. Prior to susceptibility testing, frozen strains were subcultured by at least two serial transfers on yeast malt (YM) agar (2.0% agar, 1.0% dextrose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract; pH 6.2) for 72–96 h at 25°C, so as to check them for purity. Yeast suspensions were prepared in sterile distilled water, adjusted to the density of a 0.5 McFarland standard (1–5·10^6 cells ml^{-1}) and further diluted 1/10 in sterile distilled water. Columns 1–10 of the test plate contained 100 μl of two-fold serial dilutions of the antifungals in double strength RPMI+2%G, column 11 contained 100 μl of double strength drug-free medium and column 12 corresponded to the sterility controls. One hundred microliters of the working yeast cell suspension were inoculated per well in columns 1–11, and 100 μl of sterile distilled water per well in column 12. Plates were then covered with a sterile lid to prevent the medium from evaporating and incubated at 25°C. Although the EUCAST method recommends an incubation temperature of 35 ± 2°C, we selected 25°C as this is a common growth temperature for *Metschnikowia* species, and most tested strains were unable to grow or only showed poor growth at 35 ± 2°C (Lachance 2011; de Vega *et al.* 2012, 2014; Pozo, Lachance and Herrera 2012). Assay plates were read spectrophotometrically (530 nm) after 24, 48 and 72 h of incubation. For the slow-growing species *M. caudata* (de Vega *et al.* 2014), incubation was extended for 24 additional hours (i.e. 96 h in total; see Results). All strains were tested at least twice on different days and *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019, which are two quality control strains recommended by the EUCAST method, were included in each series of experiments. Additionally, in order to assess possible differences in the performance of the EUCAST method related to the test medium, susceptibility tests were repeated for a selection of 37 strains (the seven *M. caudata* strains available and six strains representative of different regions and/or hosts for each of the remaining five species) using non-synthetic YM broth (1.0% dextrose, 0.5% peptone, 0.3% yeast extract, 0.3% malt extract; pH 6.2) instead of synthetic RPMI + 2%G.

Data analysis

Since no reference MIC end point has yet been established for susceptibility testing of azoles against *Metschnikowia* clade strains, end points of ≥50% and ≥90% of reduction in turbidity compared to the azole-free control well (i.e. partial and almost complete inhibition of growth, respectively) were determined. Essential agreement (EA) between the MIC values determined at different incubation times, or by using different test media (i.e. RPMI + 2%G and YM broth) and reading end points (50% vs. 90%) was defined as discrepancy of no more than ± 2 two-fold dilutions (Cuenca-Estrella *et al.* 2010). When necessary, high off-scale MIC results were converted to the next highest concentration and low off-scale MIC results were left unchanged (Pfaller *et al.* 2011). Discrepancies between MIC values were classified as non-substantial differences (NSD, discrepancies of three or four two-fold dilutions) or substantial differences (SD, discrepancies of >4 two-fold dilutions) (Cuenca-Estrella *et al.* 2010). Where relevant, differences among the MIC data distributions were evaluated by the Friedman's test followed by Bonferroni post-hoc comparisons, as implemented in Statgraphics Centurion XVII (Statpoint Technologies, Inc., Warrenton, VA, USA). The critical *P*-value was set at <0.05.

RESULTS

Optimization of the EUCAST method for yeast strains from the *Metschnikowia* clade

The EUCAST method for antifungal susceptibility testing recommends incubating microdilution plates for 24 ± 2 h, after which the absorbance at 530 nm of azole-free control wells should be >0.2 (Arendrup *et al.* 2012). If required, test plates can be further incubated for 12–24 h, but failure to reach the threshold absorbance after 48 h is considered to represent a failed test (Arendrup *et al.* 2012). A strict application of these stringent criteria was not possible in the present study, as most tested strains (including those used for quality control) displayed poor growth in RPMI + 2%G medium after 24 h of incubation at 25°C and, in some cases, an absorbance value >0.2 was not reached until 72 h (Fig. 1). In addition, although all *C. rancensis* strains and the quality control strains displayed enough growth in RPMI + 2%G after 48 h (Fig. 1; Table 1; Table S2, Supporting Information), absorbance values for some strains were still rather low (e.g. mean ± S.D. = 0.30 ± 0.02 for *C. rancensis* SA16, and 0.31 ± 0.03 for isolate SA25). Notably, none of the seven *M. caudata* isolates included in the study consistently grew in RPMI + 2%G even after extended incubation up to 96 h post-inoculum (Fig. 1).

In contrast, when tested in YM broth, the quality control strains and all strains tested except those belonging to *M. caudata* reached absorbance values >0.2 in just 24 h (Fig. 1). Further incubation in YM broth resulted in most cases in saturated absorbance values in the drug-free wells (data not shown), thus resulting in unreliable MIC determination. For *M. caudata*, enough growth level for reliable MIC determination was not reached until 48–96 h, depending on the strain and test plate (Fig. 1).

Voriconazole MIC values obtained in RPMI + 2%G medium for the quality control strains fell within the acceptable ranges provided in the EUCAST reference document (0.03–0.25 mg l^{-1} for *C. krusei* ATCC 6258 and 0.015–0.06 mg l^{-1} for *C. parapsilosis* ATCC 22019; Arendrup *et al.* 2012) or, for a minority of tests, differed in ≤2 two-fold dilutions (Table 1; Table S2, Supporting Information). Obviously, due to the methodological modifications described in previous paragraphs, this comparison of voriconazole MICs for the quality control strains is tentative (the acceptable ranges given by the EUCAST document only refer to the 50% inhibition end point and incubation at 37°C for 24 h). The reliability of epoxiconazole, imazalil and ketoconazole MIC determinations in RPMI + 2%G could not be assessed, as acceptable MIC ranges are not yet available for these antifungals; nevertheless, repeated assays for these compounds yielded consistent results (data not shown). The same can be said for MIC determinations in YM broth.

In view of these results, it was concluded that for all species except *M. caudata* the optimal test conditions for azole MIC determination by the EUCAST procedure are 72 h of incubation in RPMI + 2%G or 24 h of incubation in YM broth. In the particular case of *M. caudata*, MIC values can only be reliably determined after 96 h of incubation in a nutrient rich medium such as YM broth.

In vitro susceptibility to azole antifungals of yeasts of the *Metschnikowia* clade

Table 2 shows the azole MIC distributions for the studied strains in RPMI + 2%G medium (or YM broth, in the case of *M. caudata*). In general, epoxiconazole, ketoconazole and voriconazole were very active against all species tested and, regardless of the

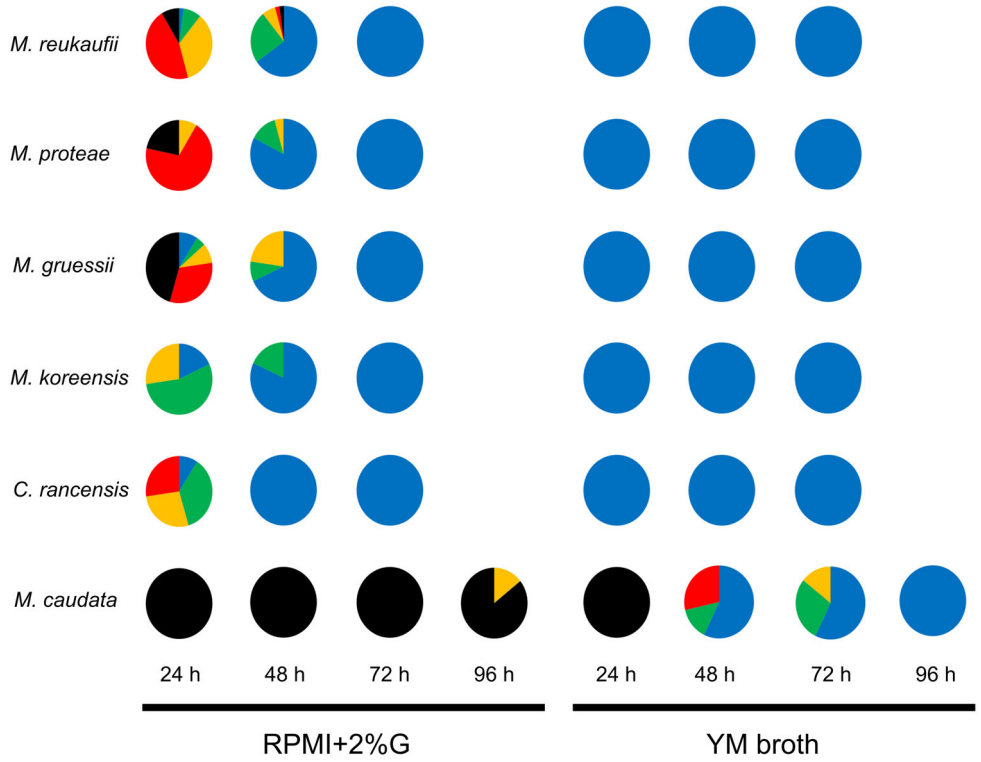

Figure 1. Pie charts showing the percentages of isolates of the tested species which displayed enough growth for reliable azole susceptibility determination by the EUCAST method (i.e. absorbance at 530 nm >0.2 in the positive control well) at each reading time (24, 48 and 72 h for most species, and also 96 h for *M. caudata*, see Results) in 100% (blue sectors), ≥75% but <100% (green), ≥50% but <75% (orange), <50% but >0% (red) and 0% (black) of the test plates. Total numbers of isolates (*N*) and tests (*n*, mean ± S.D.) per species are as follows: (i) experiments using RPMI-1640 medium supplemented with 2% (w/v) glucose (RPMI + 2%G): *M. reukaufii*, $N = 46$, $n = 423$ (9.2 ± 1.6); *M. proteae*, $N = 23$, $n = 193$ (8.4 ± 0.7); *M. gruessii*, $N = 22$, $n = 181$ (8.2 ± 0.8); *M. koreensis*, $N = 11$, $n = 102$ (9.3 ± 1.1); *C. rancensis*, $N = 11$, $n = 108$ (9.8 ± 1.8); and *M. caudata*, $N = 7$, $n = 56$ (8 ± 0); (ii) experiments using yeast malt (YM) broth: *M. reukaufii*, $N = 6$, $n = 48$ (8 ± 0); *M. proteae*, $N = 6$, $n = 51$ (8.5 ± 1.1); *M. gruessii*, $N = 6$, $n = 61$ (10.2 ± 1.7); *M. koreensis*, $N = 6$, $n = 66$ (11 ± 3.2); *C. rancensis*, $N = 6$, $n = 52$ (8.7 ± 1.5); and *M. caudata*, $N = 7$, $n = 88$ (12.6 ± 0.5).

end point considered for MIC determination, >95% of strains were susceptible at concentrations ≤0.125 mg l^{-1}. Notably, in most cases there were no significant differences in the median MICs of epoxiconazole, ketoconazole and voriconazole ($P > 0.05$ in all pair-wise comparisons except epoxiconazole versus ketoconazole and epoxiconazole versus voriconazole for *M. reukaufii* and the 90% inhibition end point). In contrast, median MICs for imazalil were generally higher ($P < 0.05$ in all pair-wise comparisons except imazalil versus voriconazole for *M. caudata* and the 90% inhibition end point), and MIC distributions depended largely on the species and end point criteria. For example, only 68.3% and 27.5% of the total number of isolates were susceptible to ≤0.125 mg l^{-1} of imazalil when the partial (≥50%) and almost complete (≥90%) inhibition end points were considered, respectively.

An excellent EA (100%) was observed for most species-azole combinations between the MIC values obtained by the two end point criteria considered (Table 2). A notable exception was *M. reukaufii*, which yielded discrepant results for all tested antifungals: 1 NSD and 2 SD for epoxiconazole; 8 NSD for imazalil; and 1 SD for ketoconazole and voriconazole (Table 2). Interestingly, one particular isolate (6.3.2-Y2, obtained in 2012 in Belgium from floral nectar of *Pulmonaria officinalis*) displayed discrepant results for all tested antifungals. In addition, two NSD were observed for *M. caudata* and imazalil.

Regarding the comparison of test media, for most species, azole antifungal and end point combinations, the EA between the MIC results determined after 72 h of incubation in plates containing RPMI + 2%G or 24 h in plates containing YM broth was 100% (Table 3). Discrepancies in MIC results due to the test medium when the partial inhibition end point criterion was considered were only observed for imazalil and the species *M. gruessii* and *M. koreensis*, for which three out of the six isolates tested in each case yielded NSDs (Table 3). Non-significant differences were also obtained for the same three *M. gruessii* and a single *M. reukaufii* isolate when tested for imazalil susceptibility considering the almost complete inhibition end point (Table 3). Notably, isolate 6.3.2-Y2 of *M. reukafii* yielded SDs in the 90% end point MIC results when tested for epoxiconazole, ketoconazole and voriconazole susceptibility (Table 3).

DISCUSSION

Controlling fungal pathogens is paramount to ensuring human and animal health, food security and preservation of wood and other materials (Hof 2001; ECDC 2013; Price *et al.* 2015). However, the widespread use of azole antifungals in agriculture and medicine is leading to a significant accumulation of azole residuals in the environment (Kahle *et al.* 2008; Battaglin *et al.* 2011), which poses a threat for the composition and/or functioning of fungal communities harboring non-target fungi (Dijksterhuis *et al.* 2011; Dimitrov *et al.* 2014). In spite of this, current knowledge about yeast antifungal susceptibility profiles is mostly limited to species responsible for human infections (Desnos-Ollivier *et al.* 2012), and there is very scarce information on the tolerance of environmental fungi to antifungal compounds. To contribute

Table 1. Range of MICs obtained for quality control strains.[a]

			≥50% inhibition end point			≥90% inhibition end point		
Strain	Test medium[b]	Antifungal (*n*)[c]	24 h	48 h	72 h	24 h	48 h	72 h
C. krusei ATCC 6258	RPMI + 2%G	EPZ (23)	ND(65.2%), ≤0.016–0.125	0.031–0.125	0.063–0.25	ND(65.2%), 0.125–0.25	0.25–0.5	0.5–1
		IZL (22)	ND(54.5%), 0.063–0.5	0.5–2	1–4	ND(54.5%), 1–2	4	8
		KTZ (22)	ND(63.6%), ≤0.016–0.25	0.031–0.25	0.063–0.25	ND(63.6%), 0.25–0.5	0.5–1	0.5–1
		VCZ (22)	ND(45.5%), 0.031–0.125	0.125–0.25	0.125–0.5	ND(45.5%), 0.25	0.25–0.5	0.5–1
	YM broth	EPZ (9)	0.125–0.25	1	1–2	0.5	1–2	1–2
		IZL (10)	1–4	4–8	8–>8	2–8	4–8	8–>8
		KTZ (9)	0.25–1	0.5–2	0.5–4	0.5–2	0.5–2	0.5–4
		VCZ (9)	0.5	1–2	1–2	0.5–1	1–2	2
C. parapsilosis ATCC 22019	RPMI + 2%G	EPZ (33)	ND(45.5%), ≤0.016–0.031	0.063–0.125	0.063–0.25	ND(45.5%), ≤0.016–0.25	0.25–0.5	0.25–0.5
		IZL (30)	ND(36.7%), 0.125–0.25	0.25–0.5	0.5–1	ND(36.7%), 1–2	2–4	4–8
		KTZ (33)	ND(36.4%), ≤0.016–0.031	≤0.016–0.031	≤0.016–0.063	ND(36.4%), 0.031–0.063	0.063–0.125	0.125–0.25
		VCZ (33)	ND(30.3%), ≤0.016	≤0.016–0.031	0.031	ND(30.3%), 0.031–0.063	0.031–0.063	0.063–0.125
	YM broth	EPZ (8)	0.031–0.063	0.125–0.25	0.25–0.5	0.25	1	2–4
		IZL (6)	0.25–1	1–4	2–8	1–4	4–8	8–>8
		KTZ (7)	≤0.016	≤0.016–0.063	0.031–0.125	≤0.016–0.063	0.031–0.25	0.063–1
		VCZ (8)	0.031	0.063	0.063–0.125	0.063–0.125	0.125–0.25	0.25–0.5

[a]For each quality control strain and combination of test conditions (test medium; antifungal compound; incubation time –24, 48 and 72 h–; and inhibition end point), the range of MIC values (in mg l^{-1}) obtained in this study is given. In some cases, the percentage of tests in which the actual MIC value could not be determined (ND) due to scarce growth (i.e. absorbance at 530 nm ≤0.2; Arendrup *et al.* 2012) is also provided.
[b]RPMI + 2%G, RPMI 1640 supplemented with glucose and buffered with 3-(N-morpholino) propanesulfonic acid (see main text); YM broth, yeast malt broth.
[c]EPZ, epoxiconazole; IZL, imazalil; KTZ, ketoconazole; VCZ, voriconazole. The number of tests performed (*n*) in each case is shown within parentheses.

Table 2. Distribution of MICs for azole antifungals determined by the EUCAST broth microdilution method for yeast strains of the *Metschnikowia* clade.

Species (no. of strains tested)	Antifungal[a]	End point[b]	MIC distribution (mg/l)[c]										EA[d]	NSD[e]	SD[e]
			≤0.016	0.031	0.063	0.125	0.25	0.5	1	2	4	8			
M. reukaufii (46)	EPZ	50%	42*	3	1								93.5%	1 (2.2%)	2 (4.3%)
		90%	20	14*	7	2	1	1	1						
	IZL	50%	1	2	2	22*	18	1					82.6%	8 (17.4%)	0
		90%			2	1	11	21*	11						
	KTZ	50%	46*										97.8%	0	1 (2.2%)
		90%	39*	4	2					1					
	VCZ	50%	46*										97.8%	0	1 (2.2%)
		90%	41*	3	1							1			
M. proteae (23)	EPZ	50%	23*										100%	0	0
		90%	23*												
	IZL	50%			7	15*	1						100%	0	0
		90%				13*	10								
	KTZ	50%	23*										100%	0	0
		90%	21*	2											
	VCZ	50%	23*										100%	0	0
		90%	22*	1											
M. gruessii (22)	EPZ	50%	21*		1								100%	0	0
		90%	19*	2		1									
	IZL	50%		1	16*	4			1				100%	0	0
		90%			1	14*	6			1					
	KTZ	50%	21*		1								100%	0	0
		90%	21*			1									
	VCZ	50%	21*		1								100%	0	0
		90%	20*	1			1								
C. rancensis (11)	EPZ	50%	2	5*	4								100%	0	0
		90%	1	2	5*	3									
	IZL	50%				1	3	4*	3				100%	0	0
		90%					1	7*		2	1				
	KTZ	50%	4	7*									100%	0	0
		90%		6*	4	1									
	VCZ	50%	5	5*	1								100%	0	0
		90%	2	3	6*										
M. koreensis (11)	EPZ	50%	11*										100%	0	0
		90%	6*	4	1										
	IZL	50%			1	7*	3						100%	0	0
		90%				2	2	7*							
	KTZ	50%	9*	2									100%	0	0
		90%	8*	3											
	VCZ	50%	10*	1									100%	0	0

Table 2. (*Continued*)

Species (no. of strains tested)	Antifungal[a]	End point[b]	MIC distribution (mg/l)[c]										EA[d]	NSD[e]	SD[e]
			≤0.016	0.031	0.063	0.125	0.25	0.5	1	2	4	8			
		90%	9*	2											
M. caudata[f] (7)	EPZ	50%	5*	2									100%	0	0
		90%		5*	2										
	IZL	50%		1	1	1	2*	2					71.4%	2 (28.6%)	0
		90%						7*							
	KTZ	50%	7*										100%	0	0
		90%	4*	3											
	VCZ	50%	5*	2									100%	0	0
–		90%		2	5*										
Total (120)	EPZ	50%	104 (86.7%)	10 (8.3%)	6 (5%)								97.5%	1 (0.8%)	2 (1.7%)
		90%	69 (57.5%)	27 (22.5%)	15 (12.5%)	6 (5%)	1 (0.8%)	1 (0.8%)	1 (0.8%)						
	IZL	50%	1 (0.8%)	4 (3.3%)	27 (22.5%)	50 (41.7%)	27 (22.5%)	7 (5.8%)	4 (3.3%)				91.7%	10 (8.3%)	0
		90%			3 (2.5%)	30 (25%)	30 (25%)	42 (35%)	11 (9.2%)	3 (2.5%)	1 (0.8%)				
	KTZ	50%	110 (91.7%)	9 (7.5%)	1 (0.8%)								99.2%	0	1 (0.8%)
		90%	93 (77.5%)	18 (15%)	6 (5%)	2 (1.7%)				1 (0.8%)					
	VCZ	50%	110 (91.7%)	8 (6.7%)	2 (1.7%)								99.2%	0	1 (0.8%)
		90%	94 (78.3%)	12 (10%)	12 (10%)		1 (0.8%)					1 (0.8%)			

[a]EPZ, epoxiconazole; IZL, imazalil; KTZ, ketoconazole; VCZ, voriconazole.
[b]Percentage of growth inhibition relative to a positive control (i.e. test medium without azoles) set as end point for antifungal susceptibility testing.
[c]Number (and percentage, only for total results) of strains falling into each MIC value.
[d]Percentage of EA (i.e. discrepancy of no more than ± 2 two-fold dilutions) between MIC values obtained for the different end points.
[e]Number (and percentage) of strains showing non-substantial differences (NSDs) or substantial differences (SDs) between MIC values obtained for the different end points.
[f]Azole susceptibility testing of *M. caudata* strains was performed using yeast maltose (YM) broth instead of RPMI 1640 supplemented with 2% (w/v) of glucose (RPMI + 2%G) as culture medium and after 96 h of incubation at 25°C instead of 72 h (see details in the main text).
*Median MIC value for each yeast species, antifungal and end point combination.

Table 3. Comparison of the results obtained for a selection of yeast strains from the *Metschnikowia* clade when tested for azole susceptibility in different culture media.

Species (no. of strains tested)	Antifungal[a]	≥50% inhibition end point					≥90% inhibition end point				
		MIC distribution[b]					MIC distribution[b]				
		RPMI + 2%G	YM broth	%EA[c]	NSD[d]	SD[d]	RPMI + 2%G	YM broth	%EA[c]	NSD[d]	SD[d]
C. rancensis (6)	EPZ	≤0.016(1), 0.031(3), 0.063(2)	0.031(2), 0.063(3), 0.125(1)	100	0	0	≤0.016(1), 0.063(3), 0.125(2)	0.031(1), 0.063(1), 0.125(3), 0.25(1)	100	0	0
	IZL	0.25(1), 0.5(3), 1(2)	1(2), 2(2), 4(2)	100	0	0	0.5(4), 2(1), 4(1)	1(1), 2(2), 4(1), 8(1)	100	0	0
	KTZ	≤0.016(2), 0.031(4)	0.031(2), 0.063(2), 0.125(2)	100	0	0	0.031(4), 0.063(1), 0.125(1)	0.031(1), 0.063(2), 0.125(3)	100	0	0
	VCZ	≤0.016(2), 0.031(4)	0.031(2), 0.063(2), 0.125(2)	100	0	0	≤0.016(1), 0.031(2), 0.063(3)	0.031(1), 0.063(3), 0.125(1), 0.25(1)	100	0	0
M. gruessii (6)	EPZ	≤0.016(6)	≤0.016(5), 0.031(1)	100	0	0	≤0.016(6)	≤0.016(3), 0.031(2), 0.063(1)	100	0	0
	IZL	0.031(1), 0.063(4), 0.125(1)	0.063(1), 0.125(1), 0.25(1), 0.5(2), 1(1)	50	3 (50%)	0	0.125(5), 0.25(1)	0.125(1), 0.25(1), 0.5(1), 1(2), 2(1)	50	3 (50%)	0
	KTZ	≤0.016(6)	≤0.016(5), 0.031(1)	100	0	0	≤0.016(6)	≤0.016(4), 0.031(2)	100	0	0
	VCZ	≤0.016(6)	≤0.016(5), 0.031(1)	100	0	0	≤0.016(6)	≤0.016(3), 0.031(2), 0.063(1)	100	0	0
M. koreensis (6)	EPZ	≤0.016(6)	≤0.016(1), 0.031(5)	100	0	0	≤0.016(2), 0.031(3), 0.063(1)	≤0.016(1), 0.063(5)	100	0	0
	IZL	0.125(5), 0.25(1)	0.25(2), 0.5(1), 1(3)	50	3 (50%)	0	0.25(2), 0.5(4)	0.25(1), 0.5(1), 1(4)	100	0	0
	KTZ	≤0.016(4), 0.031(2)	≤0.016(2), 0.031(4)	100	0	0	≤0.016(4), 0.031(2)	≤0.016(2), 0.031(4)	100	0	0
	VCZ	≤0.016(5), 0.031(1)	≤0.016(1), 0.031(5)	100	0	0	≤0.016(5), 0.031(1)	0.031(2), 0.063(4)	100	0	0
M. proteae (6)	EPZ	≤0.016(6)	≤0.016(6)	100	0	0	≤0.016(6)	≤0.016(6)	100	0	0
	IZL	0.063(1), 0.125(4), 0.25(1)	0.25(5), 0.5(1)	100	0	0	0.125(2), 0.25(4)	0.25(1), 0.5(5)	100	0	0
	KTZ	≤0.016(6)	≤0.016(6)	100	0	0	≤0.016(5), 0.031(1)	≤0.016(5), 0.031(1)	100	0	0
	VCZ	≤0.016(6)	≤0.016(5), 0.031(1)	100	0	0	≤0.016(6)	≤0.016(5), 0.031(1)	100	0	0
M. reukaufii (6)	EPZ	≤0.016(6)	≤0.016(6)	100	0	0	≤0.016(2), 0.031(2), 0.063(1), 1(1)	≤0.016(1), 0.031(4), 0.063(1)	83.3	0	1 (16.7%)
	IZL	0.063(1), 0.125(2), 0.25(3)	0.125(6)	100	0	0	0.25(2), 0.5(2), 1(1), 2(2)	0.125(4), 0.25(1), 0.5(1)	83.3	1 (16.7%)	0
	KTZ	≤0.016(6)	≤0.016(6)	100	0	0	≤0.016(5), 2(1)	≤0.016(6)	83.3	0	1 (16.7%)
	VCZ	≤0.016(6)	≤0.016(4), 0.031(2)	100	0	0	≤0.016(5), 8(1)	≤0.016(1), 0.031(5)	83.3	0	1 (16.7%)
TOTAL (30)	EPZ	≤0.016(25), 0.031(3), 0.063(2)	≤0.016(18), 0.031(8), 0.063(3), 0.125(1)	100	0	0	≤0.016(17), 0.031(5), 0.063(5), 0.125(2), 1(1)	≤0.016(11), 0.031(7), 0.063(8), 0.125(3), 0.25(1)	96.7	0	1 (3.3%)
	IZL	0.031(1), 0.063(6), 0.125(12), 0.25(6), 0.5(3), 1(2)	0.063(1), 0.125(7), 0.25(8), 0.5(4), 1(6), 2(2), 4(2)	80	6 (20%)	0	0.125(7), 0.25(9), 0.5(10), 1(1), 2(2), 4(1)	0.125(5), 0.25(4), 0.5(8), 1(8), 2(3), 4(1), 8(1)	86.7	4 (13.3%)	0
	KTZ	≤0.016(24), 0.031(6)	≤0.016(19), 0.031(7), 0.063(2), 0.125(2)	100	0	0	≤0.016(20), 0.031(7), 0.063(1), 0.125(1), 2(1)	≤0.016(17), 0.031(8), 0.063(2), 0.125(3)	96.7	0	1 (3.3%)
	VCZ	≤0.016(25), 0.031(5)	≤0.016(15), 0.031(11), 0.063(2), 0.125(2)	100	0	0	≤0.016(23), 0.031(3), 0.063(3), 8(1)	≤0.016(9), 0.031(11), 0.063(8), 0.125(1), 0.25(1)	96.7	0	1 (3.3%)

[a]EPZ, epoxiconazole; IZL, imazalil; KTZ, ketoconazole; VCZ, voriconazole.

[b]For each species and combination of test conditions (test medium, antifungal compound, and inhibition end point), the number of strains displaying each MIC value (in mg/l) is given. RPMI + 2%G, RPMI 1640 supplemented with glucose and buffered with 3-(N-morpholino) propanesulfonic acid (see main text); YM broth, yeast malt broth.

[c]Percentage of EA (i.e. discrepancy of no more than ±2 two-fold dilutions) between MIC values obtained in different culture media.

[d]Number (and percentage) of strains showing non-substantial differences (NSDs) or substantial differences (SDs) between MIC values obtained in different culture media.

to fill this research gap, this study has provided novel information on the azole susceptibility of plant- and insect-associated strains from the *Metschnikowia* clade. To do so, we first had to optimize the EUCAST broth microdilution method of antifungal susceptibility testing for *Metschnikowia* clade yeasts.

Apart from setting the incubation temperature to 25°C, which is optimal for members of the *Metschnikowia* clade (see Materials and Methods), the scarce growth displayed by most tested species in RPMI + 2%G necessitated extended incubation of test plates (72 h, instead of the 24 h recommended by the EUCAST method) for reliable determination of azole MICs. Alternatively, adequate growth for MIC determination was obtained in just 24 h when RPMI + 2%G was substituted for nutrient rich YM broth. Nevertheless, *M. caudata* was particularly recalcitrant to azole susceptibility testing, and MIC values for this species could only be determined when YM broth was used as test medium, and plates were read after 96 h of incubation.

In general, most strains included in the present study were highly susceptible to broad-spectrum imidazole and triazole antifungals of widespread use in clinical and agricultural settings. These findings are in line with the observation of Desnos-Ollivier *et al.* (2012), who tested 62 *Metschnikowia* isolates (belonging to 36 different species) from reference culture collections and found no resistance to the medical azoles fluconazole, itraconazole, posaconazole and voriconazole. Nevertheless, for a few *M. reukaufii* strains included in our study the azole MICs determined at a 90% inhibition end point were several two-fold dilutions higher than those obtained using the partial inhibition criterion. This observation points to the occurrence of a 'trailing' phenotype in some *Metschnikowia* strains, which is defined as the manifestation of reduced but persistent growth in broth dilution tests with azole agents at antifungal concentrations above the MIC (Lee *et al.* 2004). Curiously, the trailing phenotype of *M. reukaufii* only appeared when susceptibility tests were performed in RPMI + 2%G but not when these were carried out in YM broth, thus confirming that this effect depends on species and strain-specific characteristics, as well as on different methodological aspects (Arthington-Skaggs *et al.* 2002; Agrawal *et al.* 2007; Coenye *et al.* 2008).

It is worth noting that MICs for the imidazole imazalil for our strain collection were generally higher than those observed for the other azoles tested. A similar result was reported by Dijksterhuis *et al.* (2011) after performing toxicity tests to determine the effects of azoles and other fungicides on aquatic fungi and oomycetes. The reason for this lower susceptibility to imazalil is still unknown, but it might be due to a longer exposure to imazalil residues and/or the presence of higher concentrations of these in the environment. Indeed, imazalil has been extensively used in agriculture since the 1970s, while epoxiconazole was introduced 20 years later (Morton and Staub 2008; Price *et al.* 2015). Typical uses of imazalil include field, glasshouse and indoor application by diverse methods (e.g. spraying, dipping and waxing) for the pre- and post-harvest control of diverse fungal pathogens (EFSA 2010). Moreover, apart from its agricultural applications, imazalil is used (sometimes under the synonym enilconazole) in veterinary medicine as a topical broad-spectrum antimycotic and also in some countries as a fungicide formulation for the disinfection of farm buildings (EMEA 1998), which constitute potential additional sources for environmental contamination (Kahle *et al.* 2008). Although most strains tested in this study were obtained from natural plant communities located relatively far from agricultural fields and human settlements, the presence of azole residues in these environments cannot be excluded and should be evaluated in future.

Floral nectar is a valuable reward for pollinators, and extensive research work has been carried out to understand its composition, availability and secretion patterns (Nicolson and Thornburg 2007; Brandenburg *et al.* 2009; Heil 2011; Lievens *et al.* 2015). More recently, there has been a growing interest in studying the role of floral nectar as a habitat for eukaryotic and prokaryotic microorganisms, and the effects these might have on nectar chemistry, pollinator behavior and sexual plant reproduction (see Pozo, Lievens and Jacquemyn 2015 for an updated review). In particular, it was found that *Metschnikowia* yeasts are widespread in the floral nectar of diverse plant families, and some species such as *M. reukaufii* could have a relevant role in attracting pollinators and influencing their foraging behavior (Herrera, Pozo and Medrano 2013; Schaeffer and Irwin 2014; Schaeffer *et al.* 2014). Another emerging focus of interest is the study of the presence of anthropogenic contaminants in floral nectar, and the impact of these on declining pollinator populations and, eventually, on plant reproduction. For example, it has been demonstrated that some insecticides such as the neonicotinoids are relatively common in nectar and can alter the physiology and behavior of pollinators (Blacquière *et al.* 2012; Stanley *et al.* 2015). Although some studies have reported the presence of trace amounts of certain azoles in pollen and nectar collected by foraging honey bees shortly after field applications, and over a prolonged time afterwards (e.g. Wallner 2009), to the best of our knowledge, no study has analyzed so far the possible effect of these antifungal compounds on the nectar microorganisms-plant-pollinator system. In any case, given the high susceptibility to azoles of nectar yeasts from the *Metschnikowia* clade found in this study, it seems clear that future risk assessments of the use of antifungals should pay attention to the nectar microbiota.

In summary, results of this study provide compelling evidence that exposure to azoles may pose a risk for ecologically important yeasts from the *Metschnikowia* clade, and thus could potentially have detrimental effects on ecosystem dynamics and key services including plant pollination. This adds yet another source of concern for the long-term persistence of healthy plant-pollinator systems in natural communities. A next step would be to study the *in planta* effects of azoles on *Metschnikowia* yeasts, as well as to determine the actual ecological consequences of the *in vitro* results here reported.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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