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Abstract

BACKGROUND: *Aspergillus fumigatus*, the causal agent of aspergillosis in humans, is commonly present as saprophyte in various organic substrates, such as spoiled silages. Aspergillosis is generally combated with demethylation inhibitor (DMI) fungicides, but the recent appearance of

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resistant medical and environmental strains made current treatment strategies less reliable. The goal of this study was to determine the evolution of *A. fumigatus* populations during the ensiling process of whole-crop corn, high moisture corn and wet grain corn, and to monitor the sensitivity of isolates from treated and untreated fields to one medical and one agricultural DMI fungicide.

RESULTS: *A. fumigatus* was isolated from fresh forage at harvest at rather low concentrations (10^2 cfu/g). The low frequency lingered during the silage process (at 60 and 160 days), whereas it significantly increased during air exposure (at 7 and 14 days of air exposure). Field treatment of corn with a mixture of prothioconazole and tebuconazole did not affect the sensitivity of *A. fumigatus* isolates. One isolate out of 29 coming from the untreated plot was resistant to voriconazole. A unique amino acid substitution (E427K) was detected in the *cyp51A* gene of 10 out of 12 sequenced isolates, but it was not associated to DMI resistance.

CONCLUSION: *A. fumigatus* significantly increased during aerobic deterioration of ensilaged corn after silo opening, compared to the low presence in fresh corn and during ensiling. Field treatment of corn with DMI fungicides did not affect the sensitivity of *A. fumigatus* isolates collected from fresh and ensiled corn.

Keywords: *Aspergillus fumigatus*; corn; ensiling; environmental samples; fungicide treatment; quantification; sensitivity to DMIs

INTRODUCTION

Aspergillus fumigatus (Fresen., syn. *Neosartorya fumigata*, O'Gorman, Fuller and Dyer), common soil inhabiting fungus and saprophyte of decomposing organic matter, is an important human pathogen provoking serious to life-threatening diseases in immunocompromised patients, such as invasive pulmonary aspergillosis.[1,2] It propagates by releasing into the atmosphere a large amount of asexual spores (conidia) which stay viable for a prolonged period of time.[3] A teleomorph of *A. fumigatus* was recently found and named as *N. fumigata*.[4] The conidia are resistant to high temperatures, with the ability to germinate from 20 °C to 50 °C.[5] The fungus can survive in extreme environments at high concentrations of CO_2 and N_2, limited nutrients and oxidative stress.[6] Besides, it represents an important threat for bred animals, especially cattle, due to its capacity to colonize feed and fibre entering the ensiling process.[5,7]

Ensiling is one of the most effective techniques to conserve forages, cereals and other feeds in dairy and beef farm. The conservation of feed is due to an acidification by lactic acid bacteria (LAB) that convert water soluble carbohydrates into lactic and other acids under anaerobic conditions. The acidification and the anaerobic conditions inhibit the growth of aerobic microorganisms, such as yeast and moulds. During the feed-out, silages are exposed to the air and, after an initial stable phase, the silages begin to deteriorate as a result of the activity of aerobic microorganisms, first yeast and later moulds.[8,9] High concentration of *A. fumigatus* capable of producing thermogenic mycotoxins have been found repeatedly in aerobic deteriorated silages and forages for dairy cows.[10-13]

Demethylation inhibitor (DMI) fungicides, chemically known as “azoles”, are the main antifungals used for prophylactic and therapeutic treatments of diseases caused by *A. fumigatus* in humans and animals.[14] DMIs are also commonly used in agriculture as fungicides against a wide range of fungal pathogens of plants.[15] DMIs bind the cytochrome P450 mediated lanosterol 14-α demethylase, encoded by two paralogues (A and B) of the *cyp51* gene, thus preventing the biosynthesis of ergosterol, the principal fungal cell membrane sterol.[16,17]

Soon after the introduction of medical DMIs for therapy and prophylaxis of aspergilloses, resistant isolates were recorded.[18] Resistance was attributed to few mutations in the *cyp51A* gene and started to expand in DMI treated patients in several countries. The main mutations involved in DMI resistance occurred in *cyp51A* gene causing amino acid changes at positions 98, 121 and 289 (L98H, Y121F and T289A), together with modifications of the promoter region by the presence of a tandem repeat (TR_{34} or TR_{46}). DMI-resistant strains recently isolated from DMI naïve patients indicated that resistance might originate not only from clinical treatments, but may also have environmental sources.[19,20] DMI resistant isolates carrying *cyp51A* mutations and TR promoter modifications were also reported to occur in different environmental matrices, such as soil and compost enforcing the suggestion that DMI resistance in patients may originate also from the application of similar DMIs in agriculture which were used to control plant diseases but have hit also *A. fumigatus* as collateral effect thereafter producing resistant conidia which were inhalated by the patient.[21]

Since DMI sensitivity evaluation of *A. fumigatus* from agricultural and environmental habitats, e.g. compost[22,23] is still limited, we carried out this study in corn silage, one of the preferred habitats of *A. fumigatus* on dairy farms. Silage is considered one of the major sources of conidia release of *A.*

fumigatus into air[24] and emphasis has been given to different corn silage types (whole-crop corn, high moisture corn and wet grain silages) from corn harvest to conservation and aerobic deterioration of silage during feed-out phase.

MATERIALS AND METHODS

Field experiment and corn harvest

The experiment was carried out at the experimental farm of the University of Turin in the western Po plain, northern Italy (44°53' N, 7°41' E, altitude 232 m a.s.l.) on corn (*Zea mays* L.). The field was cultivated with maize under fungicide spray programme on the previous year. Corn (P1517W, Pioneer Hi-Bred Italia Srl, Cremona, Italy) was sown in April 2016, at expected planting density of 75,000 seeds/ha. At the beginning of flowering (61 BBCH scale), half of the plots were treated with a commercial fungicide (Prosaro®, Bayer Crop Science: 12.7 g prothioconazole and 12.7 g tebuconazole per 100 g) applied at the dose of 1.0 l/ha (twice the commercial dose). Corn was harvested at around 2/3 milk line stage as whole-crop corn and at the black line stage for high moisture corn and wet grain silages. Fresh forage for whole-crop corn and high moisture corn were harvested, using a precision forage harvester (Claas Jaguar 950, Claas, Harsewinkel, Germany) and then ensiled in 20 l plastic silos. Wet grain was harvested with a grain harvester (Wintersteiger Quantum plot combine, Wintersteiger AG, Ried, Austria).

Sample preparation and analyses

Corn plants were harvested and conserved as: a) whole-crop corn silage **(WCC)** = ensiling of the whole chopped plant; b) high moisture corn silage **(HMC)** = ensiling of the chopped ear (cob and grain); c) wet grain silage **(WG)** = ensiling of the whole wet grain.

Fresh forages were sampled and ensiled (about 10 to 12, 13 to 15, 16 to 18 kg of wet forage for WCC, HMC and WG, respectively) into 20 l plastic silos equipped with a lid that only enabled gas release. The forages were hand-packed and final packing densities, on a wet basis, were 490 ± 33 kg fresh matter (FM)/m^3, 698 ± 23 kg FM/m^3, 852 ± 22 kg FM/m^3 for WCC, HMC and WG, respectively. All silos were filled within three hours. The silos were weighed, conserved at ambient temperature and opened after 60 and 160 days. At opening, the content of each silo was mixed thoroughly and sub-sampled to determine the DM content, the fermentative and chemical characteristics and the microbial counts. After sampling, the silages were subjected to an aerobic stability test and the silages were sampled after 7 d and 14 d of aerobic exposure in order to quantify the microbial changes in the silages during air exposure.

Pre-ensiled material and silages were split into four subsamples. One sub-sample was immediately analysed for DM content by oven drying at 80 °C for 24 h. Dry matter was corrected according to Porter and Murray[25], in order to consider the losses of volatile compounds that can take place at 80 °C. The second fresh sub-sample was used to determine the water activity (a_w), pH and nitrate (NO_3^-) concentration. The water activity was measured at 25 °C on a fresh sample using an AquaLab Series 3TE (Decagon Devices Inc., Pullman, WA), which adopts the chilled-mirror dew point technique. The fresh forage was extracted for pH, and NO_3^- determination, using a Stomacher blender (Seward Ltd, Worthing, UK), for 4 min in distilled water at a 9:1 water-to-sample material (fresh weight) ratio. The total nitrate concentration was determined in the water extract, through

semi-quantitative analysis, using Merckoquant test strips (Merck, Darmstadt, Germany; detection limit 100 mg NO_3/kg). The pH was determined using specific electrodes. The third subsample was used for the microbial analyses. For the microbial counts, 30 g of sample was transferred into a sterile homogenization bag, suspended 1:10 w/v in a peptone salt solution (1 g of bacteriological peptone and 9 g of sodium chloride per litre) and homogenized for 4 min in a laboratory Stomacher blender (Seward Ltd, London, UK). Serial dilutions were prepared and the yeast and mould numbers were determined using the pour plate technique with 40.0 g/l of Yeast Extract Glucose Chloramphenicol Agar (YGC agar, DIFCO, West Molesey, Surrey, UK) after incubation at 25 °C for 3 and 5 d for yeasts and moulds, respectively. Yeast and mould colonies were divided in morphotypes, based on the macro and micro morphological features observed. Subsequently, yeast and moulds were counted separately on plates that yielded 1 to 100 colony forming units (cfu). The LAB were determined on MRS agar (Merck, Whitehouse Station, NY) with added natamycin (0.25 g/l), by incubating Petri plates at 30 °C for 3 d under anaerobic conditions. Since LAB are facultative anaerobic, the choice of anaerobic incubation was made to improve the selectivity of the media against *Bacillus* spp. For each type of matrix, sampling was carried out in 6 replicates, 3 replicates for treated plant material (T) with DMIs, and 3 replicates for untreated plants (NT) (Table 1). The fourth sub-sample was used to identification, quantification and selection of *Aspergillus fumigatus*. Sub-amples from which *A. fumigatus* was isolated are indicated in Table 2.

Morphological and molecular identification, and quantification of *Aspergillus fumigatus* isolates

Morphological identification was performed by isolation of *Aspergillus fumigatus* according to the protocol by Franceschini *et al.*[26] Samples (30 g) were collected from each of the three corn mixtures (DMI-treated and untreated) at pre-silage and silage conservation periods. The samples were resuspended (1:10 w/v) in a peptone salt solution (1 g of bacteriological peptone and 9 g of sodium chloride/l) and placed into a sterile bag for a 4 min homogenization by laboratory Stomacher blender (Seward Ltd, London, UK). One hundred µl of suspension and its serial dilutions (with a factor 10) were grown in triplicate onto potato dextrose agar (PDA, Merck, Darmstadt, Germany) amended with streptomycin (50 mg/l; Merck). Plates were sealed with parafilm and incubated at 50 °C for 4-5 days to select *A. fumigatus* from other fungal species.

Identification of Aspergillus-like colonies was based on the selection of the grey-green and powdery colonies and observation of their macro- and micro-morphological characteristics. Surviving colonies of *A. fumigatus* were quantified by considering the dilution factor and the average of three replicates.

Three monoconidial cultures were prepared per each isolate and they were stored at –80 °C in 30% glycerol. Fifty isolates of *A. fumigatus* initially isolated from the different matrices were chosen for further study: molecular characterization of the β-tubulin (*tub2*), and *cyp51A* genes, molecular identification of the mating type and DMI sensitivity assays (Table 3). Fungal DNA was extracted from 100 mg fungal mycelium using EZNA® Fungal DNA extraction kit (Omega Bio-Tek, Darmstadt, Germany) according to the manufacturer protocol. The *tub2* gene portion was amplified following the PCR protocol described by Glass and Donaldson[27], and subsequent sequencing of the tub2 amplicons.

Mating types identification

A. fumigatus mating types were determined by multiplex PCR as decribed by Paoletti *et al.*[28] using specific primers *MAT-1* (AFM1, 5′-CCTTGACGCGATGGGGTGG-3′) and *MAT-2* (AFM2, 5′-CGCTCCTCATCAGAACAACTCG-3′), along with common primer AFM3 (5′-CGGAAATCTGATGTCGCCACG-3′). The PCR-25 µl reaction included 10 ng of fungal DNA, 1× PCR buffer, 1.4 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer (AFM1 and AFM2), 0.8 µM primer AFM3, and 1 U Taq DNA polymerase (Qiagen, Hilden, Germany). PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products (5 µl) were stained with RedGel (Biotium, Hayward, CA, USA) in a 1.2% agarose gel and following the electrophoresis visualized under UV light. The mating type of isolates was determined by the amplicon size: 834 bp (MAT-1), and 438 bp (MAT-2).

DMI sensitivity of *A. fumigatus* by *in vitro* assays

Sensitivity of *A. fumigatus* against imazalil (agricultural/veterinary DMI), and voriconazole (medical DMI) was evaluated by using the EUCAST protocol[29] with some modifications applying the FRAC standard protocol regarding plant pathogens (http://www.frac.info/monitoring-methods). Three reference environmental isolates were also included in the study: a wild-type (WT) and resistant isolate (TR_{34}+L98H) from NL, and resistant isolate (TR_{46}+Y121F+T289A) from UK (kindly provided by B. Fraaije, Rothamsted Research, UK).

Imazalil (Pestanal® analytical standard; Sigma-Aldrich, Milan, Italy) and voriconazole (Vetranal™ analytical standard; Sigma-Aldrich) were applied in five concentrations (50, 10, 2, 0.4 and 0.08

mg/l) and in two replicates. The PMI 1640/L-glutamine medium (Sigma-Aldrich) supplemented with 2% glucose and 3-(N-morpholino) propanesulfonic acid (MOPS) at a 0.165 mol/l concentration (pH 7.0), was loaded together with each fungicide in flat-bottom Nunc™ 96-well microplate (100 µl/well; Thermo Fisher Scientific, Wilmington, USA). Then, 100 µl of the fungal spore suspension at 2 to 5 × 10^5 conidia/ml was loaded per well. The substrates without fungicide and without *A. fumigatus* were used as controls. The microplates were kept at 37 °C for 48 hours and then the mycelial growth was estimated visually[29] by a turbidity-grade scale (0-5), where 0 indicates optically clear well and 5 refers to no turbidity change with respect to the turbidity of the fungicide-free control.

Percent growth inhibition (GI) was determined by formula as % GI = (G_c – G_f / G_c) x 100 where G_c refers to the control growth and G_f refers to the growth percentage at each concentration of fungicide. A log/logit dose response was chosen for EC_{50} calculation (concentrations inducing 50 % of growth inhibition) by GraphPadPrism® software (7.02 v.; La Jolla, CA, USA). A fungicide concentration (log) vs. normalized response-variable (percentage of the growth inhibition) was calculated as: Y = Bottom + (Top-Bottom) / {1+10[(LogEC_{50}-X) × HillSlope]} where Y indicates the response (GI) and X the fungicide concentration. Top and Bottom refers to the plateaus of the Y axis units. Hillslope indicates the steepness of the curve.[30] According to the sensitivity of *A. fumigatus* to imazalil and voriconazole, an isolate was considered as less sensitive if its EC_{50} was at least five times the mean EC_{50} of sensitive (S) isolates (0.26 and 0.31 µg/ml for imazalil and voriconazole, respectively), and resistant (R) if the EC_{50} showed 50 times the mean EC_{50} of S) isolates. The sensitivity distribution of all 50 isolates were then estimated for both fungicides and compared to the reference isolates.

Molecular characterization of the *cyp51A* gene sequences

PCR amplification was carried out for the *cyp51A* coding sequence of 12 *A. fumigatus* isolates. The primers P450-A1 (5'-ATGGTGCCGATGCTATGG-3') and P450-A2 (5'-CTGTCTCACTTGGATGTG-3')[31] were used for the PCR applying the conditions described by Snelders *et al.*[32] Initial denaturation at 95 °C for 5 min, was followed by 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 2 min for 40 cycles, and by a final extension at 72 °C for 7 min.

Sequence analyses

The PCR products of the *tub2* and *cyp51A* genes were sequenced at BMR Genomics (Padua, Italy) and deposited in GenBank with the accession numbers reported in Table 3 for *tub2* and for *cyp51A*. The sequences were compared with those at NCBI GenBank database. Nucleotide and amino-acid alignments were performed with Vector NTI Advance 11 software (InforMax, North Bethesda, Maryland, USA) by the Clustal W algorithm.[33] Phylogenetic analyses were carried out by MEGA 7 software[34] by creating the neighbour-joining (NJ) trees at 1000 bootstrap replications.

RESULTS

Silage characteristics

The chemical characteristics and microbial counts on corn plants and in silages at harvest, silo opening and after air exposure are summarized in Table 1. At harvest, the DM content was on average around 46%, 65% and 75% for WCC, HMC and WG, respectively. The WG showed

slightly higher pH and lower LAB, yeast and mould counts compared to WCC and HMC. After 60 d of conservation the pH reached values below 4 in WCC and HMC, in which the LAB counts increased whereas the yeast and mould counts decreased. The pH of WG at opening did not show significant differences compared to harvest although an increase of LAB was detected. After 160 d of conservation, the silages showed characteristics similar to those detected at 60 d except for a further reduction of the mould counts (on average from 3.16×10^2 cfu/g to below 0.32×10^2 cfu/g). After 7 days of air exposure, both for 60 and 160 d of conservation, yeast counts increased in all the matrices. A further development of aerobic microorganisms was detected after 14 d of air exposure, with mould counts higher than 1.00×10^7 cfu/g in WCC and WG. After 7 days of air exposure, HMC had a pH close to that at silo opening; mould counts were below the detection limit, whereas in WCC and WG, these parameters increased after 14 d of air exposure.

Quantification of *A. fumigatus* in silages

A. fumigatus frequency was expressed as cfu/g dry weight of silage (Fig. 1). *A. fumigatus* was isolated from the corn samples at harvest in rather low concentrations: 2.60×10^3 cfu/g in WCC and 0.20×10^3 cfu/g in WG, while in HMC it was below the detection limit (<10 cfu/g). During silage conservation, *A. fumigatus* counts was stable after 60 and 160 days of conservation, whereas it significantly increased during air exposure (at 7 and 14 days of air exposure after silo opening). In the 60 days of conservation samples, the fungal presence was quite high in WCC and HMC (3.50×10^7 and 1.37×10^7 cfu/g, respectively) after 14 days of air exposure. The highest concentration of *A. fumigatus* was measured after 160 days of conservation, on the samples after 14 days of air exposure in WCC (2.63×10^8 cfu/g), followed by wet grain (1.28×10^7 cfu/g).

Molecular identification of *A. fumigatus* isolates and mating type

Fifty *Aspergillus*-like colonies were chosen for molecular identification based on morphological characterization and survival at 50 °C. One hundred percent of isolates collected from corn samples at harvest and after ensiling were confirmed as *A. fumigatus* by BLASTn analysis of the β-tubulin sequences (Table 3), and selected for further studies.

The isolates were further analysed by mating-type specific PCR resulting in 26% MAT1-1 and 74% MAT1-2 idiomorph (Table 3). No isolate showed simultaneously amplification of both mating-types.

Sensitivity of *A. fumigatus* isolates to imazalil and voriconazole

Fifty *A. fumigatus* isolates collected at harvest, silage opening and subsequent air exposure were evaluated for sensitivity to imazalil and voriconazole (Table 3). The intrinsic antifungal activity of the two DMI fungicides was similar: for imazalil the mean EC_{50} was 0.31 mg/l, ranging from <0.01 to 1.47 mg/l, and for voriconazole the mean EC_{50} was 0.26 mg/l, ranging from <0.01 to 1.48 mg/l.

The sensitivity distribution of *A. fumigatus* isolates was rather continuous for both fungicides without a clear separation between sensitive (EC50 < 1.50 mgl) and less sensitive isolates (EC50 > 1.51 mg/l), only one resistant isolate against voriconazole (EC50 > 10 mg/l) was detected (Fig. 2). No significant differences in sensitivity to either imazalil or voriconazole were found between the isolates coming from the treated and those coming from the untreated plots (Fig. 2). The resistant reference isolate (TR_{34}+L98) was clearly separated from all silage isolates for imazalil, but it grouped together with one silage isolate (HMC.N.60S.1C) for voriconazole (Fig. 2 and Table 3).

Ten (for imazalil) and three (for voriconazole) isolates were distributed in an intermediate group between sensitive and resistant isolates, together with another reference isolate (TR_{46} +Y121F +T289A) representing the group of intermediate resistant isolates. The resistant reference isolate TR_{34}+L98 was 34 fold less sensitive against voriconazole and 138 fold less sensitive against imazalil, with respect to the value of the WT reference isolate (Table 3).

The *cyp51A* molecular characterization

Out of 12 isolates, the cyp51A amino acid substitution (E427K) was found in 10 corn-silage *A. fumigatus* isolates (either DMI sensitive or less sensitive isolates, originating from either treated or untreated plots), including the silage isolate HMC.N.60S.1C that showed resistance to voriconazole. No other cyp51A mutations including those coding for DMI resistance (L98H, Y121F, and T289A) present in the resistant reference isolates[21,35] were found in the studied *A. fumigatus* isolates from corn silage.

The E427K polymorphism was found in the isolates of one subcluster of the main cluster, while the second subcluster included two other silage isolates (WCC.N.H.3B and HMC.T.60S.2B) with the reference resistant and sensitive isolates (Fig. 3).

DISCUSSION AND CONCLUSIONS

Emphasis in this study has been given to corn harvest as whole-crop corn, high moisture corn and wet grain which was conserved as silage for different time periods since deteriorated silages could be considered a major source of release of *A. fumigatus* spores into air in the farm environment.[13,36,37] *A. fumigatus* is a saprophytic fungus that thrives on organic debris. It is

ubiquitous worldwide and is frequently present in silage,[7,37,38] mainly under aerobic conditions (Dolci et al., 2011), where the heat derived from degradation of the organic matter favours the development of thermophilic microorganisms. The occurrence of *A. fumigatus* in whole-crop corn silage has already been documented in different reports,[13,22,36,37] but the growth evolution of the fungus through different conservation periods in high moisture corn and wet grain silages is still poorly investigated.

In this study, *A. fumigatus* was detected both at crop harvest and after ensiling. In the fresh forage at harvest, it was found at rather low concentrations in wet grains (0.11×10^3 cfu/g) and whole-crop corn (2.60×10^3 cfu/g). It was not detected in the high moisture corn. This was probably due to insufficiently sensitive detection levels of the used technique (10 cfu/g). There is still limited information on the presence of *A. fumigatus* on corn plants and grains in the field, where it was detected only by more sensitive methods like molecular techniques, however without fungal quantification.[39]

Thermophilic competence of *A. fumigatus* is evident through surviving 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 absence of competition with other microbial species.[13] Thus, in the present study, the highest number of contaminated samples by *A. fumigatus* was found after 60 days of silage conservation, indicating that the fungus was able to survive 60 days under anaerobic conditions. Whereas, after longer conservation period (160 days of anaerobic condition), the number of samples containing *A. fumigatus* was low as previously reported by Ferrero *et al.*[40]. The occurrence of *A. fumigatus* in corn silages at opening was previously described by Dolci *et al.*[10] and Spadaro *et al.*[13] after 110 and 146

days of conservation. The highest counts of *A. fumigatus* were detected during aerobic deterioration of silages when the inhibiting conditions to the growth of the fungus (absence of oxygen and low pH) were depleted by the previous activity of yeast allowing the fungus to develop reaching frequencies over 1.00×10^8 cfu/g. These data are in accordance with a high amount of *A. fumigatus* found in corn silage by Santoro *et al.*[22]. During the experiments, a fungicide treatment was performed in the corn field at the beginning of flowering with a mixture of prothioconazole and tebuconazole, both are DMIs. The DMI mixture was applied at twice the commercial dose. The aim was to evaluate if a high-dose fungicide treatment would affect the occurrence of *A. fumigatus* and the sensitivity of the isolates coming from treated plots. Neither a lower frequency of *A. fumigatus*, nor a significantly different sensitivity profile of the isolates to the two fungicides, imazalil and voriconazole, were detected in the treated plots. The only isolate resistant to voriconazole was found in silage sample originating from a plot which was not treated with DMI. The low number of treatments of field crops like corn with DMIs per cropping season does not seem to be a factor favouring the selection of fungal pathogens or *A. fumigatus* resistant to DMIs. In other crops, such as grapevine or apple, up to four DMI sprays per cropping season are made (FRAC, www.frac.info), potentially representing a higher risk ofresistance selection.

The presence of *A. fumigatus* teleomorph stage seems rare in nature.[4] The genes related to the mating type expression were found in the *A. fumigatus* genome. Mating type 1 seems to be attributed to a higher invasiveness of *A. fumigatus* in human populations, while the mating type 2 is found more frequently in environmental habitats.[22,41] In this study, the isolates did not show a simultaneous expression of both mating types, most of them belonged to mating type 2. The isolate resistant to voriconazole belonged to mat 2. This could be related to a higher aggressiveness of

silage isolates as was also described previously for compost isolates (both environmental origin), where mat 2 was predominant and a high sensitivity to DMI fungicides was reported in northern Italy.[23]

Out of 50 silage isolates evaluated, nine and three were less sensitive to imazalil and voriconazole, respectively, while only one isolate showed resistance to voriconazole. However, none of these isolates harbored the mutations known to code for DMI resistance in *A. fumigatus* as reported from other European countries and India for DMI resistant environmental isolates (TR_{34}+L98H, TR_{46}+Y121F and T289A).[21,42-45] In this survey, ten silage *A. fumigatus* isolates contained the *cyp51A* E427K amino acid mutation. The polymorphism E427K has been already found in our previous reports in *A. fumigatus* deriving from composts of kitchen and garden wastes, and orange compost[23,26] and brown compost[22] from Italy and Spain. This mutation, detected now also in several corn silage isolates, is not related to DMIs resistance, it rather represents a specific genotype existing at certain geographic sites.The silage isolate 9378C resistant to voriconazole probably possesses some additional mechanisms associated with DMI resistance such as overexpression of *cyp 51* gene.[46]

While field treatment with DMIs did not influence the sensitivity of *A. fumigatus* isolates from fresh and ensiled corn, more investigation should be done on the presence and sensitivity of *A. fumigatus* in corn silage and samples from other crops including vegatables, due to the risk of contamination of food samples.

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REFERENCES

1. Heinekamp T, Schmidt H, Lapp K, Pähtz V, Shopova I, Köster-Eiserfunke N *et al.*, Interference of *Aspergillus fumigatus* with the immune response. *Semin Immunopathol* **37**:141–152 (2015).
2. Fiore M, Cascella M, Bimonte S, Maraolo AE, Gentile I, Schiavone V, et al., Liver fungal infections: an overview of the etiology and epidemiology in patients affected or not affected by oncohematologic malignancies. *Infect Drug Resist* **11**:177-186 (2018).
3. Debeaupuis JP, Sarfati J, Chazalet V and Latgé JP, Genetic diversity among clinical and environmental isolates of *Aspergillus fumigatus*. *Infect Immun* **65**:3080-3085 (1997).
4. O'Gorman CM, Fuller H and Dyer PS, Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Nature* **457**:471–474 (2009).
5. Millner PD, Marsh PBR, Snowden RB and Parr JF, Occurrence of *Aspergillus fumigatus* during composting of Sewage Sludge. *Appl Env Microbiol* **34**:765-772 (1997).

6. Alonso V, Cavaglieri L, Ramos AJ, Torres A and Marin S, Modelling the effect of pH and water activity in the growth of *Aspergillus fumigatus* isolated from corn silage. *J Appl Microbiol* **122**:1048-1056 (2017).
7. Dos Santos V, Dorner J and Carreira F, Isolation and toxigenicity of *Aspergillus fumigatus* from moldy silage. *Mycopathologia* **156**:133–138 (2003).
8. Borreani G, Tabacco E, Schmidt RJ, Holmes BJ and Muck RE, Silage review: Factors affecting dry matter and quality losses in silages. *J Dairy Sci* **101**:3952–3979 (2018).
9. Muck RE, Nadeau EMG, McAllister TA, Contreras-Govea FE, Santos MC and Kung Jr L, Silage review: Recent advances and future uses of silage additives. *J Dairy Sci* **101**:3980-4000 (2018).
10. Dolci P, Tabacco E, Cocolin L and Borreani G, Microbial dynamics during aerobic exposure of corn silage stored under oxygen barrier or polyethylene films. *Appl Environ Microbiol* **77**:7499–7507 (2011).
11. González Pereyra ML, Chiacchiera SM, Rosa CAR, Sager R, Dalcero AM, Cavaglieri LR, Comparative analysis of mycobiota and mycotoxins contaminating corn trench silos and silo bags. *J Sci Food Agric* **91**:1474–1481 (2011).
12. Pena G. A., Pereyra M., Armando M. R., Chiacchiera S. M., Magnoli C. E., Orlando J. L., *et al., Aspergillus fumigatus* toxicity and glio-toxin levels in feedstuff for domestic animals and pets in Argentina. *Lett Appl Microbiol*, **50**:77–81 (2010).
13. Spadaro D, Bustos-Lopez MP, Gullino ML, Piano S, Tabacco E and Borreani G, Evolution of fungal populations in corn silage conserved under polyethylene or biodegradable films. *J Appl Microbiol* **119**:510–520 (2015).

14. Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA *et al.*, Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. *Clin Infect Dis* **46**:327–360 (2008).
15. Brancato A, Brocca D, De Lentdecker C, Erdos Z, Ferreira L, Greco L, *et al.*, Reasoned opinion on the review of the existing maximum residue levels for imazalil according to Article 12 of Regulation (EC) No 396/2005. *EFSA J* **15** (9):4977 (2017).
16. Ghannoum MA and Rice LB, Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin Microbiol Rev* **12**:501–517 (1999).
17. Mellado E, Garcia-Effron G, Buitrago MJ, Alcazar-Fuoli L, Cuenca-Estrella M and Rodriguez-Tudela JL, Targeted gene disruption of the 14-α sterol demethylase (*cyp51A*) in *Aspergillus fumigatus* and its role in azole drug susceptibility. *Antimicrob Agents Chemother* **49:**2536–2538 (2005).
18. Denning DW, Venkateswarlu K, Oakley KL, Anderson MJ, Manning NJ, Stevens DA *et al.*, Itraconazole resistance in *Aspergillus fumigatus. Antimicrob Agents Chemother* **41**:1364–1368 (1997).
19. Stensvold CR, Jørgensen LN and Arendrup MC, Azole-Resistant Invasive Aspergillosis: Relationship to Agriculture. *Curr Fungal Infect Rep* **6:**178-191 (2012).
20. Snelders E, Camps SM, Karawajczyk A, Rijs AJ, Zoll J, Verweij PE, *et al.*, Genotype-phenotype complexity of the TR46/Y121F/T289A cyp51A azole resistance mechanism in *Aspergillus fumigatus*. *Fungal Genet Biol*. **82**:129-35 (2015).
21. Snelders E, Huis In 't Veld RA, Rijs AJ, Kema GH, Melchers WJ and Verweij PE, Possible

environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl Environ Microbiol* **75**:4053–4057 (2009).

22. Santoro K, Matić S, Gisi U, Spadaro D, Pugliese M and Gullino ML, Abundance, genetic diversity and sensitivity to demethylation inhibitor fungicides of *Aspergillus fumigatus* isolates from organic substrates with special emphasis on compost. *Pest Manag Sci* **73**:2481-2494 (2017).
23. Pugliese M, Matić S, Prethi S, Gisi U and Gullino ML., Molecular characterization and sensitivity to demethylation inhibitor fungicides of *Aspergillus fumigatus* from orange-based compost. *PLoS One* **13**:e0200569. (2018).
24. Gisi U, Assessment of selection and resistance risk for demethylation inhibitor fungicides in *Aspergillus fumigatus* in agriculture and medicine: a critical review. Pest Manag Sci **70**:352–364 (2014).
25. Porter MG and Murray RS, The volatility of components of grass silage on oven drying and the inter-relationship between dry-matter content estimated by different analytical methods. *Grass Forage Sci* **56**:405-411 (2001).
26. Franceschini S, Chitarra W, Pugliese M, Gisi U, Garibaldi A and Gullino ML, Quantification of *Aspergillus fumigatus* and enteric bacteria in European compost and biochar. *Compost Sci Util* **24**:20–29 (2016).
27. Glass NL and Donaldson GC, Development of Primer Sets Designed for Use with the PCR To Amplify Conserved Genes from Filamentous Ascomycetes. *Appl Environ Microbiol* **61**:1323–1330 (1995).
28. Paoletti M, Rydholm C, Schwier EU, Anderson MJ, Szakacs G, Lutzoni F, *et al.*, Evidence

for sexuality in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Curr Biol* **15**:1242-1248 (2005).

29. Lass-Flörl C, Cuenca-Estrella M, Denning DW and Rodriguez-Tudela JL, Antifungal susceptibility testing in *Aspergillus* spp. according to EUCAST methodology. *Med Mycol* **44**:S319–S325 (2006).
30. Motulsky H, GraphPad Prism Regression Guide. San Diego, CA, USA, GraphPad Software Inc. Available at: http://www.graphpad.com (verified May 5, 2019) (2007).
31. Diaz-Guerra TM, Mellado E, Cuenca-Estrella M and Rodriguez-Tudela JL, A point mutation in the 14α-sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother* **47:**1120–1124 (2003).
32. Snelders E, Karawajczyk A, Schaftenaar G, Verweij PE and Melchers WJ, Azole resistance profile of amino acid changes in *Aspergillus fumigatus* CYP51A based on protein homology modeling. *Antimicrob Agents Chemother* **54**:2425–2430 (2010).
33. Thompson JD, Higgins DG and Gibson TJ, CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**:4673–4680 (1994).
34. Kumar S, Stecher G and Tamura K, MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol* **33**:1870–1874 (2016).
35. Kuipers S, Brüggemann RJ, de Sévaux RG, Heesakkers JP, Melchers WJ, Mouton JW *et al.*, Failure of posaconazole therapy in a renal transplant patient with invasive aspergillosis due to *Aspergillus fumigatus* with attenuated susceptibility to posaconazole. *Antimicrob Agents Chemother* **55**:3564–3566 (2011).

36. Alonso V, Díaz Vergara L, Aminahuel C, Pereyra C, Pena G, Torres A, *et al.*, Physiological behaviour of gliotoxigenic *Aspergillus fumigatus sensu stricto* isolated from maize silage under simulated environmental conditions, *Food Addit Contam A* **32**:236-244 (2005).
37. Dogi C, Alonso V, Fochesato A, Poloni V and Cavaglieri L, Comparison of toxicogenic and immunosuppressive capacity of *Aspergillus fumigatus* strains isolated from clinical and corn silage samples. *J Appl Microbiol* **118**:175-181 (2015).
38. González Pereyra ML, Alonso VA, Sager R, Morlaco MB, Magnoli CE, Astoreca AL, *et al.*, Fungi and selected mycotoxins from pre- and post-fermented corn silage. J Appl Microbiol **104**:1034–1041 (2008).
39. Zulkifli NA and Zakaria L, Morphological and molecular diversity of *Aspergillus* from corn grain used as livestock feed. *HAYATI Journal of Biosciences* **24**:26e34 (2017).
40. Ferrero F, Prencipe S, Spadaro D, Gullino ML, Cavallarin L, Piano S, *et al.*, Increase in aflatoxins due to *Aspergillus* section *Flavi* multiplication during the aerobic deterioration of corn silage treated with different bacteria inocula. *J Dairy Sci* **102**:1176-1193 (2019).
41. Alvarez-Perez S, Blanco JL, Alba P and Garcia ME, Mating type and invasiveness are significantly associated in *Aspergillus fumigatus*. *Med Mycol* **48**:273–277 (2010).
42. Van der Linden JW, Camps SM, Kampinga GA, Arends JP, Debets-Ossenkopp YJ and Haas PJ, Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clin Infect Dis* **57**:513–520 (2013).
43. Van Ingen J, van der Lee HA, Rijs TA, Zoll J, Leenstra T, MelchersWJ et al., Azole, polyene and echinocandin MIC distributions for wild-type, TR34/L98H and TR46/Y121F/T289A *Aspergillus fumigatus* isolates in the Netherlands. *J Antimicrob*

Chemother **70**:178–181 (2015).

44. Mortensen KL, Mellado E, Lass-Flörl C, Rodriguez-Tudela JL, Johansen HK and Arendrup MC, Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrob Agents Chemother* **54**:4545–4549 (2010).
45. Chowdhary A, Kathuria S, Xu J, Sharma C, Sundar G, Singh PK *et al.*, Clonal expansion and emergence of environmental multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR34/L98H mutations in the cyp51A gene in India. *PLoS One* **7**:e52871 (2012).
46. Verweij PE, Zhang J, Debets AJ, Meis JF, van de Veerdonk FL, Schoustra SE *et al.*, In-host adaptation and acquired triazole resistance in *Aspergillus fumigatus*: a dilemma for clinical management. *Lancet Infect Dis* **16**:e251–e260 (2016).

FIGURE CAPTIONS

Figure 1. Abundance (cfu/g dry weight) of *Aspergillus fumigatus* in different types of silage and its period of conservation, at silo opening, and at 7 days and 14 days of air exposure (n.d. = not detected or below 10 cfu/g; n.m. = not measured).

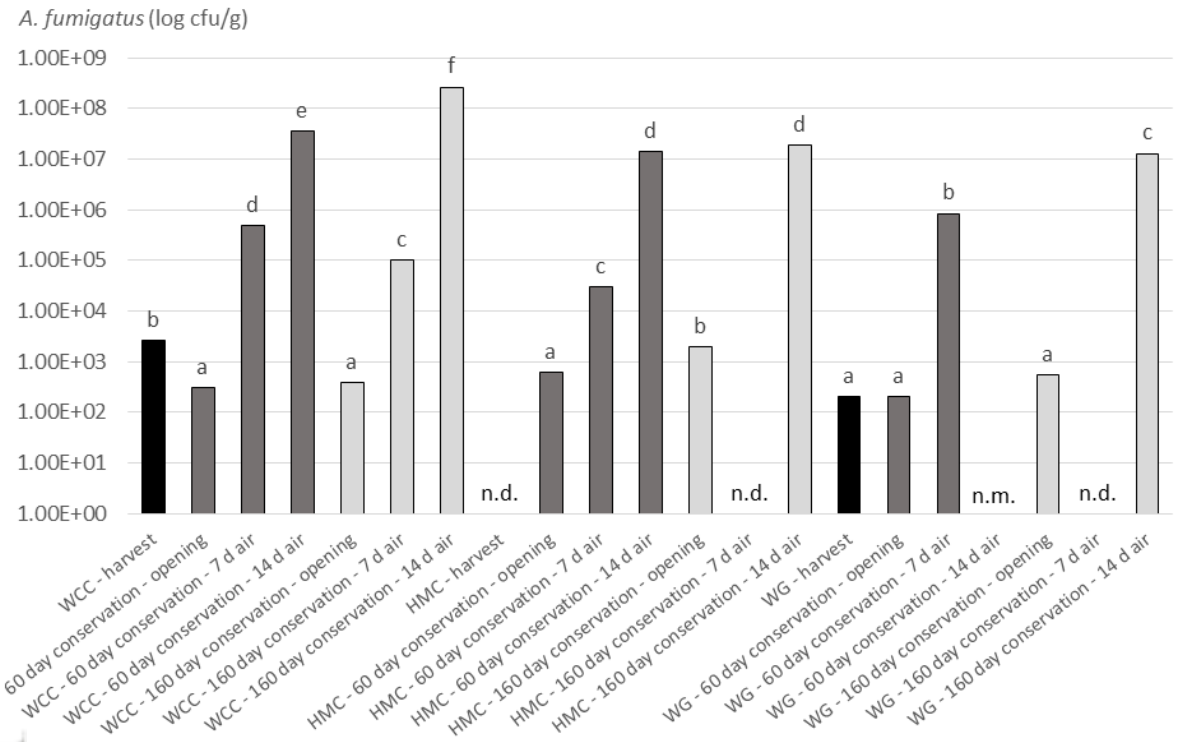


Figure 2. Sensitivity distribution of *A. fumigatus* silage isolates and reference isolates to voriconazole and imazalil. Mean EC_{50} is shown by inverted triangle.

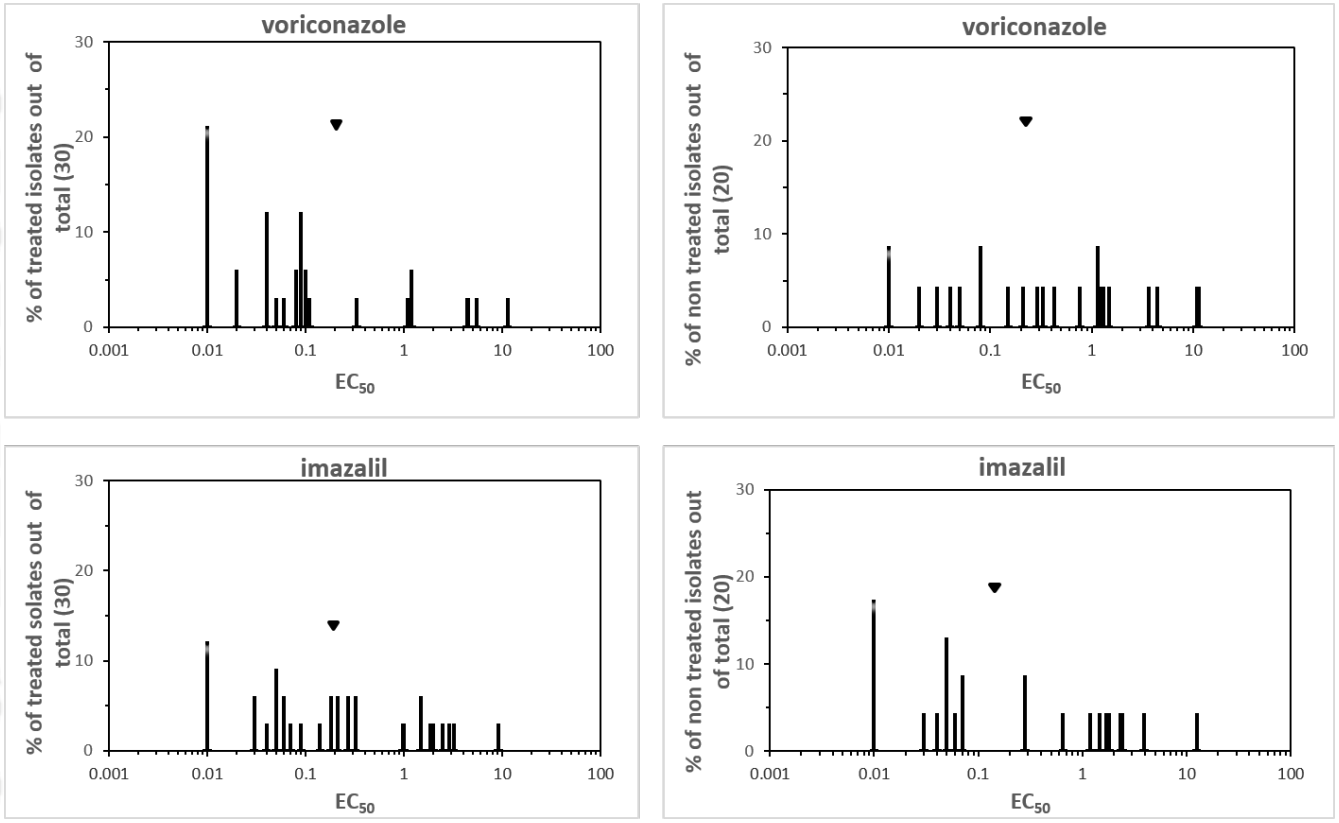
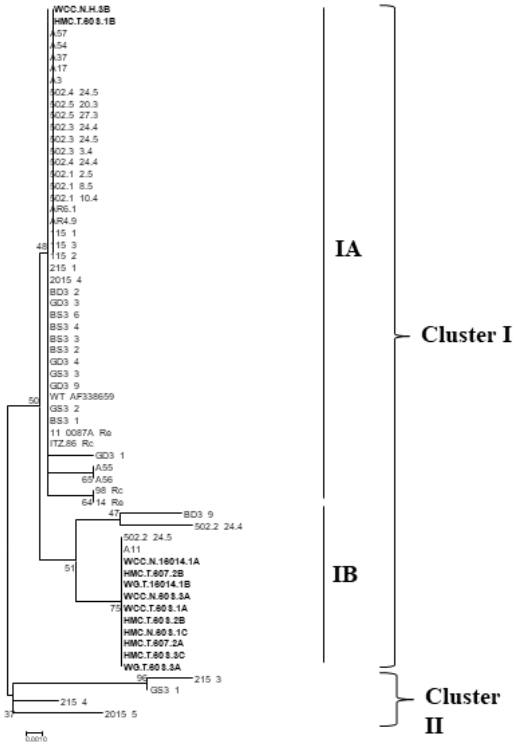


Figure 3. Phylogenetic relatedness of 12 *Aspergillus fumigatus* isolates from corn silage and reference environmental isolates based on the *cyp51A* gene sequence inferred by Neighbour-joining analysis. Bootstrap analysis is supported with 1000 replications.



Tables

Table 1. Characteristics of whole-crop corn (WCC), high moisture corn (HMC) and wet grain (WG) corn silages at harvest, silo opening and after 7 and 14 days of air exposure

Sampling time	Matrices	Treatment*	DM (%)	a_w	pH	LAB (cfu × 10^5/g)	Yeast (cfu × 10^6/g)	Mould (cfu × 10^6/g)
Harvest	WCC	T	46.0	0.985	5.87	15.85	2.63	1.70
		NT	47.3	0.984	5.89	26.30	5.50	2.24
	HMC	T	64.2	0.976	5.93	19.50	3.80	1.12
		NT	67.7	0.978	5.96	7.08	3.72	1.62
	WG	T	73.3	0.967	6.35	0.05	0.33	0.04
		NT	76.3	0.954	6.33	0.01	0.26	0.03

			Silo opening						7 days of air exposure				14 days of air exposure			
			DM (%)	a_w	pH	LAB (cfu × 10^7/g)	Yeast (cfu × 10^3/g)	Mould (cfu × 10^3/g)	DM (%)	pH	Yeast (cfu × 10^7/g)	Mould (cfu × 10^3/g)	DM (%)	pH	Yeast (cfu × 10^7/g)	Mould (cfu × 10^5/g)
60 d of conservation	WCC	T	45.4	0.982	3.79	0.30	0.79	0.19	48.7	4.97	30.9	0.09	52.7	6.33	44.7	501.2
		NT	47.1	0.980	3.82	0.98	0.28	0.32	47.9	4.10	16.22	7.59	54.0	6.57	54.95	691.8
	HMC	T	64.1	0.969	3.78	1.05	2.00	0.68	64.6	3.81	0.63	0.01	68.6	5.35	19.50	77.62
		NT	66.4	0.963	3.82	1.20	0.55	0.35	67.2	3.83	0.04	0.00	68.6	4.09	2.69	0.21
	WG	T	71.7	0.973	5.93	1.02	955.0	1.38	73.4	5.98	15.85	275.4	75.3	5.95	8.13	478.6
		NT	74.6	0.971	6.16	0.38	794.3	0.66	76.2	5.84	10.47	208.9	76.6	5.94	13.49	181.9
160 d of conservation	WCC	T	46.3	0.984	3.79	0.20	2.95	0.15	48.0	4.02	15.5	2.51	50.4	6.26	5.01	3630
		NT	46.6	0.978	3.81	0.03	10.96	0.03	48.1	4.29	25.12	20.89	46.9	6.79	4.27	5011
	HMC	T	62.7	0.963	3.80	0.42	0.05	0.01	64.0	3.83	0.35	n. d.	63.6	4.73	11.48	7.76
		NT	65.8	0.953	3.86	0.46	0.13	0.01	67.0	3.86	0.01	n. d.	68.4	3.95	2.04	0.01

WG	T	71.8	0.964	5.56	0.21	575.44	n. d.	74.8	6.14	13.80	1174.9	76.9	6.01	11.75	537.0
	NT	74.0	0.957	5.46	0.09	691.83	n. d.	76.5	5.80	10.23	0.06	77.7	5.92	14.79	436.5

*T= treated with Prosaro® (Bayer Crop Science: 12.7 g prothioconazole and 12.7 g tebuconazole per 100 g); NT= not treated; WCC = whole-crop corn; HMC = high moisture corn; WG = wet grain; DM = dry matter; aw = activity water; LAB = lactic acid bacteria; cfu = colony forming unit; n.d. = not detected or below 10 cfu/g.

Table 2. List of samples in different corn mixtures (DMI-treated and non-treated) through the different stages: pre-silage and silage conservation (selected samples for molecular characterization and *in vitro* sensitivity assays are highlighted in grey)

Silage type		Harvest	60 d conservation			160 d conservation		
			Silo opening	7 d air exposure	14 d air exposure	Silo opening	7 d air exposure	14 d air exposure
Whole-crop corn	T*	WCC.TH	WCC.T.60S	WCC.T.607	WCC.T.6014	WCC.T.160S	WCC.T.1607	WCC.T.16014
	NT	WCC.N.H	WCC.N.60S	WCC.N.607	WCC.N.6014	WCC.N.160S	WCC.N.1607	WCC.T.16014
High misture corn	T	HMC.T.H	HMC.T.60S	HMC.T.607	HMC.T.6014	HMC.T.160S	HMC.T.1607	HMC.T.16014
	NT	HMC.N.H	HMC.N.60S	HMC.N.607	HMC.N.6014	HMC.N.160S	HMC.N.1607	HMC.N.16014
Wet grain	T	WG.T.H	WG.T.60S	WG.T.607	WG.T.6014	WG.T.160S	WG.T.1607	WG.T.16014
	NT	WG.N.H	WG.N.60S	WG.N.607	WG.N.6014	WG.N.160N	WG.N.1607	WG.N.16014

*T= treated twice with Prosaro® (Bayer Crop Science: 12.7 g prothioconazole and 12.7 g tebuconazole per 100 g), NT= not treated

Table 3. Sensitivity to two DMI fungicides (EC_{50}) of *Aspergillus fumigatus* isolates collected from different silage samples

				EC_{50} (mg/L)		EC_{50} (mg/L)		Mating type	
Isolate	**Period**	**Accession no. (*β*-tubulin)**	**Accession no. (*cyp51A*)***	**Imazalil**	**Reaction****	**Voriconazole**	**Reaction****	**1**	**2**
WCC.T.H.1D	**Harvest**	MK879472		0.04	S	1.09	S	+	
WCC.T.H.3B		MK879473		<0.01	S	<0.01	S		+
WCC.N.H.1C		MK879474		0.27	S	0.02	S	+	
WCC.N.H.3B		MK879475	MK879460 (-)	3.21	LS	1.29	S		+
WG.T.H.1A		MK879476		0.06	S	<0.01	S		+
WG.T.H.2A		MK879477		<0.01	S	<0.01	S	+	
WCC.T.60S.1A	**60 d conservation**	MK879478	MK879466 (+)	0.18	S	0.09	S	+	
WCC.T.60S.2C		MK879479		0.65	S	0.06	S		+
WCC.T.60S.3B		MK879480		1.45	S	1.20	S	+	
WCC.N.60S.1C		MK879481		2.50	LS	0.29	S	+	
WCC.N.60S.3A		MK879482	MK879465 (+)	0.09	S	0.08	S		+
HMC.T.60S.1B		MK879483	MK879461 (-)	0.09	S	0.08	S		+
HMC.T.60S.2B		MK879484	MK879467 (+)	3.92	LS	5.45	LS		+
HMC.T.60S.3C		MK879485	MK879470 (+)	2.34	LS	4.46	LS		+
HMC.N.60S.1C		MK879486	MK879468 (+)	2.42	LS	10.84	R	+	
HMC.N.60S.2A		MK879487		0.20	S	0.08	S	+	
HMC.N.60S.3B		MK879488		0.28	S	0.21	S		+
WG.T.60S.3A		MK879489	MK879471 (+)	0.26	S	0.09	S		+
WG.N.60S.3A		MK879490		0.32	S	0.15	S	+	
WCC.T.607.1A	**60 d conservation (+ 7 d air exposure)**	MK879491		0.21	S	0.04	S	+	
WCC.T.607.2A		MK879492		0.05	S	0.02	S		+
WCC.T.607.3A		MK879493		<0.01	S	0.04	S		+
WCC.N.607.1A		MK879494		0.01	S	<0.01	S	+	
WCC.N.607.3A		MK879495		<0.01	S	0.04	S		+
HMC.T.607.1A		MK879496		<0.01	S	0.02	S		+
HMC.T.607.2B		MK879497	MK879463 (+)	0.32	S	0.10	S		+
HMC.T.607.2A		MK879498	MK879469 (+)	2.00	LS	1.16	S	+	
WG.T.607.1B		MK879499		0.04	S	0.08	S	+	
WG.T.607.2B		MK879500		0.03	S	0.09	S		+

WG.T.607.3C		MK879501		0.05	S	0.10	S	+	
WG.N.607.1C		MK879502		0.03	S	0.03	S		+
WCC.T.6014.1A		MK879503		1.19	S	1.12	S		+
WCC.N.6014.1A		MK879504		1.51	LS	1.47	S		+
WCC.N.6014.2D	**60 d conservation**	MK879505		2.86	LS	0.43	S		+
HMC.T.6014.1B	**(+ 14 d air exposure)**	MK879506		0.03	S	0.04	S		+
HMC.T.6014.3A		MK879507		0.05	S	0.05	S		+
WCC.T.160S.1A		MK879508		0.28	S	0.11	S		+
WCC.T.160S.2A		MK879509		0.01	S	0.04	S		+
WCC.N.160S.2B		MK879510		0.06	S	<0.01	S		+
WCC.N.160S.3A		MK879511		0.99	S	3.67	LS		+
HMC.T.160S.1A		MK879512		0.05	S	<0.01	S		+
HMC.T.160S.2A	**160 d conservation**	MK879513		0.06	S	<0.01	S		+
HMC.N.160S.2A		MK879514		0.05	S	0.05	S	+	
HMC.N.160S.3A		MK879515		1.48	S	1.25	S	+	
WCC.T.1607.2D	**160 d conservation (+ 7 d air exposure)**	MK879516		0.14	S	0.08	S	+	
WCC.T.16014.3A		MK879517		0.07	S	<0.01	S		+
WCC.N.16014.1A		MK879518	MK879462 (+)	3.05	LS	1.14	S		+
WCC.N.16014.3C	**160 d conservation**	MK879519		1.41	S	0.76	S		+
HMC.N.16014.2B	**(+ 14 d air exposure)**	MK879520		1.81	LS	1.20	S	+	
WG.T.16014.1B		MK879521	MK879464 (+)	0.05	S	<0.01	S	+	
	Mean EC_{50}*			**0.26**		**0.31**			
	WT			**0.09**	S	**0.33**	S	nt	
	TR_{34} +L98H			**12.49**	R	**11.33**	R	nt	
	TR_{46} +Y121F +T289A			**1.71**	LS	**4.42**	LS	nt	

* The presence of the E427K mutation is indicated by a +, while the absence by a -.

**Less sensitive isolates (EC_{50} between 1.51 and 10.0) and resistant isolates (EC_{50} higher than 10.0) were not included in calculation of mean EC_{50}.