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Azole-resistant *Aspergillus fumigatus* in the Italian environment

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ABSTRACT

Objectives: Azole resistance in *Aspergillus fumigatus* environmental and clinical isolates is recognised as an emerging problem worldwide. Development of azole resistance may be environmentally driven because of the massive use of azole fungicides in agriculture. The mechanism of azole resistance is mostly related to mutations in the *cyp51A* gene.

Methods: *A. fumigatus* azole resistance in the environment was previously documented in northern Italy. This study extended the research in the agricultural environment also in central and southern Italy and investigated differences in the Italian geographical areas and in the different types of crops.

Results: A total of 177 samples (173 soil samples and 4 Dutch bulbs) collected in the period 2014–20117 in 14 Italian regions were analysed. Itraconazole-resistant *A. fumigatus* isolates grew in 16.9% of the screened samples. Differences were observed in soil samples from the three Italian geographic areas: 12.5% in the north, 15.2% in the centre and 24.1% in the south. Resistant isolates were from different cultivations, treated or officially not treated with azole fungicides. Sequencing of the *cyp51A* gene confirmed that resistance was mainly associated with the TR_{34}/L98H mutation (29/30 isolates); 1 isolate showed the G54E mutation.

Conclusions: The risk for patients to acquire multi-azole-resistant strains from the environment could have a serious impact on the management of life-threatening invasive infections. The azole resistance rate of 16.9% found in Italy requires suitable monitoring of antifungal susceptibility of clinical isolates.

1. Introduction

Aspergillus is a filamentous fungal genus present in several ecological niches worldwide, especially in soil and decaying vegetation. Asexual spores (conidia) produced by the fungus are dispersed in the air, reaching a concentration up to 100 conidia/m^3 [1], and may be inhaled and reach the upper/lower airways and alveoli. Among the more than 300 species of *Aspergillus*, *Aspergillus fumigatus* is the most common cause of a broad range of human diseases (allergic, chronic or invasive aspergillosis), the severity of which depends on the host's immune system. Global estimates range from 200 000 individuals affected by invasive aspergillosis to 1.2 million living with chronic pulmonary aspergillosis [2]. In Italy, ca. 9400 cases of invasive aspergillosis and 109 000 cases of allergic bronchopulmonary aspergillosis and chronic pulmonary aspergillosis have been estimated annually [3].

Fungi are also important pathogens for plants such as grapes and maize, although the primary consequence of their presence is the contamination of foods and feeds by mycotoxins [4]. According to an evaluation published by Fisher et al., the trend for plant-infecting fungi, including *Aspergillus* spp., grew from 0.4% in the 1990s to 5.4% in 2010, with the risk of ca. 20–30% losses in crops, a drop in the quality of products and a greater quantity of mycotoxins, threatening food security [5,6].

Antifungal drugs are used to treat fungal infections both in humans and plants. In the medical field, different classes of antifungals (polyenes, azoles, echinocandins, allylamine and pyrimidine) are employed, but only four triazoles (itraconazole, voriconazole, posaconazole and isavuconazole) are recommended for the treatment and prophylaxis of aspergillosis [7,8]. In contrast, the number of antifungals employed in agriculture is much wider, including inorganic products (copper, sulphur), carbamates and dithiocarbamates, benzimidazoles, morpholines, imidazoles and triazoles (difenoconazole, epoxiconazole, fenbuconazole, flusilazole, exaconazole, propiconazole, tebuconazole, tetraconazole) (https://eur-lex.europa.eu/legal-content/IT/ALL/?uri=CELEX%3A52006PC0778).

Fungicides represent 72% of pesticides used agriculture in Italy [European Crop Protection Association (ECPA) statistical review 2013] and triazoles represent 1.4%, with an increase of 128% in

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consumption in the period 2003–2016 (ISTAT 2016; http://agri.istat.it/sag_is_pdwout/jsp/dawinci.jsp?q=pl06a00000100000110000&an=2016&ig=1&ct=411&id=3A|45A|67A). Their large use is due to their relatively low cost, broad spectrum of activity and persistence in the environment, allowing them to remain active in soil or water for several months acting to prevent or treat fungal infections [9]. Five of the most used azole fungicides in agriculture (propiconazole, bromuconazole, epoxiconazole, difenoconazole and tebuconazole) show a molecular structure very similar to medical azoles. Since the 1990s, when these compounds were approved for crop protection, azole resistance has been increasingly reported in the environment and in humans [10].

Azole resistance in *A. fumigatus* is mainly associated with several point mutations in the *cyp51A* gene, encoding lanosterol 14α-demethylase, the target of azole antifungals [11]. Azole-resistant *A. fumigatus* have been isolated both in patients under chronic azole therapy, harbouring mutations mainly in codons G54, G138, M220, Y431 and G448, and in azole-naïve patients in whom the TR_{34}/L98H mutation appears to be prevalent [10].

In the environment, the dominant mechanism of resistance is the TR_{34}/L98H mutation, suggesting an environmental origin of resistance in clinical isolates [12–15]. More recently, other mutations in the *cyp51A* gene, such as TR_{46}/Y121F/T289A, M220 and G54, have been identified worldwide both in environmental and clinical isolates [16–20]. All these data confirm that the large use of antifungals in different fields, such as agriculture and clinical and veterinary practices, causes a selective pressure leading to the development of new mechanisms of resistance as a natural response to environmental stress.

In a previous study conducted in the period 2011–2012 on soil samples from northern Italy, azole-resistant *A. fumigatus* isolates were found in 13% of samples and the TR_{34}/L98H mutation was found in the majority of these isolates [21].

As the Italian peninsula extends for 1291 km, from latitude 47°05′31″ to 35°29′24″, with different climatic zones and with a wide variety of cultivations, the aims of the present study were to extend the research of azole-resistant *A. fumigatus* in the agricultural environment also to central and southern Italy and to investigate differences in the Italian geographical areas and in the different types of crops.

2. Materials and methods

2.1. Soil sampling and screening for azole resistance

Environmental sampling was carried out in 14 Italian regions covering the north, centre and south of Italy between 2014 and 2017.

A total of 77 sites were investigated collecting a mean of 2.2 samples (range 1–4) for a total of 173 soil samples from cereal fields (36 samples), vegetable fields (32 samples), vineyards (24 samples), olive groves (17 samples), citrus orchards (10 samples), apple orchards (6 samples), other orchards (16 samples), ornamental flowers/plants (19 samples), flowerbeds of public gardens (3 samples), hospital gardens (1 sample) and other (9 samples; tobacco, sunflower and alfalfa fields). Four Dutch bulbs were also investigated.

Samples were treated according to the method previously described by Snelders et al. [22] with some modifications. Briefly, 2 g of each sample was suspended in 8 mL of sterile distilled water supplemented with 1% Tween 20 (Sigma-Aldrich, St Louis, MO) and chloramphenicol (0.5 g/L) (Sigma-Aldrich) and was vortexed. The suspension was stored at room temperature for 60 min and then 100 μL of the supernatant was inoculated on two control plates of Sabouraud dextrose agar (SDA) (Scharlab, Mas d'En Cisa, Spain) supplemented with chloramphenicol (0.5 g/L), on two plates of SDA supplemented with chloramphenicol and itraconazole (4 mg/L) (Sigma-Aldrich), and on another two plates supplemented with chloramphenicol and voriconazole (2 mg/L) (Sigma-Aldrich). Plates were incubated at 37 °C and 42 °C and were examined after 48 h and 72 h. All of the *A. fumigatus* isolates grown on antifungal-containing agar and an equal number of isolates grown on control plates were identified by macroscopic and microscopic morphology on Czapek agar medium (Difco™; Becton Dickinson, Buccinasco, Italy).

2.2. Antifungal susceptibility testing

A. fumigatus isolates able to grow on azole-containing agar plates were tested for antifungal susceptibility to itraconazole, posaconazole and voriconazole by the broth microdilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology [23]. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were included as quality controls in each test. According to EUCAST breakpoints, isolates with minimum inhibitory concentrations (MICs) of itraconazole and voriconazole of ≥4 mg/L and those with an MIC of posaconazole of ≥0.5 mg/L were considered resistant. Those with MICs of itraconazole and voriconazole of ≤1 mg/L and those with an MIC of posaconazole of ≤0.125 mg/L were considered susceptible [24–26].

2.3. Molecular analysis of azole-resistant isolates

Azole-resistant isolates were submitted to molecular analysis. Genomic DNA was extracted from the cultured isolates using an UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). Isolates were identified by amplification and sequencing of a portion of the β-tubulin gene as described previously [27]. To identify mutations responsible for azole resistance, the *cyp51A* gene promoter was amplified using the primers PA5 and PA7 as described by Mellado et al. [11], and the *cyp51A* gene was amplified as described previously [21]. Amplicons of the *cyp51A* gene were sequenced using BigDye™ terminators (Applied Biosystems, Foster City, CA) in an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems), and nucleotide sequences were analysed using Finch TV software v.1.4.0 (Geospiza Inc.; https://digitalworldbiology.com/FinchTV). Sequence alignment of Cyp51AF1/R3, Cyp51AF2/R2 and consensus Cyp51AF1R3/AF2R2 to obtain the entire sequence of the *cyp51A* gene fragment (1168 bp) was performed using EMBOSS explorer (http://www.bioinformatics.nl/emboss-explorer). The Cyp51AF1/R2 sequence, obtained from resistant strains, has been aligned using ClustalW algorithm (https://www.ebi.ac.uk/Tools/msa/clustalo) with the *cyp51A* gene sequence of *A. fumigatus* strain 237 (GenBank accession no. **AF338659**) used as wild-type reference.

2.4. Statistical analysis

The χ^2 test was employed to compare frequencies. A *P*-value of <0.05 was considered statistically significant.

3. Results

A. fumigatus isolates grew in 44.1% (78/177) of investigated samples from all kind of fields/soil, except from hospital gardens and tobacco, sunflower and alfalfa fields.

A total of 44 samples yielded *A. fumigatus* on itraconazole-containing agar, whereas no *A. fumigatus* grew on voriconazole-containing agar. Broth microdilution and mutation in the *cyp51A* gene confirmed itraconazole resistance in isolates from 30/177 (16.9%) screened samples (Table 1). Itraconazole MICs of these

Table 1
Environmental origin of azole-resistant *Aspergillus fumigatus* isolates.

Sample source	Sites investigated (*n*)	Samples examined (*n*)	Samples with growth of *A. fumigatus* on ITR-containing agar plates (*n*)	Samples with isolates with *cyp51A* mutation/ samples investigated
Olive groves	6	17	7	7/17 (41%)
Citrus orchards	4	10	3	2/10 (20%)
Vineyards	8	24	8	4/24 (17%)
Apple orchards	2	6	4	3/6 (50%)
Other orchards	8	16	4	4/16 (25%)
Cereal fields	13	36	2	1/36 (3%)
Vegetable fields	14	32	8	8/32 (25%)
Public gardens	3	3	1	1/3 (33%)
Ornamental flowers/plants	11[a]	19[b]	6	0
Hospital garden	1	1	0	0
Others[c]	3	9	0	0
Dutch bulbs	4	4	1	0
Total	77	177	44	30/177 (16.9%)

ITR, itraconazole.
[a] Nine from botanical gardens.
[b] Seventeen from botanical gardens.
[c] Tobacco, sunflower and alfalfa fields.

isolates ranged from 4 mg/L to >16 mg/L. Twenty-two isolates were also resistant to posaconazole (MIC range 0.5–2 mg/L) (Table 2). Among the isolates resistant to both itraconazole and posaconazole, 5 were susceptible to voriconazole (MIC < 2 mg/L) and 14 showed intermediate susceptibility (MIC = 2 mg/L). Only three isolates were resistant to all azoles tested, one recovered from a public garden in the north of Italy and two from vegetable fields (one in the centre and one in the south of Italy) (Table 2). The geographical distribution of the investigated samples and of the samples with growth of azole-resistant isolates is shown in Fig. 1. The percentage of positive Italian soil samples ($n = 173$) ranged from 12.5% (5/40) in the north to 15.2% (12/79) in the centre and 24.1% (13/54) in the south. These differences were not statistically significant.

The *A. fumigatus* isolates grown on control plates (medium without antifungals) were susceptible to itraconazole (MIC range 0.06–1 mg/L), posaconazole (MIC range ≤0.03 mg/L to 0.12 mg/L) and voriconazole (MIC range 0.12–1 mg/L).

Regarding the molecular analysis, all 30 resistant isolates were identified as *A. fumigatus sensu stricto* by amplification of a fraction of the β-tubulin gene.

Sequencing of the *cyp51A* gene of the 30 resistant isolates showed the 364T → A point mutation, resulting in the L98H amino acidic substitution, associated with the 34-bp tandem repeat in the promoter region (TR_{34}/L98H) in 29 isolates. These isolates were recovered from fields treated with azoles (4 fields) or other non-azole antifungals (2 fields) but also from officially untreated fields (14 fields). For the remaining isolates no information was available (Table 2). The mutation 161A → G, resulting in the G54E amino acidic substitution, was detected only in one isolate (15-0123) recovered from a vineyard in Valpolicella (Veneto region) treated with sulfuric nitrogen but not with azole fungicides. This isolate was resistant to itraconazole and posaconazole but was susceptible to voriconazole (MIC = 0.12 mg/L).

4. Discussion

Azole-resistant *A. fumigatus* in soil samples were first detected in the Netherlands and Denmark with rates of 20.4% and 8%, respectively [22,28]. Since then, environmental azole resistance has been reported worldwide [29–32]. The first study conducted in northern Italy in the period 2011–2012 revealed the presence of 13% azole resistance in soil.

The present study, conducted in the period 2014–2017, revealed a 16.9% prevalence of resistance among the environmental samples investigated (Italian soil samples and Dutch bulbs), including 12.5% in the north, 15.2% in the centre and 24.1% in the south of Italy from soil samples. Adding the present data regarding northern Italy to those of the previous study (2011–2012) conducted exclusively in the north, we obtain a prevalence 16.1% (14/87) resistance in northern Italy, comparable with that of the central regions (15.2%) but lower, although not statistically significant, than the rate of southern regions (24.1%).

The highest Italian annual consumption of azoles in agriculture was reported in the northern regions (304 649 kg), followed by southern (138 893 kg) and central (64 295 kg) regions (ISTAT 2016; http://agri.istat.it/sag_is_pdwout/jsp/dawinci.jsp?q=pl06a0000010000011000&an=2016&ig=1&ct=411&id=3A|45A|67A). Therefore, we would have expected to find the highest rate of azole resistance in northern Italy where azoles are more used because of rainier and humid weather together with numerous apple orchards and vineyards, notoriously extensively treated with fungicides. Unfortunately, the current data about treatment of crops are likely incomplete to investigate the real correlation between the amount of azoles used and *A. fumigatus* azole resistance development. Resistant isolates were detected in crops certainly not treated with azoles as they were household vegetable gardens. Probably, *A. fumigatus* resistant conidia spread in the air reaching everywhere or, as suggested by Gisi, the simple application of azole fungicides rather than the amount is critical for the risk of resistance selection in *A. fumigatus* [33].

In the present study, apple orchards and olive groves harboured the highest number of *A. fumigatus* isolates with a *cyp51A* mutation, reaching 50% and 41% of samples, respectively. Different to our previous study where resistant *A. fumigatus* isolates were isolated from pot compost [21], no resistant isolates were found in ornamental flowers despite the practice of treating compost and of dipping plant bulbs in fungicides [34]. The absence of resistant isolates in botanical gardens is in contrast with the high rate detected in South Wales, UK (25.9%) [35].

We observed that environmental resistance is mainly associated with the TR_{34}/L98H mutation, confirming data previously obtained in Italy and worldwide. This mutation confers resistance to itraconazole and a variable pattern of susceptibility to voriconazole (intermediate or resistant) and posaconazole (susceptible, intermediate or resistant) [10]. As expected, all of the

Table 2
Results of European Committee on Antimicrobial Susceptibility Testing (EUCAST) susceptibility testing and analysis of mutations in the *cyp51A* gene.

Isolate no.	Sample source	Italian region	MIC (mg/L) [a]			Mutation	Field treatment [b]
			ITR	VRZ	PCZ	*cyp51A*	
14-0196	Citrus orchard	Piedmont (N)	8	2	0.5	TR_{34}/L98H	Not known
14-0208	Apple orchard	Lombardy (N)	8	2	1	TR_{34}/L98H	Triazoles
14-0271	Olive grove	Lombardy (N)	4	2	1	TR_{34}/L98H	Not known
15-0098	Public garden	Friuli-Venezia Giulia (N)	>16	4	0.5	TR_{34}/L98H	Not known
15-0123	Vineyard	Veneto (N)	>16	0.12	2	G54E	Other fungicide
14-0107	Other orchard	Tuscany (C)	>16	2	0.5	TR_{34}/L98H	None
14-0112	Other orchard	Tuscany (C)	>16	2	0.25	TR_{34}/L98H	None
14-0114	Other orchard	Tuscany (C)	>16	0.25	1	TR_{34}/L98H	None
14-0117	Vegetable field	Tuscany (C)	>16	4	0.5	TR_{34}/L98H	None
14-0118	Vegetable field	Tuscany (C)	4	2	0.5	TR_{34}/L98H	None
14-0120	Vineyard	Tuscany (C)	>16	2	0.25	TR_{34}/L98H	Triazoles
14-0122	Vineyard	Tuscany (C)	>16	2	0.5	TR_{34}/L98H	Triazoles
14-0126	Vineyard	Tuscany (C)	>16	2	0.5	TR_{34}/L98H	Triazoles
14-0178	Apple orchard	Marche (C)	8	2	0.25	TR_{34}/L98H	Other fungicide
14-0179	Apple orchard	Marche (C)	8	2	0.5	TR_{34}/L98H	Other fungicide
16-0082	Olive grove	Umbria (C)	>16	1	1	TR_{34}/L98H	None
16-0083	Olive grove	Umbria (C)	>16	1	1	TR_{34}/L98H	None
14-0075	Vegetable field	Puglia (S)	>16	2	0.25	TR_{34}/L98H	Not known
14-0077	Olive grove	Sicily (S)	>16	2	0.25	TR_{34}/L98H	None
14-0078	Other orchard	Sicily (S)	>16	2	0.25	TR_{34}/L98H	None
14-0079	Olive grove	Sicily (S)	>16	2	0.25	TR_{34}/L98H	None
14-0085	Vegetable field	Sicily (S)	>16	2	1	TR_{34}/L98H	None
14-0087	Cereal field	Sicily (S)	>16	2	1	TR_{34}/L98H	None
14-0135	Citrus orchard	Calabria (S)	>16	2	0.25	TR_{34}/L98H	Not known
14-0147	Olive grove	Calabria (S)	4	2	1	TR_{34}/L98H	None
14-0149	Olive grove	Calabria (S)	4	2	1	TR_{34}/L98H	None
14-0205	Vegetable field	Puglia (S)	4	2	0.5	TR_{34}/L98H	Not known
14-0212	Vegetable field	Puglia (S)	16	0.5	0.5	TR_{34}/L98H	Not known
14-0256	Vegetable field	Puglia (S)	4	2	1	TR_{34}/L98H	Not known
14-0257	Vegetable field	Puglia (S)	8	4	1	TR_{34}/L98H	Not known

MIC, minimum inhibitory concentration; ITR, itraconazole; VRZ, voriconazole; PCZ, posaconazole; N, north; C, centre; S, south.
[a] Green, susceptible; yellow, intermediate; orange, resistant.
[b] None indicates officially untreated; not known indicates data not available.

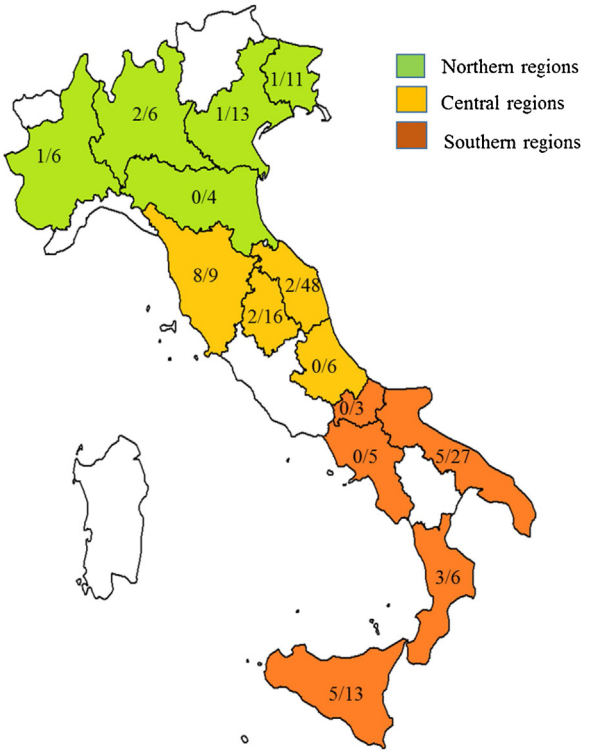

Fig. 1. Map of Italy showing the number of soil samples ($n = 173$) with growth of *Aspergillus fumigatus* resistant isolates/samples investigated.

isolates in the current study were itraconazole-resistant, and cross-resistance to posaconazole (22 isolates) and voriconazole (3 isolates) was observed. In the present study, only one isolate showed a G54E mutation. Up to now in Italy this mutation was found only in patients treated with long-term azole therapy [36]. The G54 mutants, as well as TR_{34}/L98H, are supposed to be induced in agriculture under the selective pressure of long-term exposure to fungicides [16]. However, the G54E isolate in the current came from a vineyard officially untreated with triazoles.

In the hypothesis of an environmental origin of *A. fumigatus* azole resistance, the risk for patients to acquire multi-azole-resistant strains from the environment could have a serious impact on the management of life-threatening invasive infections [6,18]. The threshold of environmental resistance at which first-line therapy with azole monotherapy should be avoided has been widely debated and most experts consider giving up azole monotherapy in geographical areas with a resistance rate >10% [18]. Therefore, the rate of 16.9% found in Italy in this study requires suitable monitoring of antifungal susceptibility of clinical isolates. Among the measures to reduce the risk of azole resistance, banning the use of specific azoles in agriculture might cause an important drop in food production. However, future new classes of medical antifungals should not be allowed in food production and particularly in non-food crop production such as that of tulips [37].

An international and multidisciplinary collaboration involving different professional roles would be required to improve the surveillance of antifungal resistance in the clinic and in the environment as well as to search for alternative fungicides minimising the resistance developing from their use.

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Competing interests

None declared.

Ethical approval

Not required.

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