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Abundance, genetic diversity and sensitivity to demethylation inhibitor fungicides of *Aspergillus fumigatus* isolates from organic substrates with special emphasis on compost

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Keywords: *Aspergillus fumigatus*, environmental substrates, quantification, compost, molecular characterization, sensitivity to DMIs

ABSTRACT

Aspergillus fumigatus is a widespread saprophytic fungus colonizing all kind of dead organic substrates (e.g. compost) but it can also cause fatal disease in animals and immunosuppressed patients. Aspergilloses are treated mainly with demethylation inhibitor (DMI) fungicides; however, resistant isolates appeared in the last twenty-five years in the medical but also environmental area. The origin of DMI resistant isolates found in the environment is still not clear. The present study aims at quantifying *A. fumigatus* in major environmental habitats and determining its sensitivity to medical and agricultural DMI fungicides. Different environmental substrates were analyzed and the genetic diversity of selected *A. fumigatus* isolates was determined by using simple sequence repeat (SSR, microsatellite) markers and β-tubulin and *cyp51A* gene sequences. *A. fumigatus* was isolated only rarely and at low frequencies from soil and organic matter from meadows and forestry but high concentrations (10^3 to 10^7 cfu/g) were detected in substrates subjected to elevated temperature processes, such as compost and silage. High genetic diversity of *A. fumigatus* isolates collected from compost was found based on SSR markers, while only moderate and no genetic diversity was observed with *cyp51A* and β-tubulin gene sequences, respectively. The phylogenic relatedness based on SSR markers yielded major clusters reflecting geographic origin (countries) and distinguished among isolates even when coming from the same substrate sample. Several amino acid substitutions were found in the *cyp51A* gene sequence but they were not associated with DMI resistance, since all isolates were fully sensitive to the four tested DMI fungicides, with exception of one isolate in combination with one fungicide. Imazalil and posaconazole were (intrinsically) most active, difenoconazole about 6 times and epoxiconazole about 20 times less active against the tested isolates. The sensitivity range (between least and most sensitive isolate) was 6 to 36 fold; there was clear cross-sensitivity/resistance amongst the four DMIs for the tested isolates. In our study, *A. fumigatus* isolates collected in Italy, Spain and Hungary from the fungus' major living habitats (compost) and commercial growing substrates were not found as potential carriers for DMI resistance in the environment.

INTRODUCTION

Aspergillus fumigatus (Fresen., syn. *Neosartorya fumigata*, O'Gorman, Fuller and Dyer) is a saprophytic filamentous fungus widespread in soil and decaying matter; it is also capable of causing serious infections in immunocompromised human hosts as invasive aspergillosis (Pringle et al.,

2015; Heinekamp et al., 2015). As all the other species of *Aspergillus*, it is ubiquitous in the environment and participates in recycling of carbon and nitrogen via degradation of organic matter (Bignell, 2014). Because of its thermo-tolerance, *A. fumigatus* is prevalent during the high-temperature phase of the composting process (Rhodes et al., 2006). It disseminates thanks to an abundant spore production; the asexual spores (conidia) are released in the environment and dispersed by air remaining viable for a long time. A sexual cycle was found in *A. fumigatus* recently and described as a teleomorph *N. fumigata* (O'Gorman et al., 2009); it seems to occur more regularly than previously thought (Teixeira et al., 2015). Generally, *A. fumigatus* conidia represent a common component of air microflora, even if concentrations rarely exceed 100 cfu/m^3 (Torpy et al., 2013). It has a global geographic distribution with presence in soil, air, and dead plant material (Warris et al., 2003; Pringle et al., 2005; Pena et al., 2015), and human exposure is unavoidable. Serious problems can occur when spores are inhaled by people with a weak immune system, due to radiation therapy, disease or immunosuppressive drug consumption (Lacey et al., 2006). *A. fumigatus* spores inhaled by susceptible hosts are deposited in bronchiolar or alveolar spaces, starting germination and invasion of lung tissues. Their small size allows the infiltration in the deepest part of the alveoli, making infection difficult to eradicate (Dagenais and Keller, 2009). The disease symptoms can range from allergies to life-threatening infections expressing acute and chronic invasive aspergillosis especially in immunocompromised patients (Heinekamp et al., 2015). High mortality in diseased patients is mainly based on limited specific diagnostic methods and insufficient treatment opportunities (Brakhage, 2005). Demethylation Inhibitor fungicides (DMIs, chemically named also as "azoles") are the major antifungal compounds used in prophylaxis and treatment of diseases caused by *A. fumigatus* (Walsh et al., 2008). DMIs prevent ergosterol biosynthesis through inhibition of the target enzyme lanosterol 14-α demethylase belonging to the cytochrome P450 family, which is encoded by *cyp51* gene (A and B paralogues) (Ghannoum and Rice, 1999; Ji et al., 2000).

Soon after DMIs (e.g. itraconazole) have been introduced for aspergillosis treatments in the 1990ies, resistance development was reported (Denning et al., 1997). Resistance was associated with several mutations in the *cyp51A* gene, and resistance evolution was considered to be based on extended DMI therapy (Dannaoui et al., 2004). Different mutations in *cyp51A* gene were found in resistant *A. fumigatus* isolates such as N22D, S52T, G54E, G138C, Q141H, P216L, F219I, M(220)different amino acids, S297T, G448S, and F495I, or in combination with a tandem repeat (TR) in the promoter region such as TR_{34}+L98H, TR_{46}+Y121F and T289A (da Silva Ferreira et al., 2004; Mellado et al., 2007; Howard et al., 2009; Snelders et al., 2009; 2011; Kuipers et al., 2011; Camps et al., 2012a; Camps et al., 2012b; Vermeulen et al., 2012). The recent isolation of DMI resistant strains from patients not previously treated with DMIs suggested that DMI resistance may not evolve exclusively in the medical area, stimulating a vivid debate about resistance origin inside

the scientific community. Resistant isolates bearing certain mutations were also detected in environmental habitats such as soil, compost and water, leading to the hypothesis that DMI resistance in patients may not be acquired exclusively from DMI treatments in medicine but also from the use of related DMI compounds in agriculture and the environmental area (Snelders et al., 2009).

Since in depth sensitivity monitoring data of *A. fumigatus* isolates collected from environmental and agricultural sites are largely missing, we decided to investigate the major living habitats of the fungus such as compost, growing substrates, organic material from meadows and forestry, soil and silage for its abundance and genetic diversity as well as sensitivity to major agricultural and medical DMIs. In this paper, the focus will be on different compost types since we consider this substrate as major source of spores released into air. However, the fungicide loads (residues) are not expected to be high enough for resistance selection in most compost types (Gisi, 2014). Several molecular tools were included for a better understanding of the genetic relationship between environmental and clinical isolates allowing assessment of potential transmission routes. A major problem with common molecular techniques such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) analyses, is the poor inter-laboratory reproducibility. Therefore, we decided to use the highly reproducible microsatellites, also referred to as simple sequence repeats (SSRs) or short tandem repeats (STRs) as non-coding molecular marker (Dutech et al., 2007; Valk et al., 2005) and to also include sequencing of the *cyp51A* gene for analysing the existence of mutations potentially coding for DMI resistance.

MATERIALS AND METHODS

Collection of environmental samples

A total of 56 samples have been analyzed in order to determine the presence and abundance of *A. fumigatus* in different agricultural and environmental habitats. From June to November 2015, samples were collected from different substrates and stored at 4° C until analysis: commercially available plant growing substrates (12), digestates (2), green and brown composts (11), organic matter from forestry and meadows (6), corn silage (6), corn plant untreated or treated with DMI fungicides (6), different soils (top 10 cm) from vegetable fields, vineyards and orchards which were untreated or treated with DMIs (13) (Table 1).

Identification, quantification and selection of *A. fumigatus* isolates

A. fumigatus was isolated and identified as describes by Franceschini et al. (2016). Briefly, 0.5 g of three subsamples were diluted in 4.5 ml Ringer solution (Merck® Darmstadt, Germany), and shaken for one hour at 100 rpm. One hundred µl of suspension and serial dilutions thereof were plated in triplicates on 15 ml of potato dextrose agar (PDA, Merck®) amended with 50 mg/l of streptomycin (Applichem, Darmstadt, Germany). Plates were sealed and incubated at 42 °C for 3-5 days. Typical grey-greenish, powdery colonies were purified, and identified by macro and micro-morphology and incubated at 50 °C in order to discriminate *A. fumigatus* and *Aspergillus lentulus*. Ten monoconidial cultures were obtained per each positive sample and stored at –80 °C in 30% glycerol solution.

Thirty-three isolates of *A. fumigatus* initially isolated from compost, originating from Italy, Spain and Hungary, were selected for further studies: molecular identification of β-tubulin gene, microsatellite analysis, DMI sensitivity testing, and molecular characterization of *cyp51A* gene (Table 2). DNA was extracted from 100 mg fungus material using EZNA® Fungal DNA extraction kit (Omega Bio-Tek, Darmstadt, Germany) according to manufacturer instructions. Molecular identification was carried out by sequencing the amplicons of the internal transcribed spacer (ITS) region and β-tubulin gene following the PCR protocols described by Glass and Donaldson (1995).

Genetic diversity assessment of *A. fumigatus* isolates

Six different microsatellite regions (STR*Af* 3A, 3B, 3C and STR*Af* 4A, 4B and 4C) of 33 *A. fumigatus* isolates were amplified using the primer pairs reported by Valk et al. (2005). Four additional environmental isolates were included in the study as reference isolates (a wild-type isolate WT, and three resistant isolates TR_{34}+L98H from UK, TR_{34}+L98H from NL, and TR_{46}+T289A from UK; kindly provided by B. Fraaije, Rothamsted Research, Harpenden, UK). Each PCR mixture (20 µl) contained 1X PCR buffer, 1 µM of forward and reverse primer, 0.2 mM DNTPs, 3.0 mM of $MgCl_2$, 1 U *Taq* DNA polymerase (Qiagen, Hilden, Germany), and 1 ng of genomic DNA. DNA amplification was performed in Bio-Rad thermocycler using the following protocol: initial denaturation step at 95 °C for 10 min, 30 cycles composed of a denaturation step at 30 s at 95 °C, an annealing step at 60 °C for 30 s and an extension step at 72° C for 1 min, followed by a final extension at 72 °C for 10 min. DNA amplification of STR*Af* 3B and STR*Af* 4B microsatellite loci required an annealing temperature equal to 65 °C. Amplified DNA fragments were separated in a 3% MetaPhor® agarose gel (Lonza, Rockland, USA). DNA fragment sizes were analyzed using PyElph 1.4 (Pavel and Vasile, 2012), and isolate weight matrices were exported in GenAlEx 6.502 software (Peakall and Smouse, 2006, 2012) for calculation of genetic distance between isolates. The dendrogram based on polymorphism of the six SSR markers was performed using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) created by MEGA 7 software (Kumar et al., 2016).

In vitro sensitivity testing of *A. fumigatus* isolates to DMIs

The antifungal activity of two agricultural DMIs (epoxiconazole, difenoconazole), one medical DMI (posaconazole) and one DMI used in agriculture, post harvest and veterinary medicine (imazalil, syn. enilconazole) was tested against 33 *A. fumigatus* isolates selected from different compost types following the EUCAST protocol with slight modifications (Lass-Flörl et al., 2006). Four environmental reference isolates were included in the study (wild-type isolate WT, and the three resistant isolates as specified above).

Epoxiconazole, difenoconazole, imazalil (PESTANAL® analytical standard) and posaconazole (VETRANAL™ analytical standard) were purchased from Sigma-Aldrich (Milan, Italy). Each fungicide was tested in five concentrations (50, 10, 2, 0.4 and 0.08 mg/l) using two replicates. 100 µl of RPMI 1640 medium (with L-glutamine, Sigma-Aldrich) supplemented with 2% glucose and 3-(N-morpholino) propanesulfonic acid (MOPS) at a final concentration of 0.165 mol/l (pH 7.0) containing a specific fungicide were dispensed per well of flat-bottom Nunc™ 96-well microplate (Thermo Fisher Scientific, Wilmington, USA). Each well was inoculated with 100 µl of *A. fumigatus* spore suspension with an inoculum density ranging from 2 to 5×10^5 conidia/ml. Fungicide-free and *A. fumigatus*-free controls were also included. The microplates were incubated at 37° C for 48 hours. Following the method described by Rodríguez-Tudela et al. (2003), the mycelial growth of each isolate was evaluated by absorbance reading (at 492 nm wavelength) using Multiskan™ FC Filter-based Microplate Photometer (Thermo Fisher Scientific). Absorbance values were transformed to growth percentage using a 0–5 scale, were 0 corresponded to the blank; 1 to 1-20% of the growth; 2 to 21-40% of the growth; 3 to 41-60% of the growth; 4 to 61-80% of the growth; and 5 to 81-100% of the growth.

Growth percentage at each fungicide concentration G_f was compared with the control (G_c) and percent growth inhibition (GI) calculated as % GI = (G_c – G_f / G_c) x 100. EC_{50} values (concentrations producing 50 % growth inhibition) were calculated using a log/logit dose response with GraphPadPrism® software (version 7.02; La Jolla, CA, USA). Log fungicide concentration vs. normalize response-variable (percent growth inhibition) procedure a logistic regression with the following formula:

$$Y = Bottom + (Top-Bottom) / (1+10\text{^}((LogEC_{50}-X) \times HillSlope))$$

where Y refers to the response (GI) and x to the fungicide concentration. Top and Bottom represent the plateaus in the units of the Y axis. Hillslope refers to the steepness of the curve (Motulsky, 2007).

The sensitivity distribution (frequency of isolates at certain EC_{50} sensitivity level) and cross-sensitivity/resistance of all 33 tested isolates were evaluated for the four fungicides as compared to

the resistant reference isolates. Cross-resistance between DMIs was assessed by regression analysis (regression coefficient r^2), comparing log EC_{50} values for individual isolates for each fungicide pair. The resistant reference isolates were included. The sensitivity (EC_{50}) distribution was plotted on a logarithmic EC_{50} scale and the median for the sensitive isolates calculated for each fungicide (resistant isolates were treated as separate group). The range of sensitivity of isolates was calculated by dividing the EC_{50} value of the least and most sensitive isolate.

Molecular characterization of the *cyp51A* gene and promoter sequences

The full coding *cyp51A* sequence of 33 *A. fumigatus* isolates was amplified by using the primers P450-A1(5'-ATGGTGCCGATGCTATGG-3') and P450-A2 (5'-CTGTCTCACTTGGATGTG-3') (Diaz-Guerra et al., 2003). PCR amplification was carried out as described by Snelders et al. (2010) with following cycling conditions: a denaturation step of 95° C for 5 min, followed by 40 cycles at 94° C for 30 s, 58° C for 45 s, and 72° C for 2 min, and a final extension step at 72° C for 7 min. Furthermore, the promoter region of the *cyp51A* gene was amplified by PCR using the primer set PA5 and PA7 as described previously by Mellado et al. (2001).

Sequence analyses

All PCR products for the ITS, β-tubulin and *cyp51A* gene sequences and its promoter region were sent for sequencing to BMR Genomics (Padua, Italy); β-tubulin and *cyp51A* gene sequences were deposited in GenBank with accession numbers as reported in Table 2. The sequences obtained were aligned and compared with those available at GenBank. GenBank *cyp51A* sequence (accession no. AF338659) was used as a control wild-type isolate, while ITZ.86, 11_0087A, 14c, and 98 sequences (Snelders et al., 2010; Prigitano et al., 2014; Van der Linden et al., 2013; Van Ingen et al., 2015, respectively) were used as resistant clinical and environmental reference isolates. Sequence alignments were carried out with Vector NTI Advance 11 software (InforMax, North Bethesda, Maryland, USA) with the Clustal W algorithm (Thompson et al., 1994). Phylogenic analyses were performed using MEGA 7. Neighbour-joining (NJ) trees were constructed with 1000 bootstrap replications.

RESULTS

Quantification of *A. fumigatus* in different substrates

The abundance of *A. fumigatus* in different environmental samples was determined and expressed as cfu/g substrate (Table 1). With the chosen method, *A. fumigatus* was detected in about half of the samples analysed. The highest concentrations were found in the three corn silage samples after 14

days of air exposure (7.4 x 10^4 and 9.0 to 23.5 × 10^6 cfu/g, respectively). However, as long as the silage material was kept under anaerobic conditions (for 270 days), concentrations were much lower (1.8 x 10^2 cfu/g) or under the detection level (10 cfu/g). Also in the starting material of corn (leaves or minced plants), the fungus was not detected. In all 11 compost types (originating from Spain, Hungary and Italy), although made of different starting material (green, brown type) and after varying processing history (static, dynamic, turning, aerated) *A. fumigatus* was detected with concentrations ranging from 1.0 × 10^2 up to 1.1 × 10^4 cfu/g of compost. In tendency, abundance was higher in green as compared to brown compost with the highest value after forced aeration. Also in 10 out of 12 commercial growing substrates (basic, mixed, peaty amendments) *A. fumigatus* was isolated successfully at concentrations ranging from 0.23 × 10^3 cfu/g in C12 (mixed growing substrate) to 1.13 x 10^3 in C11 (basic growing substrate) and about 10 fold higher values for the two substrates C1 (1.97 × 10^4 cfu/g) and C4 (2.18 × 10^4 cfu/g). Surprisingly, the concentrations were rather low (0.1 to 0.5 x 10^3) or under the detection level in organic matter from meadows and forestry, in digestates and in soil samples from vegetable fields, vineyards and orchards (Table 1).

Molecular identification and genetic similarity of *A. fumigatus* isolates

Thirty-three isolates collected from compost samples from Italy, Spain and Hungary were selected for further studies and identified as *A. fumigatus* on the basis of ITS and β-tubulin sequences (Table 2). Moreover, genetic similarity among isolates was investigated comparing the β-tubulin gene sequences. All 33 isolates were completely identical within the sequenced β-tubulin gene sequence (Table 2).

Genetic diversity of *A. fumigatus* isolates

Based on the six SSR markers, the genetic diversity of the 33 *A. fumigatus* isolates from compost was very high, every isolate was different from one another (Figure 1). Independently of the compost type (brown or green compost) and composting process (dynamic or static), the isolates could be grouped into three well separated clusters reflecting their geographic origin: Spain (E), Hungary (H) and Italy (I). The Spanish cluster was most separate but completely homogeneous, whereas the Hungarian and Italian clusters were closer together and also homogeneous with one Italian isolate (2015_1) being within the Hungarian cluster. The WT reference isolate grouped together with the Italian cluster, whereas the resistant reference isolate TR_{46}+T_{289}UK was together with the Hungarian isolates. The Hungarian isolate 215-1 and the other two resistant reference isolates (TR_{34}+L_{98}UK and NL, which were quite related) were well separated from all other isolates (Figure 1).

Based on the *cyp51A* gene with a sequence of 515 aa of the coding region, the genetic relatedness of the 33 *A. fumigatus* isolates from compost was less clear than based on SSR markers.

The phylogenetic tree obtained by Neighbor-joining analysis (Figure 2) revealed a moderate genetic diversity: a group of 29 isolates (from all three countries and compost types, including the WT isolate and four resistant reference isolates) were grouped into the main cluster, while the other 4 isolates (2015_5, BD3_9, 215_3 and GS3_1) were separated into the second cluster, and different from one another (Figure 2). It was obviously not possible to distinguish or cluster isolates based on their geographic origin, compost type or DMI sensitivity. No subclustering was observed regarding specific fungal populations.

Sensitivity of *A. fumigatus* isolates to DMIs

The sensitivity of the 33 *A. fumigatus* isolates originating from two brown and five green composts from three European countries was evaluated for the four DMI fungicides epoxiconazole, difenoconazole, imazalil and posaconazole (Table 3). In addition, one DMI sensitive WT and three DMI resistant environmental *A. fumigatus* isolates were used as controls. The intrinsic antifungal activity of the four molecules was quite different with imazalil being the most active (mean EC50 = 0.05 ± xxxx mg/L, range <0.01 – 0.08 mg/L), closely followed by posaconazole (mean EC50 = 0.08 ± xxxx mg/L, range <0.01 – 0.22 mg/L). Difenoconazole was about 5-7 times less active (mean EC50 = 0.37 ± xxxx mg/L, range 0.03 – 1.08 mg/L) and epoxiconazole about 15 – 24 times (mean EC50 = 1.21 ± xxxx mg/L, range 0.38 – 2.09 mg/L) (Table 3). The range of sensitivity (between most and least sensitive isolate) was between 5 to 8 (for epoxiconazole and imazalil) and 20 to 36 fold (for posaconazole and difenoconazole, respectively) with a more or less continuous sensitivity distribution for the isolates (Figure 3). The resistant reference isolates were clearly in a separate group quite appart from all compost isolates (Figure 3) which can be considered as fully sensitive (as is the WT isolate) against all four fungicides (Table 3), except one isolate (215_1) which was clearly outside the sensitivity distribution for posaconazole (EC50 = 1.08 mg/L) but within the sensitive isolates for the other three fungicides. This isolate originatesfrom Hungarian green compost, processed under forced aeration, and was the only isolate of five from the same substrate with an elevated EC50 against posaconazole. The three resistant reference isolates were about 25 to 70 fold less sensitive against the four fungicides than the respective value for the WT reference isolate (Table 3). Based on the tested isolates, the four fungicides were entirely cross sensitive (cross-resistant according to FRAC definition) with a rather strong correlation (r^2 = 0.65-0.95) (Figure 4).

Molecular characterization of the coding *cyp51A* gene sequence

Of the 33 *A. fumigatus* isolates, 18 did not show any cyp51A amino acid changes when compared with the wild-type isolate WT. The other 15 isolates showed amino acid substitutions ranging from 1 to 13 changes per isolate (Table 4). The most frequent amino acid changes were found at positions

27 (L to F), 47 (L to V) and 115 (F to V) (in 10 isolates), and 52 (S to T) and 120 (V to G) (in 9 isolates). Isolate 215-1 with an elevated EC50 value against posaconazole showed three amino acid changes (at 27, 33, and 38) which were present together. However, this isolate did not show any tandem repeats (34 or 46) in the promoter region, and it had the same nucleotide sequence as the wild-type reference strain (data not shown). None of the mutations L98H, Y121F, and T289A known to code for DMI resistance (Mellado et al., 2007; Kuipers et al., 2011) and present in the resistant reference isolates were detected in any of the 33 tested *A. fumigatus* isolates (Table 4).

DISCUSSION

A. fumigatus is a ubiquitous saprophytic fungus mainly colonizing dead organic material after it has gone through a thermophilic degradation process as it is typical for compost. In order to address the question raised by medical scientists (Snelders et al., 2009; Mortenesen et al., 2010; Chowdhary et al., 2012) whether DMI resistant isolates collected from patients who suffered from aspergillosis may originate also from environmental habitats, we investigated different compost types for the presence and abundance of *A. fumigatus* as compared to other substrates and characterized the genetic diversity and sensitivity to agricultural and medical DMI fungicides. *A. fumigatus* was found in all compost samples; the abundance of the fungus was similar to previously reported data (Anastasi et al., 2005; Franceschini et al., 2016). No important differences were observed among different types of compost (static vs. dynamic, green vs. brown composts), although brown composts showed a slightly lower degree of contamination than green compost. These data generally support previous reports of Franceschini et al. (2016) demonstrating that the presence of animal waste in compost substrates can lower *A. fumigatus* concentrations.

The presence of viable *A. fumigatus* propagules in corn silage samples increased rapidly at 2 weeks of air exposure leading to the highest contamination of all analysed samples (2.35×10^7 cfu/g), while it was rather low or below the detection limit as long as the material was stored anaerobically in the covered silos for 270 days. Since the abundance of the fungus was very low in original plant material and corn samples before fermentation, it is reasonable to assume that both composting and silage fermentation processes influence microbial populations by species selection. The major common feature shared by the two substrates is the high temperature during fermentation, which leads to a selection of thermo-tolerant microorganisms. *A. fumigatus* is able to grow at temperatures that approach the upper limit for eukaryotes, thanks to unique mechanisms of stress resistance, useful to bypass high-temperature processes and starting a re-colonization of the substrate in the absence of competition with other microbial species (Spadaro et al., 2015). This mechanism is particularly evident analysing *A. fumigatus* abundance in corn silage stored anaerobically for 270 days when the silo was opened and the material exposed to air. Starting from

a concentration close to the detection limit (100 cfu/g) immediately after opening, *A. fumigatus* abundance increased rapidly reaching the highest concentrations ever detected in an organic substrate (7.4×10^4 to 2.35×10^7 cfu/g).

The abundance of *A. fumigatus* in soil was very low; the fungus was not found or below the detection limit in most soil samples. Only 2 of 13 soil samples contained *A. fumigatus*, both of them collected in Moretta (North-Western Italy) in June from pepper and lettuce grown in greenhouse tunnels. These results are in contrast to some environmental surveys (Mortensen et al., 2010; Prigitano et al., 2013; Astvad et al., 2014) where *A. fumigatus* was found at high concentrations in soil. Astvad et al. (2014) suggested a seasonal dependence of *A. fumigatus* abundance in soil. Therefore, more extensive soil studies will be done in the future in order to determine whether *A. fumigatus* presence in soil depends on seasonal collecting, soil type and geographic location.

A. fumigatus isolates collected from different compost samples showed very high SSR polymorphism, confirming on one hand a high genetic variability in this species (Varga and Tóth, 2003; Bertout et al., 2001) and on the other hand the high sensitivity of microsatellite analysis (Bart-Delabesse et al., 2001; Valk et al., 2005). Genetic relatedness seems to be based primarily on geographical origin of the isolates, since microsatellite markers allowed the grouping of Spanish isolates in a homogeneous subcluster apart from Hungarian and Italian isolates. The two environmental reference isolates with the tandem repeat 34 in the promoter region from Britain and Holland were grouped together and were well separated from all other isolates. Interestingly, the other resistant reference isolate (TR_{46}+T289A) from UK was for unknown reasons closely related to the Hungarian compost isolates. A high SSR variability of *A. fumigatus* isolates from India was reported also by Chowdhary et al. (2012). Although different SSR genotypes were found inside the subclusters, all our compost isolates were sensitive to DMIs, while the two resistant reference isolates (TR_{34}+L98H) showed an identical genotype. Interestingly, the Hungarian isolate 215-1, which showed a reduced sensitivity to posaconazole, grouped closely together with these two resistant isolates.

All 33 *A. fumigatus* isolates collected from compost and tested for their sensitivity in this study were sensitive to all four tested DMI fungicides (except isolate 215-1). These results fit with previous findings from Denmark where no DMI resistant isolates were found in soil from organically grown fields and from fields where DMI fungicides were previously used (Astvad et al., 2014). However, there are several reports from Europe (including Italy) and India showing the presence of DMI resistant isolates (TR_{34}+L98H, TR_{46}+Y121F and T289A) in the environment (Snelders et al., 2009; Mortensen et al., 2010; Chowdhary et al., 2012; van der Linden et al., 2013; Prigitano et al., 2014; van Ingen et al., 2015). The reason why we did not find DMI resistant isolates in our survey could be related with the type of substrate we examined (compost) and its processing method. Although compost represents the most favourable habitat for *A. fumigatus* growth,

effective biodegradation processes might be disadvantageous for resistance evolution due to rapid degradation of fungicide residues potentially still present in treated plant material (Coppola et al., 2011).

One of the current hypothesis claims that exposure of *A. fumigatus* to DMIs largely used in crop protection and material preservation may select mutations which confer DMI resistance also in isolates producing aspergillosis (Verweij et al., 2007; Verweij et al., 2009). Isolates collected from the environment and DMI-naïve patients showed a common resistance mechanism due to an insertion of 34 or 46 base pairs in the promoter region of the *cyp51A* gene and an amino acid substitution in codon 98 or in codons 121 and 289. These changes lead to "multiple" or cross resistance (against several medical and agricultural DMIs) and may produce a high risk of disease control failures in human patients. As suggested already by others (Mansfield et al., 2010; Khan et al., 2012; Gisi, 2014), and shown again in our study, cross-resistance among all (medical and agricultural DMIs, "azoles") is not surprisung, because they share a common biochemical mode of action in sterol biosynthesis (lanosterol demethylation) encoded by *cyp51* gene. The resistance risk assessment of DMI uses in agriculture, human and vetenary medicine, material protection and other area (Gisi 2014) is very important in order to avoid further increase of resistant individuals and evolution of exixting and new mutations in *A. fumigatus* populations (Snelders et al., 2015). In isolate 215-1 from Hungarian compost found to be less sensitive against posaconazole in this study, there were no tandem repeats in the promoter region nor any of the common mutations in the *cyp51A* gene. Reduced sensitivity of this isolate may be based on three amino acid mutations found together in *cyp51A* (L27F, N33G, P38S). Furthermore, additional mechanisms could be involved in the expression of DMI resistance as mentioned byVerweij et al. (2016). To our knowledge, we found for the first time an environmental *A. fumigatus* isolate which is less sensitive only to a medical DMI (posaconazole) but not to agricultural DMIs, which might suggest a dissemination of a resistant genotype from the medical to the environmental area. This "opposite" way of genotype dissemination than previously claimed requires further attentions in the agricultural and food security sectors.

Our study clearly demonstrates a high genetic diversity in *A. fumigatus* isolates even when deriving from the same environmental sample. However, this result was visible only by using a sensitive and suitable molecular analysis technique like the (non-coding) microsatellite markers. Although sensitivity and resistance is encoded by the *cyp51* gene, the corresponding gene sequence was not able to distinguish between sensitive and resistant isolates; they were within the same clusters. The high genetic diversity in *A. fumigatus* isolates suggests rather frequent sexual and/or parasuexual (asexual) recombination at least in the compost environment requiring more profound studies in this field as already accentuated by Teixeira et al. (2015).

AUTHOR CONTRIBUTIONS

MP, DS and UG conceived and designed the project. SM and KS carried out the experiments, analysed the data and wrote the manuscript. AG, UG and MG critically revised the manuscript.

ACKNOWLEDGMENTS

The research leading to these results has received funding from the Giuseppe Boccuzzi Memorial Project and from the European Union Seventh Framework Programme (FP7/2007 2013) under grant agreement No. 289785 (Collaborative project REFERTIL: Reducing mineral fertilizers and chemicals use in agriculture by recycling treated organic waste as compost and biochar products).. We are very grateful to Dr. Bart Fraaije (Rothamsted Research, Harpenden, UK) for the supply of reference isolates of *Aspergillus fumigatus*.
Authors would like to acknowledges Fondazione CRT and AgriNewTech for supporting Lagrange Project Applied Research Scholarships for 2015 to Maria Chiara Diacono, who helped in carrying out part of the experiments.
All experimental materials were handled under a class 2 laboratory hood.

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Fig. 1 Genetic difference of stains isolated from Spanish, Hungarian and Italian composts on the basis of six loci polymorphism. Resistant isolates from England are included as reference

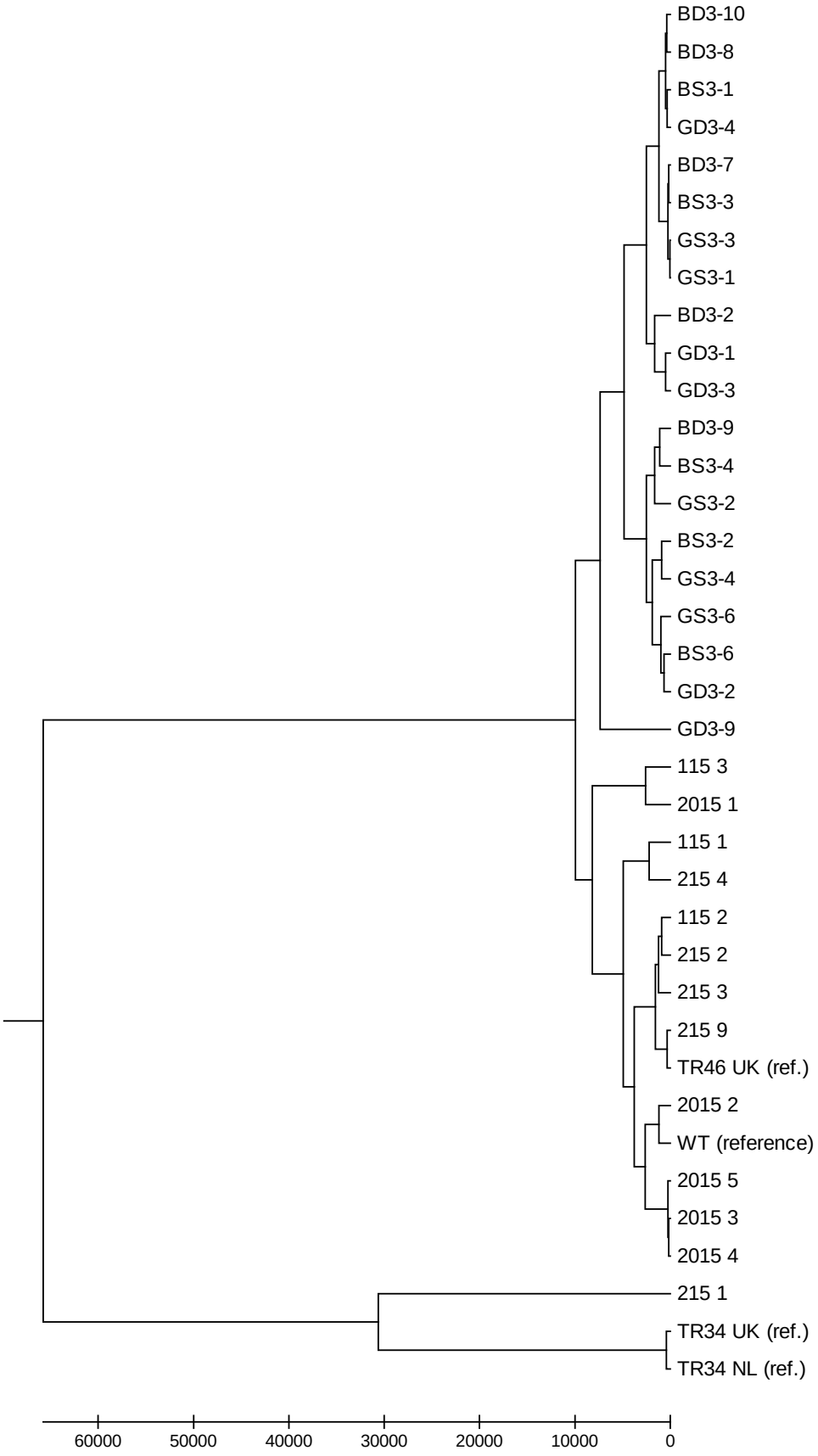

Fig. 2 Phylogenetic relationships of the environmental resistant (in red) and susceptible strains of *A. fumigatus* based on the cyp51A protein inferred by Neighbour-joining analysis. Bootstrap analysis is supported with 1000 replications.

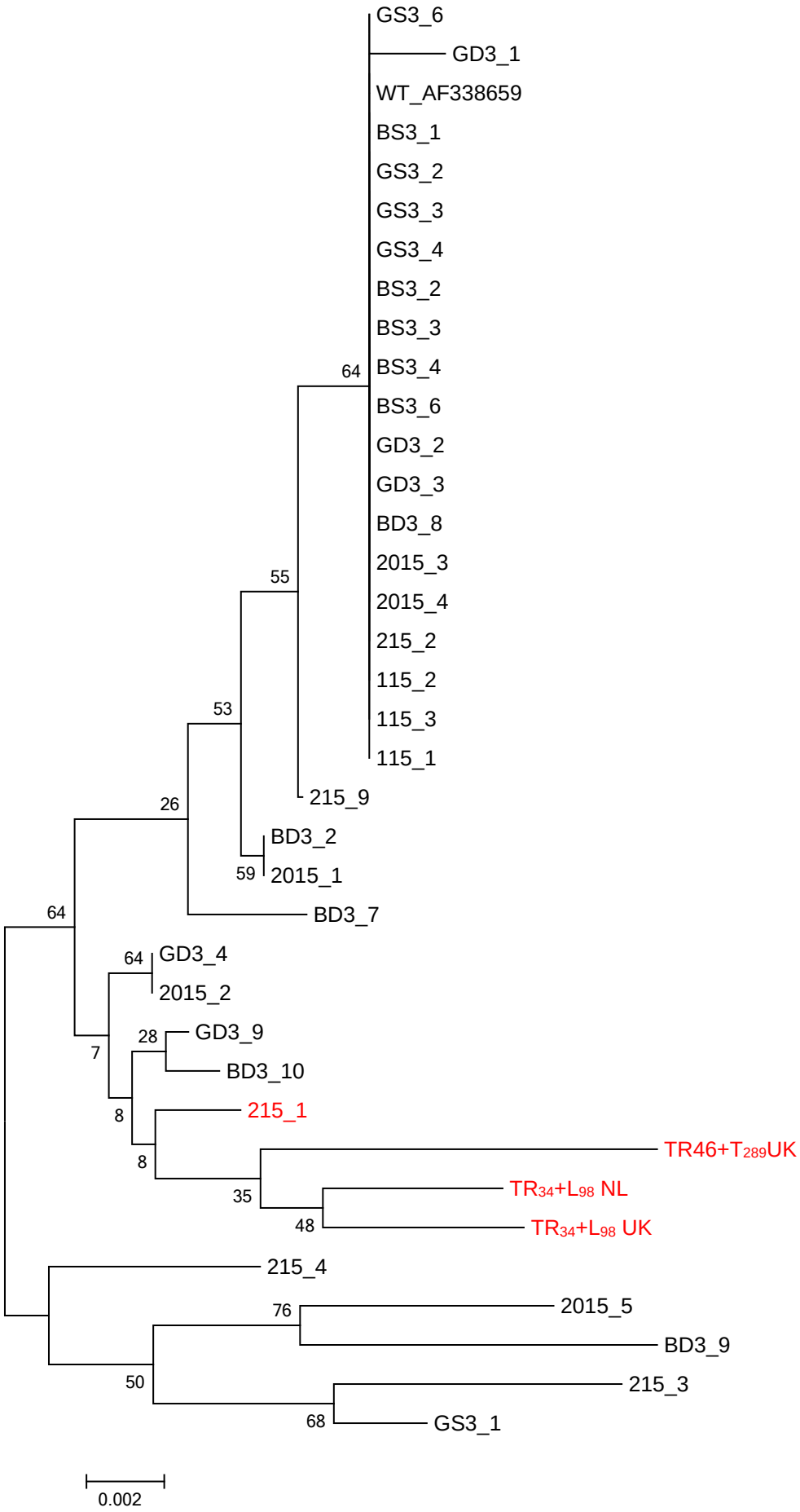

Table 1 Abundance (cfu/g) of *Aspergillus fumigatus* in different environmental substrates collected in Italy, Spain and Hungary

Sample type	Sample name	Sample description	Location of the manufacturer	*A. fumigatus* (cfu x 10^3/g)[a]
Commercial growing substrate	C1	Growing substrate (peat + green compost)	Munster, Germany	19.7 ±3.9
	C2	Growing substrate (peat)	Bergamo, Italy	n.d.
	C3	Growing substrate (peat)	Bergamo, Italy	0.5 ±0.1
	C4	Growing substrate (peat)	Munster, Germany	21.8 ±4.5
	C5	Growing substrate (peat)	Bergamo, Italy	0.7 ±0.2
	C6	Growing substrate (peat + green compost)	Bergamo, Italy	0.6±0.1
	C7	Growing substrate (peat)	Bergamo, Italy	0.35 ±0.1
	C8	Growing substrate (peat)	Munster, Germany	n.d.
	C9	Growing substrate (peat)	Bergamo, Italy	0.6±0.1
	C10	Growing substrate (peat)	Savona, Italy	0.38 ± 0.12
	C11	Growing substrate (peat)	Savona, Italy	1.13 ± 0.6
	C12	Growing substrate (peat + green compost)	Torino, Italy	0.23 ±0.1
Digestate	D1	Digestate	Novara, Italy	n.d.
	D2	Digestate	Rovigo, Italy	n.d.
Compost	GS3	Green compost from static composting	Toledo, Spain	7.5 ±1.1
	GS4	Green compost from static composting	Toledo, Spain	7.6 ±4.0
	BS3	Brown compost from static composting	Toledo, Spain	1.1 ±0.5
	BS4	Brown compost from static composting	Toledo, Spain	1.5 ±0.4
	BD3	Brown compost from dynamic composting	Toledo, Spain	2.7 ±0.6
	BD4	Brown compost from dynamic composting	Toledo, Spain	3.5 ±1.2
	GD3	Green compost from dynamic composting	Toledo, Spain	7.6 ±5.3
	GD4	Green compost from dynamic composting	Toledo, Spain	2.9 ±0.9
	COHU115	Green compost from windrow composting (turning)	Budapest, Hungary	0.1 ±0.01
	COHU215	Green compost from forced aerated process	Budapest, Hungary	10.6 ±5.6
	COIT2015	Green compost	Turin, Italy	2.65 ±0.6
Organic matter from meadow and forestry[c]	R1	Leaves in the box near the greenhouse	Grugliasco (TO), Italy	0.47±0.11
	R2	Green matrix	Grugliasco (TO), Italy	n.d.
	R3	Grass 1	Grugliasco (TO), Italy	0.50 ±0.28
	R4	Grass 2	Grugliasco (TO), Italy	n.d.
	R5	Grass/leaves next to the vineyard	Grugliasco (TO), Italy	n.d.
	R6	Wood materials next to the vineyard	Grugliasco (TO), Italy	n.d.
Corn leaves	F1	corn - control	Piedmont, Italy	n.d.
	F1T	corn – Fungicide double dosage	Piedmont, Italy	n.d.
	Fu 13T	corn – Fungicide single dosage	Piedmont, Italy	n.d.
Corn plant	P1	Minced corn plant - Control	Piedmont, Italy	n.d.
	P2	Minced corn plant - Fungicide double dosage	Piedmont, Italy	n.d.
	P3	Minced corn plant - Fungicide single dosage	Piedmont, Italy	n.d.
Corn Silage	Si1	Decaying silage. 14 day air exposure	Piedmont, Italy	74.0±9.0
	Si2	Decaying silage. 14 day air exposure	Piedmont, Italy	$9.02x10^3$±10.0
	Si3	Decaying silage. 14 day air exposure	Piedmont, Italy	$2.35x10^4$±0.2
	Si4	Silage stored for 270 days	Piedmont, Italy	n.d.
	Si5	Silage stored for 270 days	Piedmont, Italy	0.18 ±0.14
	Si6	Silage stored for 270 days	Piedmont, Italy	n.d.
Soil	S1	Soil from tomato field	Boves (CN), Italy	n.d.
	S2	Soil from pepper field	Boves (CN), Italy	n.d.
	S3	Soil from pepper field	Moretta (CN), Italy	1.2 ±0.4
	S4	Soil from lettuce field	Moretta (CN), Italy	0.1 ±0.06
	S5	Vineyard soil (row)	Grugliasco (TO), Italy	n.d.
	S6	Vineyard soil (between rows)	Grugliasco (TO), Italy	n.d.
	S7	Vineyard soil (external edge)	Grugliasco (TO), Italy	n.d.
	S8	Orchard soil (row)	Grugliasco (TO), Italy	n.d.
	S9	Orchard soil (between rows)	Grugliasco (TO), Italy	n.d.
	S10	Orchard soil (external edge)	Grugliasco (TO), Italy	n.d.
	S11	Orchard soil external edge)	Cervignasco (CN), Italy	n.d.
	S12	Orchard soil	Cervignasco (CN), Italy	n.d.
	S13	Orchard soil	Bombonina (CN), Italy	n.d.

[a] Concentrations are expressed as means per gram dry weight ± standard deviation; n.d. not detected or below 10 cfu/g; [b] Location refers to the place of the producer; [c] Organic matter from forestry and meadows were collected in Grugliasco (Italy) at University Campus of Tu

Table 2 | List of *Aspergillus fumigatus* isolates selected for molecular characterization and sensitivity assays

Strain	Origin	Host	Accession no. (β-tubulin)	Accession no. (*cyp51A*)
BD3 2	Spain	Brown compost	In arrival all	KY495895
BD3 10	Spain	Brown compost		KY495896
BD3 9	Spain	Brown compost		KY495897
BD3 8	Spain	Brown compost		KY495898
BD3 7	Spain	Brown compost		KY495899
BS3 6	Spain	Brown compost		KY495900
BS3 4	Spain	Brown compost		KY495901
BS3 3	Spain	Brown compost		KY495902
BS3 2	Spain	Brown compost		KY495903
BS3 1	Spain	Brown compost		KY495904
GS3 6	Spain	Green compost		KY495905
GS3 4	Spain	Green compost		KY495906
GS3 3	Spain	Green compost		KY495907
GS3 2	Spain	Green compost		KY495908
GS3 1	Spain	Green compost		KY495909
GD3 1	Spain	Green compost		KY495910
GD3 2	Spain	Green compost		KY495911
GD3 3	Spain	Green compost		KY495912
GD3 4	Spain	Green compost		KY495913
GD3 9	Spain	Green compost		KY495914
115 1	Hungary	Green compost		KY495915
115 2	Hungary	Green compost		KY495916
115 3	Hungary	Green compost		KY495917
215 1	Hungary	Green compost		KY495918
215 2	Hungary	Green compost		KY495919
215 3	Hungary	Green compost		KY495920
215 4	Hungary	Green compost		KY495921
215 9	Hungary	Green compost		KY495922
2015 1	Italy	Green compost		KY495923
2015 2	Italy	Green compost		KY495924
2015 3	Italy	Green compost		KY495925
2015 4	Italy	Green compost		KY495926
2015 5	Italy	Green compost		KY495927
WT	The Netherlands	Reference (E)[a]	RRC	RRC
TR_{34}+L_{98} UK	United Kingdom	Reference (E)	RRC	RRC
TR_{34}+L_{98} NL	The Netherlands	Reference (E)	RRC	RRC
TR_{46}+T_{289}UK	United Kingdom	Reference (E)	RRC	RRC

[a] (E) - environmental origin

[b] RRC - Rothamsted Research Collection

Table 3 | Sensitivity to four DMI fungicides (EC50) of *Aspergillus fumigatus* isolates collected from different compost types.

Isolate	EC_{50} (mg/L)			
	Epoxiconazole	Difenoconazole	Imazalil	Posaconazole
BD3 2	1.20	1.08	0.04	<0.01
BD3 10	2.03	0.42	0.07	<0.01
BD3 9	1.96	0.31	0.07	0.22
BD3 8	2.07	1.08	0.07	0.09
BD3 7	0.98	0.67	<0.01	<0.01
BS3 6	1.53	1.08	0.07	0.08
BS3 4	2.09	1.08	0.01	0.22
BS3 3	1.25	0.40	0.01	0.22
BS3 2	1.18	1.08	0.01	0.22
BS3 1	1.33	0.40	0.07	0.22
GS3 6	0.85	0.42	0.07	0.22
GS3 4	1.20	0.42	0.07	0.09
GS3 3	1.87	0.40	0.07	0.08
GS3 2	1.13	1.08	0.07	0.22
GS3 1	2.04	0.03	0.07	<0.01
GD3 1	0.85	0.12	<0.01	<0.01
GD3 2	1.90	0.12	0.08	<0.01
GD3 3	0.81	0.21	<0.01	<0.01
GD3 4	1.20	0.14	0.07	<0.01
GD3 9	1.65	0.29	<0.01	<0.01
115 1	0.56	0.07	0.05	0.07
115 2	1.82	0.07	0.05	0.07
115 3	0.74	0.07	0.01	0.07
215 1	1.86	0.08	0.07	1.08
215 2	0.73	0.08	0.01	0.07
215 3	0.41	0.21	0.05	0.07
215 4	0.89	0.22	0.05	0.07
215 9	0.38	0.05	0.07	0.07
2015 1	0.65	0.08	0.07	<0.01
2015 2	0.42	0.08	0.07	<0.01
2015 3	0.43	0.08	0.07	0.04
2015 4	1.15	0.18	0.07	0.07
2015 5	0.89	0.08	0.07	<0.01
WT	0.58	0.09	0.07	0.07
TR_{34}+L_{98} UK	23.54	3.54	1.88	2.05
TR_{34}+L_{98} NL	23.54	4.47	1.88	5.40
TR46+T_{289}UK	23.54	47.84	1.28	2.19

Table 4 | Amino acid changes in the *cyp51A* gene sequence of *Aspergilus fumigatus* isolates from compost.

Isolate	cyp51A mutations
BD3 2	L(27)F L(47)V
BD3 10	L(27)F P(38)S L(47)V S(52)T F(115)V V(120)G
BD3 9	N(33)S P(45)Q L(47)V S(49)T S(52)T I(71)L K(99)R E(105)Q F(115)V V(120)G S(142)P E(154)D A(204)D
BD3 8	-
BD3 7	V(24)D L(27)F L(47)V S(52)T
BS3 6	-
BS3 4	-
BS3 3	-
BS3 2	-
BS3 1	-
GS3 6	-
GS3 4	-
GS3 3	-
GS3 2	-
GS3 1	L(47)V S(52)T I(71)L F(115)V V(120)G K(127)Q E(152)G S(169)T A(204)D P(216)A
GD3 1	T(182)S
GD3 2	-
GD3 3	-
GD3 4	L(27)F L(47)V S(52)T F(115)V V(120)G
GD3 9	P(38)S L(47)V S(52)T F(115)V V(120)G
115 1	-
115 2	-
115 3	-
215 1	L(27)F N(33)G P(38)S L(47)V F(115)V V(120)G
215 2	-
215 3	L(27)F N(33)G P(45)Q S(52)T F(115)V V(120)G K(127)Q E(145)Q E(152)G S(169)T A(204)D P(216)A
215 4	L(27)F N(33)G S(52)T F(115)V V(120)G K(127)Q E(130)G S(169)T
215 9	L(27)F
2015 1	L(27)F L(47)V
2015 2	L(27)F L(47)V S(52)T F(115)V V(120)G
2015 3	-
2015 4	-
2015 5	P(45)Q S(49)T S(52)T I(71)L K(99)R F(115)V
AF338659	-
11-0087A[a] (E)[b]	L(98)H
14[c] (E)	Y(121)F T(289)A
ITZ.86+[d] (C)[e]	L(98)H
98[f] (C)	Y(121)F T(289)A

[a] Prigitano et al. (2014)
[b] (E) – environmental
[c] Van der Linden et al. (2013)
[d] Snelders et al. (2010)
[e] (C) – clinical
[f] Van Ingen et al. (2015)