Azole use in agriculture reduces *Aspergillus fumigatus* abundance but does not alter its population structure

Amelia E. Barber[1,2] #, Jennifer Riedel[3] #, Tongta Sae-Ong[1], Kang Kang[1], Werner Brabetz[4], Gianni Panagiotou[1,5], Holger B. Deising[3], Oliver Kurzai[1,2] *

# Denotes equal contribution

* Address correspondence to: okurzai@hygiene.uni-wuerzburg.de

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Author affiliations:

[1] Leibniz Institute of Natural Product Research and Infection Biology – Hans Knöll Institute. Jena, Germany.

[2] Institute for Hygiene and Microbiology. University of Würzburg. Würzburg, Germany

[3] Institute for Agriculture and Nutritional Sciences. Martin Luther University Halle-Wittenberg. Halle (Saale), Germany.

[4] Biotype GmbH. Dresden, Germany.

[5] Systems Biology and Bioinformatics Group. School of Biological Sciences. Faculty of Sciences. The University of Hong Kong. Hong Kong, China.

ABSTRACT

Antibiotic resistance is an increasing threat to human health. In the case of *Aspergillus fumigatus*, which is both an environmental saprobe and an opportunistic human fungal pathogen, resistance is suggested to arise from fungicide use in agriculture, as the azoles used for plant protection are almost identical to the frontline antifungals used clinically. However, limiting azole fungicide use on crop fields to preserve their activity for clinical use could threaten the global food supply via a reduction in yield. In this study we clarify the link between fungicide use on crop fields and resistance in a prototypical human pathogen through systematic soil sampling on farms in Germany and surveying fields before and after azole application. We observe a reduction in the abundance of *A. fumigatus* on fields following azole treatment—a finding that was not observed on an organic control field not applying azoles. However, the overall resistance frequency among agricultural isolates is low, with only 1-3% of isolates from 2016-2018 displaying resistance to medical azoles. Isolates collected after the growing season and azole exposure show a subtle, but consistent decrease in susceptibility to medical and agricultural azoles. Whole genome sequencing indicates that, despite the decrease in fungal abundance, azole application does not significantly affect the population structure and genetic diversity of *A. fumigatus* in fields. Given the low observed resistance rate among agricultural isolates, as well the lack of genomic impact following azole application, we do not find evidence that azole use on crops is significantly driving resistance in *A. fumigatus*.

IMPORTANCE

Antibiotic resistance is an increasing threat to human health. In the case of *Aspergillus fumigatus*, which is an environmental fungus that also causes life-threatening infections in humans, antimicrobial resistance is suggested to arise from fungicide use in agriculture, as the chemicals used for plant protection are almost identical to the antifungals used clinically. However, removing azole fungicides from crop fields threatens the global food supply via a reduction in yield. In this study, we survey crop fields before and after fungicide application, and find that while azole fungicide application reduces the abundance of *A. fumigatus* in the soil, we do not find evidence that fungicide use is significantly driving resistance in this organism and context.

INTRODUCTION

Aspergillus fumigatus is a globally-distributed fungus responsible for an estimated 300,000 case of invasive disease and more than 10 million cases of chronic and allergic disease globally each year (1). Humans inhale the infectious particles, or spores, on a daily basis, but are actually an accidental host for the fungus whose primary niche is soil and decaying vegetation. Management and prophylaxis against aspergillosis relies largely on the azole class of antifungals with voriconazole and isavuconazole recommended as first line therapy (2). Unfortunately, clinical resistance to the azoles in *A. fumigatus* is an increasing problem, with some medical centers reporting rates as high as 30% in specific patient populations, and similarly high rates for environmentally-isolated *A. fumigatus* (3, 4). Regrettably, the mortality for resistant infections is upwards of 90% in some patient populations (5-7). While resistance can evolve during patient therapy (8, 9), the emergence of resistance in *A. fumigatus* has mainly been linked to

68 the use of azoles in agriculture, as structurally similar and mechanistically
69 indistinguishable compounds are heavily used for plant protection (10). This resistance
70 has been described as collateral damage as *A. fumigatus* is not a plant pathogen that is
71 being directly targeted by fungicide treatments (11). The triazoles were first released for
72 agriculture in 1973, well before they were first introduced to human medicine in the early
73 1990s, and are currently the most widely-utilized antifungal compound group in
74 agriculture due to their systemic distribution in treated plants, high efficiency and broad
75 spectrum of target pathogens (12, 13). Crops, particularly cereals and fruits, are sprayed
76 multiple times each growing season at a recommended dose of 100 g/hectare to control
77 powdery mildew, rust, septoria leaf blotch, and other phytopathogenic fungi (14).
78 Currently, there are 32 azoles commercially available for plant protection (15), but only
79 five in regular use in human medicine (16).

80 The most common azole resistance mechanism in *A. fumigatus* occurs via
81 mutations in the target protein of azole fungicides, sterol 14α-demethylase (CYP51A,
82 syn ERG11), a key enzyme of the ergosterol biosynthesis pathway. In *A. fumigatus*, the
83 dominant resistant mechanism among both environmental and clinical isolates is a 34 bp
84 tandem repeat (TR_{34}) in the *cyp51a* promoter coupled with a leucine to histidine
85 substitution (L98H) in the amino acid coding sequence—the net effect of which is an
86 increase in gene expression as well as an alteration in both the stability of the target
87 enzyme and the interaction between the protein heme cofactor of *cyp51a* and the azole
88 ligand (17-19). Additional mutations that have been identified to confer azole resistance
89 in *A. fumigatus* include other variations of the tandem repeat, such as
90 TR_{46}/Y121F/T289A and TR_{53}, as well as other point mutations in the *cyp51a* coding
91 sequence (20, 21).

92 Disease-causing fungi are responsible for roughly 20% of crop yield loss, with a
93 further 10% loss postharvest (16), so the use of azoles is critical for securing the food
94 supply. However, this must be balanced against the need to preserve the activity of the
95 azoles for clinical use and, as such, there is an urgent need to identify the contributions
96 of azole fungicide use on food crops to the development of resistance in *A. fumigatus*.
97 We address this through systematic soil sampling conducted on 10 agricultural sites in
98 Germany over a three-year period—including conventionally-managed fields applying
99 azoles fungicides, as well as those practicing organic agriculture that do not use these
100 compounds. In the largest published *A. fumigatus* sequencing effort to date, and the first
101 to focus on the fungus in its natural niche, we also use whole genome sequencings
102 (WGS) to examine the impact of azole fungicides on the population genetics of 64
103 agriculturally-isolated *A. fumigatus* isolates.
104

105 **RESULTS**

106 Variable abundance of *Aspergillus fumigatus* on agricultural sites in Germany

107 To examine the depth distribution of *A. fumigatus* in agricultural soils, we
108 collected soil samples from a test field at five cm intervals down to a depth of 30 cm
109 below the surface. *A. fumigatus* was most abundant in the top five cm of soil and was
110 not significantly observed below a depth of 15 cm (Fig 1A). As a result, only the top 5 cm
111 layer of soil was collected in subsequent soil samples.

112 To identify whether our sampling areas are representative of azole and fungicide
113 usage for Germany as a whole, we calculated the fraction of each administrative district
114 that is theoretically treated with azoles in the context of agriculture using publicly
115 available data. Overall, 51% of Germany is designated as agricultural land (see Material

116 & Methods for data sources). However, not all this land is sprayed with azoles. For
117 example, meadow or pasture land is rarely treated with fungicides. Additionally,
118 approximately 7% of agriculture in Germany utilizes organic farming and does not apply
119 azoles, with a range of 3-15% between the different federal states. Using land use data
120 on arable farm land and permanent crop areas for each district in Germany, we
121 calculated that the mean fraction of potentially azole-treated area in Germany is 32%
122 (for the districts where data is available), with a range of 1-68% (Fig 1B). The districts
123 where we performed our soil sampling were almost all above the average for Germany,
124 with a mean of 52% potentially azole-treated hectares and a range of 30-65%.

125 To examine the inter- and intra-field variability in the density of *A. fumigatus* on
126 agricultural fields, soil samples were taken from nine conventional and eight organic
127 fields before the growing season in 2017. Conventional fields were also sampled again
128 after the vegetative period and application of azoles. In total, 2875 soil samples were
129 taken between 2016 and 2018 (Supplemental Table 1). The predominant crops being
130 grown were cereals such as wheat and barley, but several apple orchards were also
131 sampled. Of the fields sampled during this period in 2017, 67% of the soil samples taken
132 were positive for *A. fumigatus* with a large range between fields (28-100%) (Fig 1C). We
133 also observed a large degree of variation in the mean CFU/g of soil between different
134 fields, with some fields having 30x higher *A. fumigatus* density over others (0.7 – 18.8
135 CFU/g).

136 To examine the stability of *A. fumigatus* population sizes in agricultural soil, we
137 investigated the same farms a year later in the spring of 2018 and repeated the soil
138 sampling on eight conventional and seven organic fields. Due to crop rotation, it was not
139 possible to sample the identical fields as the previous year, except for the apple

140 orchards on Farms H and L. During the 2018 sampling period, an overall lower
141 proportion of samples were positive for *A. fumigatus* (51% with a range of 10-96%
142 between different fields) (Fig 1D), but the mean CFU/g for the 1000 samples was similar
143 to the previous year (5.18 CFU/g soil for 2017 compared to 5.74 CFU/g for 2018). As in
144 the previous year, there was a large variability in the mean CFU of *A. fumigatus* present
145 between fields. Interestingly, the farms with the highest mean *A. fumigatus* CFU/g soil in
146 2017 (*e.g.* Farms B and D) were also the highest in 2018, and the farm with the lowest
147 amount of *A. fumigatus* in 2017 (Farm K) was also the lowest in 2018, suggesting that
148 the amount of *A. fumigatus* is consistent year-to-year (Fig 1E). To investigate potential
149 factors that might support a higher abundance of *A. fumigatus*, we compared the total
150 organic carbon (TOC) content of a random selection of samples with the CFU/g *A.*
151 *fumigatus,* but did not detect a clear relationship between the two (Supplemental Fig 1).
152 Altogether, we observed a non-uniform distribution of *A. fumigatus* in soil samples taken
153 from the same field and a large degree of heterogeneity between fields, but the relative
154 amounts of *A. fumigatus* remained largely consistent year-to-year.
155
156 <u>Azole application reduces *A. fumigatus* abundance</u>
157 To examine the impact of azole fungicides on *A. fumigatus* in agricultural soil, we
158 performed additional soil sampling on the conventional fields surveyed in the spring at
159 the end of the vegetative period and after several months of fungicidal crop protection. A
160 schematic illustration of the soil sampling and fungicide application time lines for 2017
161 and 2018 can be found in Supplemental Figures 2A and B. Unfortunately, the fungicide
162 history for Farms H and L was not available to us. Fields were typically treated with
163 fungicides twice during the growing period and every application recorded contained at

164 least one azole [Note: fungicides are often applied as commercially-available cocktails of
165 different chemicals].

166 When comparing the amount of *A. fumigatus* on fields before azole exposure to
167 after azole exposure, we detected a significant reduction in the CFU/g soil on the
168 majority of fields in 2017 (Fig 2A), even though it is not being directly targeted as a plant
169 pathogen. A similar trend was observed in 2018 comparing *A. fumigatus* density before
170 and after azole treatment, but the results were less dramatic (Fig 2B), possibly due to
171 the extreme heat and drought experienced in Germany during the growing season.

172 To confirm that the reduction in agricultural *A. fumigatus* populations was the
173 result of azole application, and not a seasonal effect from comparing April to July, in
174 2017 we took monthly soil samples from a conventional field and an organic field not
175 treated with azoles as a control. Samples were taken beginning in April, before azoles
176 were applied to the conventional field, through the harvest period in July, and then
177 additional samples were taken in October and November to allow for a period without
178 azole fungicide application. From April to July, the conventional field was sprayed with
179 azoles every 3-5 weeks. When comparing the abundance of *A. fumigatus* on the organic
180 field, we did not observe any significant differences between the abundance recorded
181 monthly between April and July (Fig 2C). However, the conventional field showed a
182 significant reduction in abundance between April and May, corresponding to the
183 beginning of the azole application period, and this reduction was maintained through the
184 rest of the azole application period (Fig 2D). In summary, we observed a reduction in *A.*
185 *fumigatus* abundance on conventional fields following the application of azole
186 fungicides, suggesting that this selection pressure might be imposing a population
187 bottleneck in this niche.

188

189 Reduced susceptibility to agricultural azoles in populations isolated after the growing
190 season and azole exposure

191 To assess the susceptibility of *A. fumigatus* to commonly-applied agricultural
192 azoles, approximately 20 isolates per field and time period from 2017 and 2018 were
193 screened for their ability to grow at a set concentration of difenoconazole and
194 tebuconazole (n = 435 for 2017 and 342 for 2018). To limit potentially clonal isolates
195 from skewing the results, a maximum of two isolates per soil sample were included for
196 testing. As there are no established breakpoints for defining resistance to these
197 compounds in *A. fumigatus*, we selected concentrations that mimicked MIC90 values for
198 these azoles (1 mg/L for difenoconazole and 2 mg/L for tebuconazole). When examining
199 conventional and organic farms in the spring, we observed a wide range in the fraction
200 of isolates per field that were able to grow when challenged with agricultural azoles. For
201 difenoconazole, this ranged from 10-65% per field in 2017 (n = 17 fields, 320 isolates in
202 total) and 0-50% in 2018 (n = 15 fields, 261 isolates in total) (Fig 3A and Supplemental
203 Table 2). For tebuconazole, the rates ranged from 0-25% in 2017 and 0-20% in 2018
204 (Fig 3B and Supplemental Tables 2). We did not detect any significant differences in the
205 rates between conventional and organic fields in the spring or between fields growing
206 different crops (cereals or apples).

207 Given that we observed a reduction in the *A. fumigatus* population size on
208 conventional fields following azole application, we wanted to examine the effect of azole
209 fungicides on the local susceptibility following the vegetative period and several months
210 of azole application. We observed an increase in the proportion of isolates that were
211 able to grow at the test concentrations of difenoconazole (1 mg/L) and tebuconazole

212 (2mg/L) for fields sampled after azole exposure compared to the same field in the spring
213 prior to azole application (Fig 3C, D, and Supplemental Table 3). We detected a 1.67-
214 fold increase in the proportion of isolates that were resistant to our test concentration of
215 difenoconazole in 2017 with a range from a -1.4 to 4.5-fold for individual fields (Fig 3C).
216 In 2018, this increased to a 2.85-fold with a range of 1-4.5-fold change increase for
217 individual fields. We also saw a similar increase in the fraction of isolates with reduced
218 susceptibility to our test concentration of tebuconazole after azole exposure compared to
219 the before. In 2017, we observed a 4.9-fold in number of isolates that were able to grow
220 at the test concentration after azole exposure vs. before exposure with a range of 0-
221 25.0% for individual fields (Fig 3D). In 2018, we detected a more modest 1.8-fold with
222 range of -5 to 18.2% for individual fields.

223 For the fields growing cereals, we were not able to sample the same fields over
224 subsequent years due to crop rotation and the fields not being in use the following year.
225 However, we were able to compare the same apple fields in both 2017 and 2018. We
226 tracked the local susceptibility to agricultural azoles in these fields over two consecutive
227 years at two time points: in the spring prior to azole application and just prior to harvest
228 after ≈ 3 months of azole application. The fraction of isolates that were able to grow in
229 the presence of 1 mg/L difenoconazole or 2 mg/L tebuconazole was, in general, low in
230 the spring, and increased after azole application (Fig 3E, Supplemental Table 3).
231 Interestingly, the spring of 2018, the proportion had returned to a level comparable to
232 what we observed in the spring of 2017—indicating that the reduced susceptibility is
233 transient and recedes when the selective pressure of the azoles is removed. In
234 summary, we found a wide range of susceptibilities to agricultural azoles between
235 different fields, but a consistent decrease in susceptibility following the growing season

236 and azole exposure. However, this change is seemingly transient, and the *A. fumigatus*
237 populations from azole-treated fields typically returned to what they were prior to azole
238 application by the following spring.
239
240 Resistance to medical azoles is agricultural *A. fumigatus* isolates
241 We next examined our isolate collection for resistance to medical azoles and
242 determined the proportion that would be considered clinically resistant. Using the
243 VIPcheck™ agar-based screening method followed by broth microdilution for isolates
244 showing growth on agar-containing wells (22, 23), we determined that only a very small
245 fraction of *A. fumigatus* isolated from agriculture showed resistance to itraconazole,
246 voriconazole, or posaconazole in 2016-2018 (Figure 3F). The overall resistance rate to
247 itraconazole among all isolates collected was highest, with 3.0% (11/333) of isolates
248 being resistant in 2016, 0.7% in 2017 (4/460), and 0.6% (2/322) in 2018. We observed
249 lower resistance rates for posaconazole and voriconazole, with only 2.1% (7/333) being
250 resistant in 2016, 0.7% (4/460) in 2017, and 0.0% (0/322) in 2018.
251 To quantify what mutations were responsible for azole resistance in the
252 population analyzed, we genotyped the *cyp51a* locus of all resistant isolates using
253 Sanger sequencing (n = 18). This gene is the target of the azoles and the major source
254 of resistance among both environmental and clinical *A. fumigatus*. We found that the
255 most dominant DNA alteration observed was the well-characterized TR_{34}/L98H mutation
256 (Fig 3G). This *cyp51a* genotype accounted for 6/12 (50%) of resistant isolates in 2016
257 and 3/4 (75%) in 2017. In 2018, we identified only two resistant isolates among the 322
258 screened and both had wild type *cyp51a* loci. However, both of these isolates were only
259 weakly resistant to itraconazole, but not other azoles, with itraconazole MIC values right

260 at the breakpoint of 2-4 mg/L. For comparison, resistant isolates from other years had
261 MIC values of >8 mg/L. In total, the genetic cause of resistance remained unknown for 8
262 isolates from 2016-18.

263 Since the majority of fields had no resistant isolates, it was not possible to
264 effectively compare resistance rates among agricultural *A. fumigatus* for medical azoles
265 before and after azole treatment. In lieu of this, we examined the MIC distribution for
266 isolates collected before and after the growing season and azole application. Examining
267 40 isolates from the before period and 40 isolates from after azole exposure, we
268 observed a shift in the MIC distribution towards higher MICs for all azoles examined,
269 both medical and agricultural (Fig 3H). However, the median MICs remained
270 unchanged, indicating that the majority of the population following azole exposure does
271 not exhibit a change in MIC. Taken together, these results indicate that the rate of
272 resistance to clinical azoles among environmental *A. fumigatus* isolated from agricultural
273 environments is overall low and that exposure to agricultural azoles only causes a minor
274 increase in MIC values for some of the population, with the majority of isolates left
275 unchanged.

276

277 <u>No population structure observed for *A. fumigatus* in agricultural fields</u>

278 To better understand the population structure of *A. fumigatus* in the environment
279 and how it is impacted by the azoles, we performed whole genome sequencing on
280 isolates from four conventional farms collected before and after azole exposure. 64
281 isolates were sequenced by Illumina paired-end sequencing, representing eight isolates
282 per farm and time point. Isolates from each field and time point were randomly selected,
283 with a maximum of two per soil sample to avoid sequencing of clonal isolates. Raw

284 reads were checked for quality and then aligned to the Af293 reference genome,
285 resulting in a median depth of coverage after mapping of 31.5X (range 10-90X) and a
286 median genome coverage median of 94% (range 92.8-96.7%). Consistent with what has
287 been observed among sequenced clinical strains (24), isolates differed from the Af293
288 reference by a median of 84,690 single nucleotide variants (SNVs) or 2.88 SNVs/kb,
289 with a range of 65,854-146,055 SNVs. The analysis of copy-number variations (CNVs)
290 identified 8,277 unique CNVs in total, with a median of 3,115 CNVs per isolate (range
291 1,666-4,532 CNVs). These CNVs were further delimited into a median of 2,589 deletions
292 (range 1,247-4,405) and 5,687 insertions (range 3,872-7,030).

293 A maximum-likelihood phylogeny based on SNVs indicated no population
294 stratification among isolates from different farms and regions of Germany (Fig 4). To
295 more directly assess the association between genetic and geographic distance, we
296 performed a Mantel test correlating a geographic distance matrix with the F_{ST} genetic
297 distance matrix, and observed no significant association between the two. When
298 considering the azole-resistance status of the isolates, the two itraconazole-resistant
299 TR_{34}/L98H isolates sequenced clustered next to each other, despite originating from
300 separate farms, while the third itraconazole-resistant isolate with an undefined
301 resistance mechanism was on a distinct branch. Despite being the nearest sequenced
302 neighbors, the two TR_{34}/L98H isolates were genetically distinct, each possessing 19,439
303 and 60,841 unique SNVs not shared by the other isolate, along with 67,196 common
304 SNVs relative to Af293.

305 Comparative analysis of molecular variance (AMOVA) indicated that the majority
306 of the variation seen among the 64 isolates came from the population as a whole (within
307 sample) (94.8%) and between samples (5.0%) (Table 1). There was no significant

308 molecular variance between farms (0.2%), with the exception of modest variation
309 between Farm B and Farm C (1.2% of variation observed). Weighted Weir and
310 Cockerham's fixation indexes (F_{ST}) for each farm were essentially zero, indicating an
311 interbreeding, panmimetic population with no separation between farms (Supplemental
312 Fig 3A). V_{ST} values estimating population differentiation based on copy number variation
313 also indicated no subdivision among the farms (Supplemental Fig 3B). To examine the
314 genetic diversity within farms, nucleotide diversity (the average number of nucleotide
315 differences per site for all possible pairs in the population or π), and the number of
316 polymorphic sites (Watterson estimator or θ) were calculated along 5 kb windows with a
317 500 bp step size for each farm population. Farm C showed the greatest intra-farm
318 diversity, while Farm E showed the smallest (Supplemental Fig 3C-D). Taken together,
319 there was no population differentiation between *A. fumigatus* isolates from different
320 farms in Germany, a finding in line with the fungus' capacity for aerosol dispersal.
321
322 <u>Changes in population genetics following azole exposure vary by field</u>
323 Given the observed reduction in overall *A. fumigatus* abundance after azole
324 treatment, we examined the populations for changes in genetic diversity and evidence of
325 selective sweeps in *A. fumigatus* field populations. Using 5 kb sliding windows with a 500
326 bp step size, nucleotide diversity (π) was calculated for the individual farms before and
327 after azole treatment. No clear trend was seen regarding changes in nucleotide diversity
328 between the time points. The isolates from Farms B and E showed increased diversity
329 following azole application, while nucleotide diversity decreased on Farms C and D (Fig
330 5A). We also calculated the number of segregating sites (θ) for the same 5 kb windows
331 and observed the same lack of consensus. Farms B and C showed similar values of θ

332 before as compared to after azole application, while Farm D showed a dramatic
333 decrease in θ following azole application (Fig 5B). AMOVA analysis on isolates from
334 before and after the vegetative period and azole exposure did not indicate any
335 significant molecular variation between the time periods, with the majority of the variation
336 being between and within samples (Table 2). Finally, we measured Tajima's D to test
337 neutrality along 5 kb windows, where negative values indicate less variation than
338 expected and are indicative of a selective sweep. Positive values denote a population
339 that is more heterogenous than would be expected and suggest either a sudden
340 population contraction or balancing selection. Overall, the bulk of the Tamija's D values
341 were close to neutral and there was no clear trend between farms, indicating that there
342 was no genomic signature of a population bottleneck or selective sweep following azole
343 exposure (Fig 5C). The median Tajima's D was roughly zero for Farm B and increased
344 slightly to 0.37 following azole exposure prior to azole application, and the same
345 direction shift was seen for Farm D, but the starting Tajima's D was negative at the time
346 point before azole exposure (-0.53 to 0.53) (Fig 5C). Conversely, the median Tajima's D
347 for farms C and E shifted from positive to negative after the growing season and azole
348 exposure (0.75 to -0.57 for field C and 0.61 to -0.10 for Field E). Taken together, these
349 results indicate that despite the reduction in abundance of *A. fumigatus* on agricultural
350 fields following azole application, we were unable to detect any marked changes at the
351 population level.

352

353 **DISCUSSION**

354 The use of azole fungicides for plant protection has been previously suggested as
355 a driver of clinical resistance in the environmental saprobe and human pathogen

356 *A. fumigatus*, as well as the emergence of new fungal pathogens such as *Candida auris*
357 (11, 25, 26). However, direct evidence linking azole use in agriculture and clinical
358 resistance is missing. Additionally, delineation of specific roles for the use of azoles in
359 crops vs. ornamental plants such as flower bulbs has not been defined and is important
360 to make, as limiting azole fungicide use would significantly impact disease control and
361 yield in many crops. In this study, we provide the first systematic investigation of the
362 impact of azole fungicides on the ecology and resistance status of a human pathogen,
363 *A. fumigatus*. Through analysis of 2,875 soil samples over a three year period in central
364 Germany, we found an overall low incidence of *A. fumigatus* isolates that would be
365 considered clinically resistant (1-3%). However, we observed a reduced abundance of
366 the fungus following the growing season and azole application, which coincided with a
367 modest, but consistent decrease in azole susceptibility. Interestingly, this susceptibility
368 was transient and reset by the following spring. We also assessed the influence of azole
369 application on *A. fumigatus* population dynamics by WGS and were unable to find a
370 clear impact on the population structure or genetic diversity.

371 Despite sampling on fields that were actively treated with azole fungicides, and in
372 regions of Germany with above average fungicide exposure, only 1-3% of *A. fumigatus*
373 isolates collected were resistant to medical azoles. This incidence is in agreement with
374 clinically-reported frequencies in Germany and other European countries, where the rate
375 of triazole resistance ranged from 0.6% to 4.2% (3.2% in Germany) (27, 28). The
376 environmental resistance rates reported for Europe, however, have been much broader.
377 Some studies have found environmental resistance frequencies approaching 20%, while
378 others have reported virtually no incidence of resistant environmental isolates (29-34).
379 Part of these differences could be attributed to differences in methodology, such as the

380 usage of azole selection during the isolation procedure or inclusion of potentially clonal
381 isolates from a given sample to skew the data. In the current study, we avoided azole
382 selection during the isolation procedure, as well as only allowed two isolates per soil
383 sample, to avoid potentially clonal isolates that influence the results. Another potentially
384 contributing factor could be that while all technically "environmental" isolates, rural or
385 agricultural settings could be a completely different niche than an urban flower garden—
386 a supposition supported by a recent study where rural areas in the UK had much lower
387 resistance rates (1.1%) than urban locations (13.8%) (33).

388 Another important and novel finding from this study is that isolates from after the
389 vegetative period and azole exposure consistently showed decreased azole
390 susceptibilities to difenoconazole and tebuconazole, as well as the subtle MIC shift to
391 both agricultural and medical azoles. However, one shortcoming to our study is that we
392 did not collect isolates from organic fields at a matching time point for susceptibility
393 testing, so we cannot exclude that the changes observed in azole susceptibility are not
394 also influenced by seasonal changes. The observation that changes in susceptibility are
395 transient and reset in the period between the end of the growth period and the following
396 spring on the two field is intriguing and worthy of further study. This transformation could
397 either be the result of a naïve population coming in via aerosol dispersal, or a
398 consequence of isolates acquiring only transient resistance, a phenomena previously
399 observed in other plant pathogenic fungi, as well as the human pathogen *Mucor*
400 *circinelloides* (35-37).

401 We observed a wide range in the CFU/g soil within and between farms during
402 annual spring sampling, but the mean level for each farm was stable between years for

403 reasons that remain undiscovered. Our mean *A. fumigatus* CFU/g soil was in line with
404 what was reported recently for abundance in wheat grain, maize silage, and fruit waste
405 (38). However, this abundance is several magnitudes lower than that reported for *A.*
406 *fumigatus* in flower bulb waste and green material waste in this same study, where it
407 was not uncommon to isolate 10_4 CFU/g. Importantly, we demonstrated a reduction in
408 the *A. fumigatus* population size on fields in the context of azole application for plant
409 protection. Despite this apparent population contraction, there were no substantial or
410 consistent changes in the population genomics of *A. fumigatus* on the fields we
411 sampled. One possible confounding factor in comparing time points before and after
412 azole exposure could be the significant aerial dispersion of the fungus. Once the
413 effective fungicide concentration decreased sufficiently, but before we collected samples
414 after azole application, new isolates could be introduced to the field and add their
415 genetic diversity.

416 Our study also provides the first WGS-based study focused on *A. fumigatus* in its
417 natural niche, the environment. Previous studies have primarily concentrated on clinical
418 isolates, with particular priority given to resistant strains (24, 39). Even while sampling
419 within the same field, we found a large degree of genetic diversity, where the majority of
420 diversity came from within samples. We also did not observe a defined population
421 structure or separation between farms or regions. The degree to which this
422 environmental diversity is recapitulated in clinical isolates, and whether there are
423 enrichments for particular subgroups in the transition from environment to clinic is an
424 interesting question for further study.

425 Given our low observed resistance rate among agricultural isolates, as well the
426 lack of genomic impact of azole application on the fields surveyed, our study does not
427 find evidence that azole fungicide use in crop agriculture significantly contributes to
428 resistance in *A. fumigatus,* nor should be necessarily removed from use in this context
429 due to their crucial role in global food production. Instead, we propose a refined model,
430 where azole resistance in *A. fumigatus* is being driven by the use of these compounds in
431 the cultivation of flowers and other ornamentals. It is common practice to dip flower
432 bulbs directly in fungicide stocks prior to storage and shipment, and their use in this
433 setting should be more carefully considered and managed (40, 41). This revised model
434 is supported by the recent identification of flower bulb and green waste as environmental
435 "hotspots" for resistance selection, which possess both high overall fungal colony counts
436 as well as increased incidence of resistant *A. fumigatus* isolates compared to soil (38).
437 Unfortunately, due to massive aerial dispersal of *A. fumigatus* conidia, the use of azoles
438 in any hotspot can lead to the worldwide distribution of resistant strains.

439

440 **MATERIALS AND METHODS**

441 Site selection and soil sampling

442 During 2016 to 2018, soil sampling was conducted on agricultural sites in the federal
443 states of Thuringia, Saxony-Anhalt, and Saxony with the approval of the land owner
444 and/or relevant ministries. The majority of fields sampled were growing cereals such as
445 wheat or barley, but some apple orchards were sampled as well (see Supplemental
446 Table 1 for full details). Farms were arbitrarily assigned an alphabetic identifier (A-L) and
447 specific fields a numeric identifier as described in the supplementary Materials &

448 Methods. Due to crop rotation, the same field could not be surveyed over subsequent
449 years, with the exception of the apple orchards. Soil samples were collected at
450 beginning of the vegetation period on the conventional and organic cultivated sites and
451 after azole application an additional sampling was carried out on the conventional sites.
452 In general, 50 soil samples per site and type of farming (conventional or organic) were
453 collected, with a total of approximately 1000 soil samples per year. Soil samples were
454 selected to best cover the field with a minimum distance of 1 m between samples. For
455 each sample, the top layer of soil was collected by a metal spatula into a sterile sample
456 cup and refrigerated until processing.

457

458 Soil processing and isolation of *A. fumigatus*

459 3 g of soil from each sample cup was weighed out and resuspended in 8 mL 0.2M NaCl
460 containing 1% Tween20. Samples were vortexed vigorously and then left to settle until a
461 phase separation became apparent. 2 mL of the upper phase was transferred to a new
462 tube for plating onto Sabouraud Glucose Agar (SGA) containing 50 µg/mL
463 Chloramphenicol (Sigma-Aldrich, Taufkirchen, Germany). Of this 2 mL, 150 µL was
464 plated onto one plate and the remaining volume was plated onto a second plate in order
465 to adjust for variable fungal concentrations in samples. Plates were then incubated at
466 50°C for 5 days to select for *A. fumigatus,* which is unique among *Aspergillus* spp in its
467 ability to grow at this temperature. On day 5, the incubator temperature was reduced to
468 42°C to allow for sporulation and plates grown for another 2 days. The number of *A.*
469 *fumigatus* colonies was counted and up to two colonies per soil sample transferred to
470 new plates for isolation.

471

472 Antifungal susceptibility testing

473 Quick screening of susceptibility to itraconazole, voriconazole, and posaconazole were
474 assessed using the agar-based VIPcheck™ Assay (Mediaproducts BV, Groningen,
475 Netherlands) following the manufacturer's directions. For testing, isolates were grown on
476 SGA for 2-4 days at 37°C. Plates were then swabbed with a damp, sterile cotton swab
477 to prepare a conidial suspension of 0.5-2 McFarland. 25 µL of this suspension was then
478 plated onto the 4 wells of the VIPcheck plate containing 4 mg/L itraconazole, 2 mg/L
479 voriconazole, 0.5 mg/L posaconazole, or a control well containing no drug. To assess
480 susceptibility to agricultural azoles during isolates collected during 2017 and 2018,
481 RPMI+2% glucose agar plates containing either 1 mg/L difenoconazole or 2 mg/L
482 tebuconazole were prepared as in (42). Resistance was defined as significant inhibition
483 of germination and hyphal growth compared to the no drug control. Isolates that showed
484 resistance to any of the medical azoles in the VIPcheck assay were subject to broth
485 microdilution following EUCAST methodology.

486

487 DNA extraction

488 For WGS and PCR-based amplification, isolates were grown shaking in SG broth at
489 37°C and genomic DNA isolated using the Quick-DNA Fungal/Bacterial miniprep kit
490 (Zymo Research, Ivrine, CA) according to the manufacturer's suggested protocol.

491

492 *cyp51a* genotyping

493 The *cyp51a* coding sequence and upstream region containing the tandem repeat was
494 amplified using the primers described in Supplemental Table 4. Cleaned up PCR

495 products were sequenced and the *cyp51a* genotype determined using FunResDB
496 (https://elbe.hki-jena.de/FunResDb/index.php).
497
498 Genome sequencing, quality assessment, and alignment
499 Library preparation and 2x150bp paired-end sequencing was performed on a NextSeq
500 500 v2 by LGC Genomics (Berlin, Germany) following the manufacturer's recommended
501 protocols. Sequence data quality control and filtering were performed using an in-house
502 script and FastQC (v0.11.5). Quality reads were mapped to the *A. fumigatus* Af293
503 reference genome (version 2015-09-27; retrieved from FungiDB (43)) using BWA-MEM
504 (version 0.7.8-r779-dirty) (44). PCR duplicates were marked using MarkDuplicate from
505 Picard version 2.18.25 embeded in the Genome Analysis Toolkit (GATK; version
506 4.1.0.0). All WGS samples included for analysis possessed greater than 10 fold genome
507 coverage after mapping and more than 90% of reads mapped to the reference genome.
508
509 Variant identification and SNV-based phylogeny
510 Short variants, including single nucleotide variants (SNVs) and short insertions and
511 deletions (InDels) were detected using GATK Haplotype Caller following their
512 recommended best practices for single calling (45). Copy number variants (CNVs) were
513 identified using Control-FREEC (46). For the phylogenetic analysis, nucleotide
514 consensus sequences were extracted from vcf files using VCFtools (47) and an in-house
515 script used to translate nucleotide to protein-coding sequences. Multiple sequence
516 alignment was performed using MUSCLE v3.8.31 (48) using 7,771 conserved core
517 genes and an approximately-maximum-likelihood phylogeny constructed using

518 FastTree2 (version 2.1.10) (49). Interactive Tree of Life (iTOL) v4 was used for
519 visualization (50).
520
521 Genetic diversity analyses
522 Analysis of molecular variance (AMOVA) was determined using the R package ade4
523 (nrep = 999). Nucleotide diversity (π) was calculated by VCFtools (version 0.1.6) using
524 5kbp windows with a step size of 500bp. Nucleotide polymorphism (θ) and Tajima's D
525 were calculated using ANGSD (51) using a window size of 5 kbp and a step size of 500
526 bp. Weighted Weir and Cockerham's F_{ST} values were calculated using VCFtools, while
527 V_{ST} values were calculated as in (52). A Mantel test correlating geographic distance
528 matrices with pairwise FST matrices was performed using the R package ade4 (nrep =
529 9999).
530
531 Estimation of fungicide treatment areas and rates in Germany
532 The fraction of each district theoretically treated with fungicides was calculated using
533 publicly reported data from 2016 available from the German Statistical Offices
534 (statistikportal.de) using the following equation:

535 $$\frac{(Ha_{Ackerland} + Ha_{Dauerkulturen}) \times Fraction_{Conventional}}{Ha_{Total}}$$

536
537 The total area per district was obtained from Table 33111-01-02-4: Ground area by
538 actual use. The sum of arable farmland and permeant crop areas was calculated for
539 each administrative district using Table 41141-01-01-4: Farms and their agricultural
540 used area by crop type. To accommodate that some percentage of this area is cultivated
541 under organic agriculture methods, the hectares of crop land was then multiplied by the

542 fraction of non-organic agriculture for the federal state in which the district is located to
543 estimate the number of hectares potentially treated with fungicides (as calculated using
544 data on “Farms, agricultural areas, and workers”; accessed on November 1, 2019 on
545 https://www.statistikportal.de/node/254). Unfortunately, no data was available on the
546 breakdown of agricultural methods at the district level to allow for more exact estimation.
547 Finally, this value of estimated treated area per district was divided by the total area of
548 the district for visualization as a choropleth map.

549

550 Box and whisker plots

551 Box and whisker plots presented in this paper are in the style of Tukey, where the bold
552 line indicates the 50th percentile, and the hinges represent the 25th and 75th percentiles.
553 The lower whisker extends from the lower hinge to the lowest datum within 1.5
554 interquartile range (IQR), while the upper whisker represents the highest datum still
555 within 1.5 IQR. Outliers are marked with points.

556

557 Data and isolate availability

558 Isolates generated within this study were submitted to and are publicly available in the
559 Jena Microbial Resource Collection. Raw FASTQ files were uploaded to the NCBI
560 Sequence Read Archive and are publicly available under BioProject XXXXXXX.

561

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AUTHOR CONTRIBUTIONS

O.K. and H.B.D. conceived the study. A.E.B. and J.B. collected samples and performed experiments. W.B. provided reagents and experimental advice. A.E.B., J.B., T.S.E., K.K, G.P., H.B.D., and O.K. analyzed the data and interpreted the results. A.E.B. wrote the manuscript with input from all authors.

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Table 1. Analysis of molecular variance (AMOVA) between farms. Calculated using isolates from before and after azole application. n = 16 isolates per farm.

	Total variation	Variation within samples (%)	p-value	Variation between samples (%)	p-value	Variation between farms (%)	p-value
All farms	289735.7	274622.7 (94.8)	<0.001	14629 (5)	<0.001	484 (0.2)	0.303
B vs. C	147358.0	139257.7 (94.5)	<0.001	6386.2 (4.3)	<0.001	1714.1 (1.2)	0.028
B vs. D	190425.2	181470 (95.3)	<0.001	9002.4 (4.7)	<0.001	-47.2 (0.0)	0.429
B vs. E	162874.7	155745.5 (95.6)	<0.001	6879.3 (4.2)	<0.001	249.9 (0.2)	0.211
C vs. D	130625.7	122692.8 (93.9)	<0.001	7932 (6.1)	<0.001	1 (0.0)	0.355
C vs. E	105529.1	99521.4 (94.3)	<0.001	6098.3 (5.8)	<0.001	-90.6 (-0.1)	0.414
D vs. E	143684.1	135847.0 (94.5)	<0.001	8382.7 (5.8)	<0.001	-545.6 (0.4)	0.890

Table 2. Analysis of molecular variance between isolates collected before and after azole exposure. n = 16 isolates per farm field (eight pre-azole and eight post-azole).

	Total variation	Variation within samples (%)	p-value	Variation between samples (%)	p-value	Variation between time periods (%)	p-value
Farm B	106712.9	103295.3 (96.8)	<0.001	3729.5 (3.5)	<0.001	-311.9 (-0.3)	0.617
Farm C	48438.0	45393.1 (93.7)	<0.001	3341.7 (6.9)	<0.001	-296.8 (-0.6)	0.593
Farm D	85724.6	80522.3 (93.9)	<0.001	5592 (6.5)	<0.001	-389.7 (-0.5)	0.564
Farm E	59366.5	55941.4 (94.2)	<0.001	3001.2 (5.1)	<0.001	423.9 (0.7)	0.149

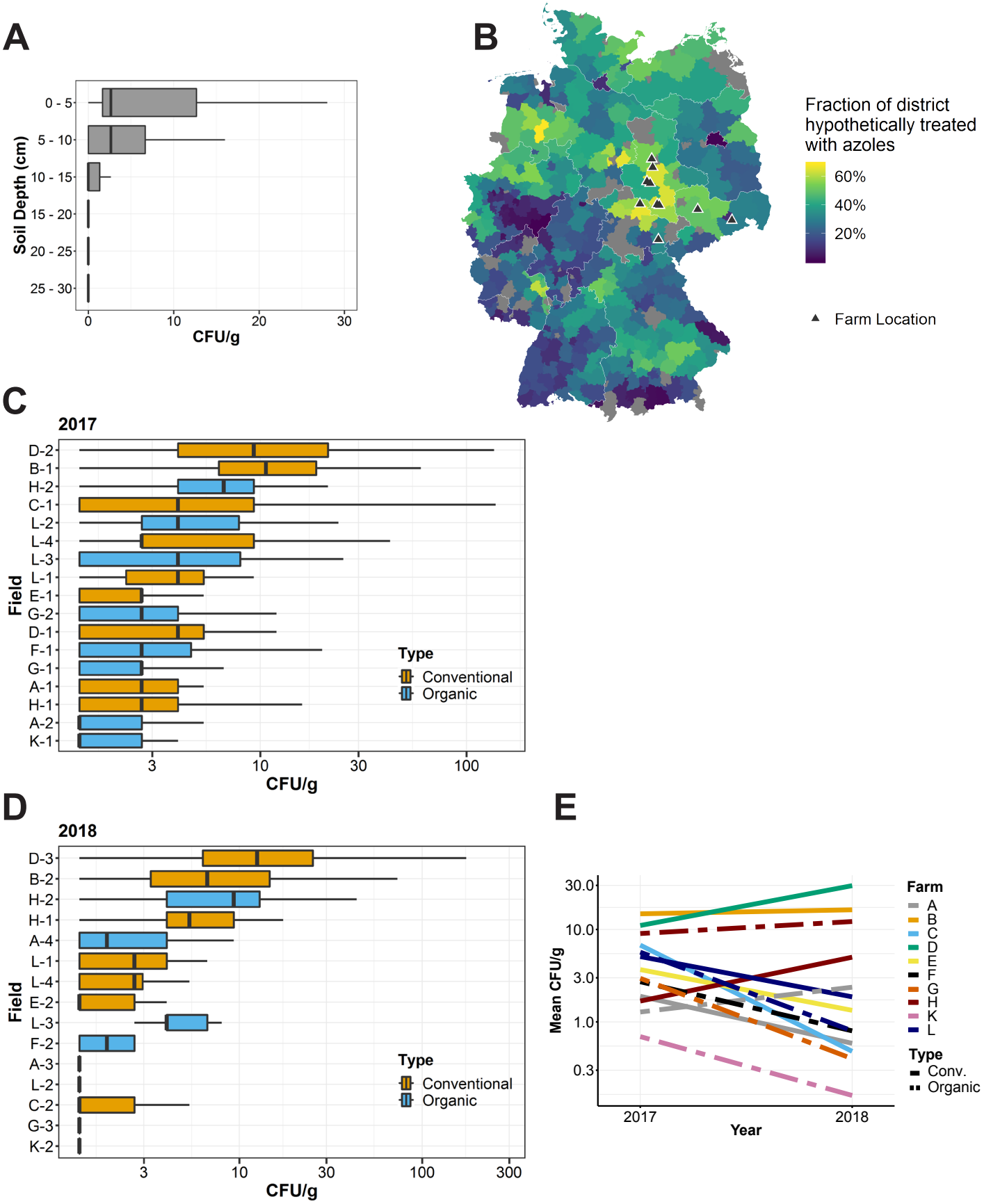


Figure 1. Abundance of *A. fumigatus* in the soil of conventional and organic farms.
(A) CFU/g *A. fumigatus* at varying soil depths. n = 10 samples per depth. (B) Estimated fungicide treatment rates and areas in Germany. The fraction of each district that is theoretically treated with fungicides was calculated using land use and organic agriculture share data reported by the Statistical Office of Germany in December 2016. Districts where no data on land use was available are shaded grey. (C-D) Abundance of A. fumigatus in the spring as measured by CFU/g soil in the spring of 2017 (C) and 2018 (D). For 2017, boxplots represent n = 50 soil samples per field for farms A, B, C, E, F, H, K and n = 25 for farms D, G, L. For 2018, n = 50 soil samples per field for farms A, B, C, D, E, G, H, K and n = 25 for farms F and L. (E) Comparison of the mean CFU/g soil on farms between 2017 and 2018.

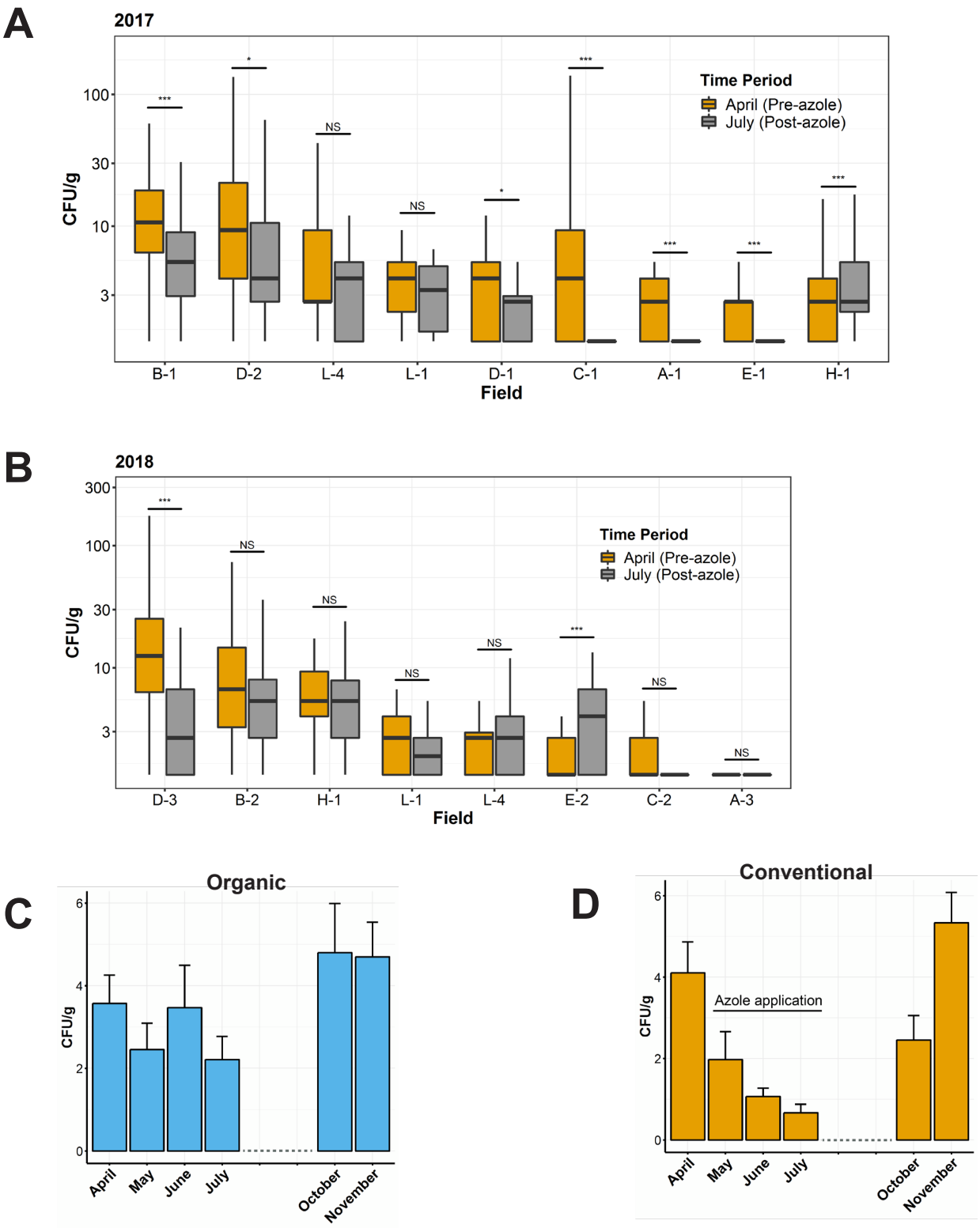
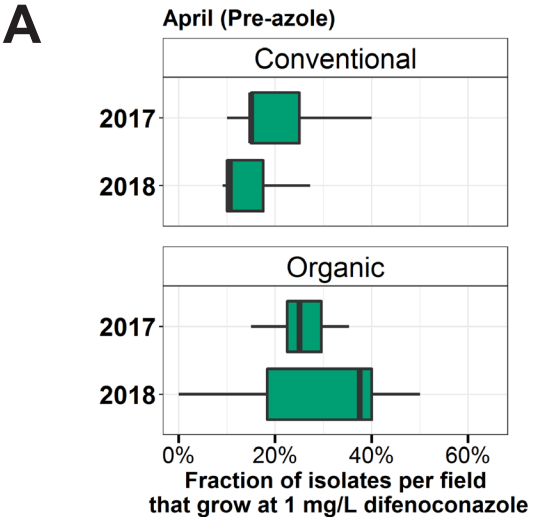
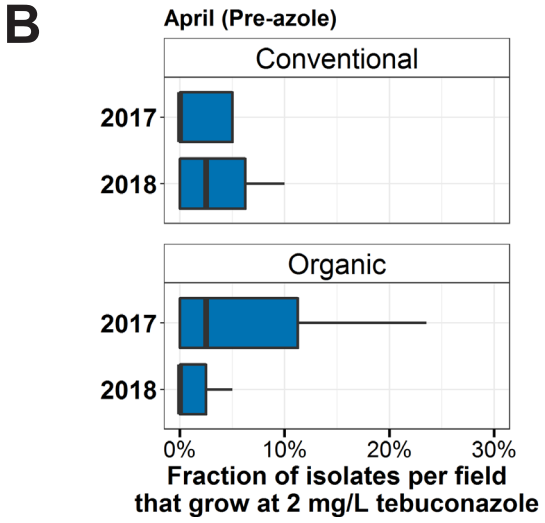
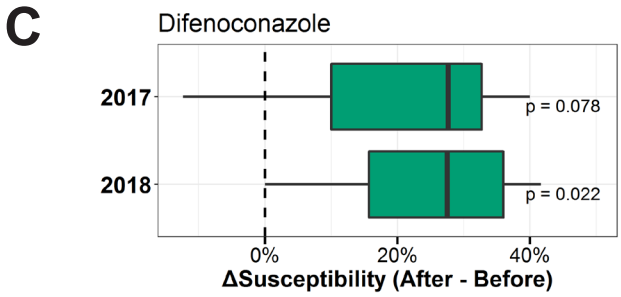
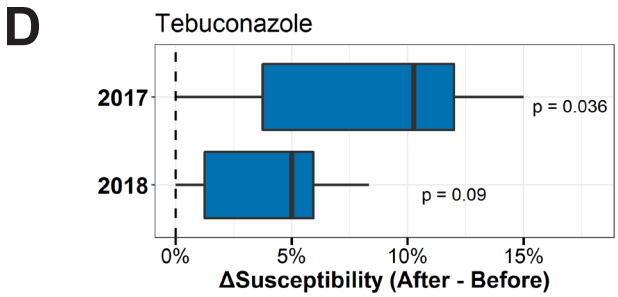


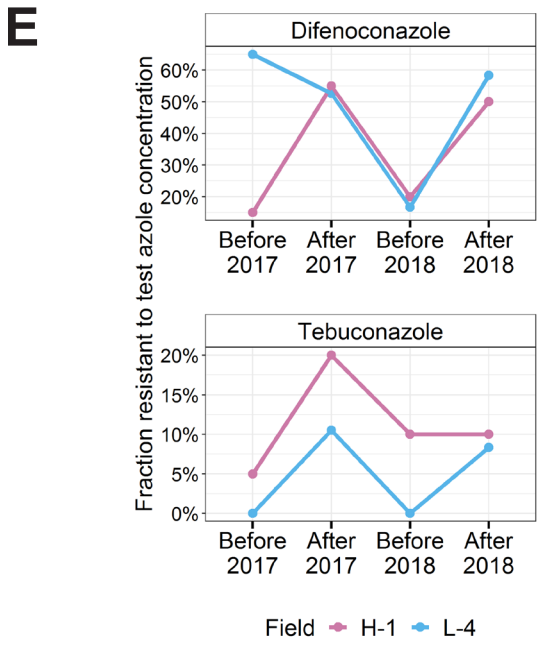
Figure 2. Abundance of *A. fumigatus* in the soil of conventional farms before and after the vegetative period and azole application.
(A, B) CFU/g *A. fumigatus* on conventional fields sampled in April, prior to the vegetative period and azole application (orange), and in July, after the vegetative period and three months of azole application (gray) in 2017 (A) and 2018 (B). *, $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ as determined by Mann-Whitney U test. (A) Boxplots represent n = 50 samples per field and time point for farms A, B, C, E, H and n = 25 samples for farms D and L. (B) n = 50 samples per field and time point for farms A, B, C, D, E, H and n = 25 samples for farm L. (C-D) CFU/g A. fumigatus during the months of April, May, June, July, October and November of a conventional field applying azoles from May-July (D) and an organic field not applying azole fungicides (C). Bars represent mean ± SEM of 50 soil samples per month. No significant difference was found in abundance between the months of April, May, June, and July for the organic field using a Kruskal-Wallis test. In contrast, we found a significant difference (P = 0.004) for the abundances in this time period on the conventional field. Pairwise Wilcoxon signed-rank test confirmed a significant difference between April and all subsequent months until the cessation of azoles.





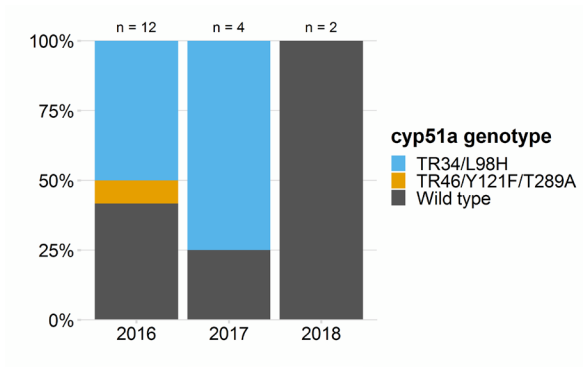






F

		Resistance rate (%)		
Year	n	ITR	POS	VOR
2016	333	3.0	2.1	2.1
2017	460	0.7	0.7	0.7
2018	322	0.6	0.0	0.0



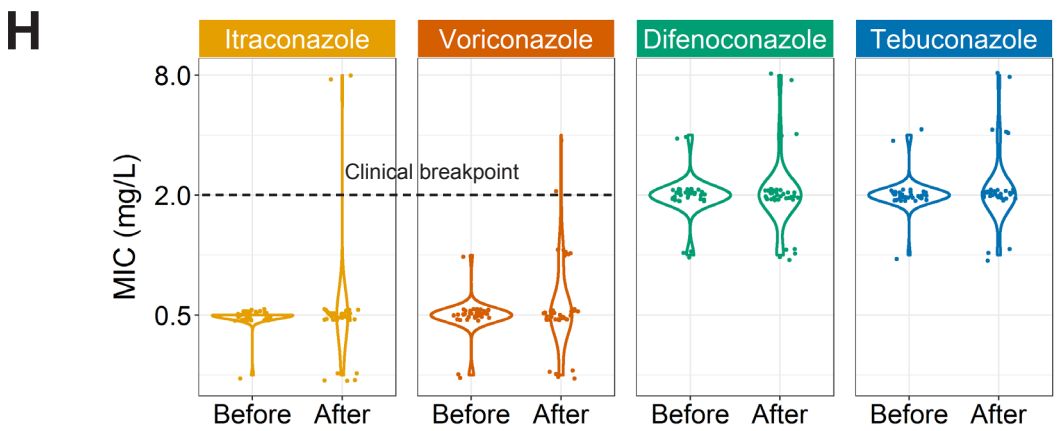


Figure 3. Azole resistance among agricultural *A. fumigatus.*
(A-B) Fraction of the ≈ 20 isolates tested per field that grow at 1 mg/L difenoconazole (A) and 2 mg/L tebuconazole (B). For 2017, n = 340 isolates from 9 conventional and 8 organic fields. For 2018, n = 213 isolates from 8 conventional and 7 organic fields (full summary in Supplemental Tables 2 & 3). (C-D) Comparison of the proportion of isolates that grow at the 1 mg/L difenoconazole (C) and 2 mg/L tebuconazole (D) before and after the vegetative period and azole application. For 2017, n = 275 isolates from 7 fields and for 2018, n = 261 isolates from 8 fields; n ≈ 20 isolates per field tested (full summary in Supplemental Tables 4 & 5). Positive values indicate an increased proportion for after compared to before; negative values indicate a decreased proportion for after compared to before. P values calculated by Wilcoxon signed-rank test between before and after values for each field. (E) Local resistance/tolerance in agricultural *A. fumigatus* populations shifts in response to azole application. Fraction of isolates that can grow at 1 mg/L difenoconazole (top) and 2 mg/L tebuconazole (bottom). n = 12-20 isolates/field and time point. (F) Medical azole resistance rates among agriculturally-isolated *A. fumigatus* isolates. Isolates were screened for potential azole resistance using the VIPcheck agar-based screening. Resistance was confirmed and MICs determined via EUCAST broth microdilution testing. (G) cyp51a genotypes of isolates resistant to one or more medical azole. (H) MIC of agricultural *A. fumigatus* isolated before and after azole exposure from 2 fields (n = 20 before and n = 20 after from each field, n = 79 in total).

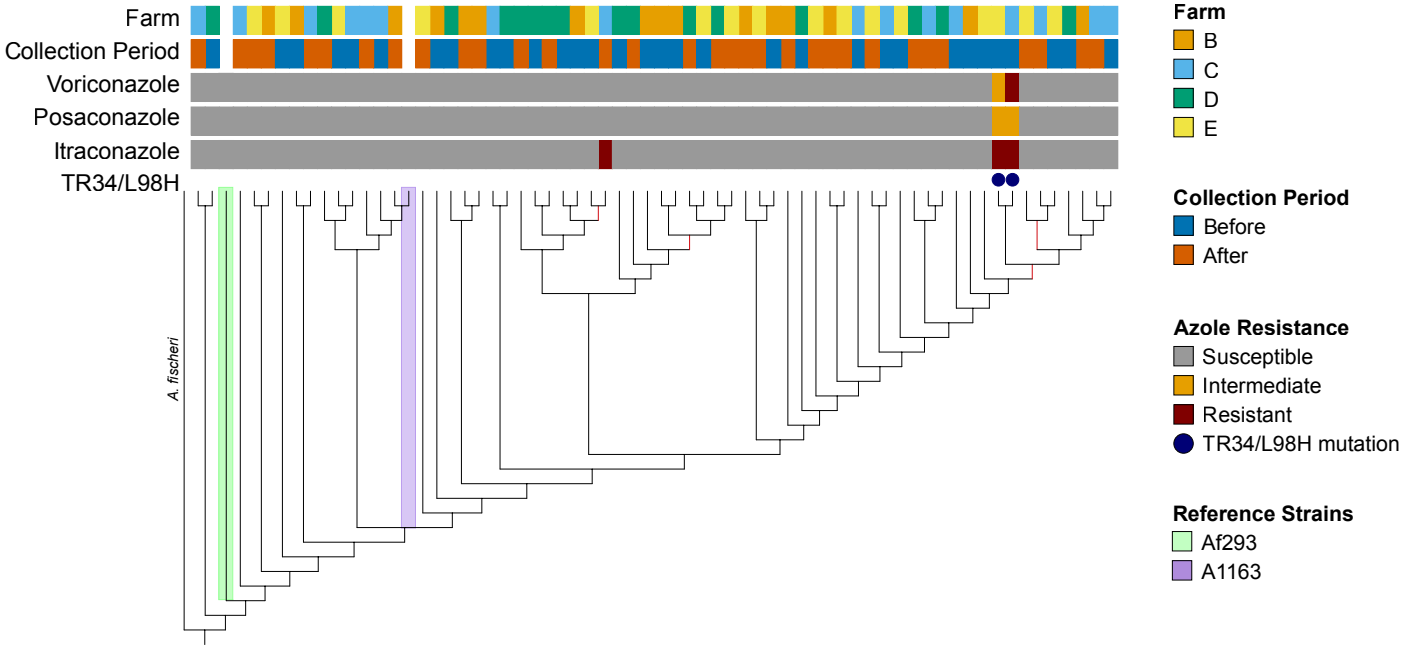


Figure 4. Phylogeny of agricultural *A. fumigatus* isolates from before and after the vegetative period and azole application.
From top to bottom, the colored bars indicate: 1) the farm where the isolate was collected, 2) the collection period, 3) voriconazole resistance (susceptible, intermediate, or resistant, according to EUCAST definitions), 4) posaconazole resistance, 5) itraconazole resistance, and 6) the presence of the cyp51a TR34/L98H allele. *A. fischerei* is indicated as an out group and the two *A. fumigatus* reference strains, Af293 and A1163 (CEA10), are also marked. Branches with support values less than 0.9 are marked in red.

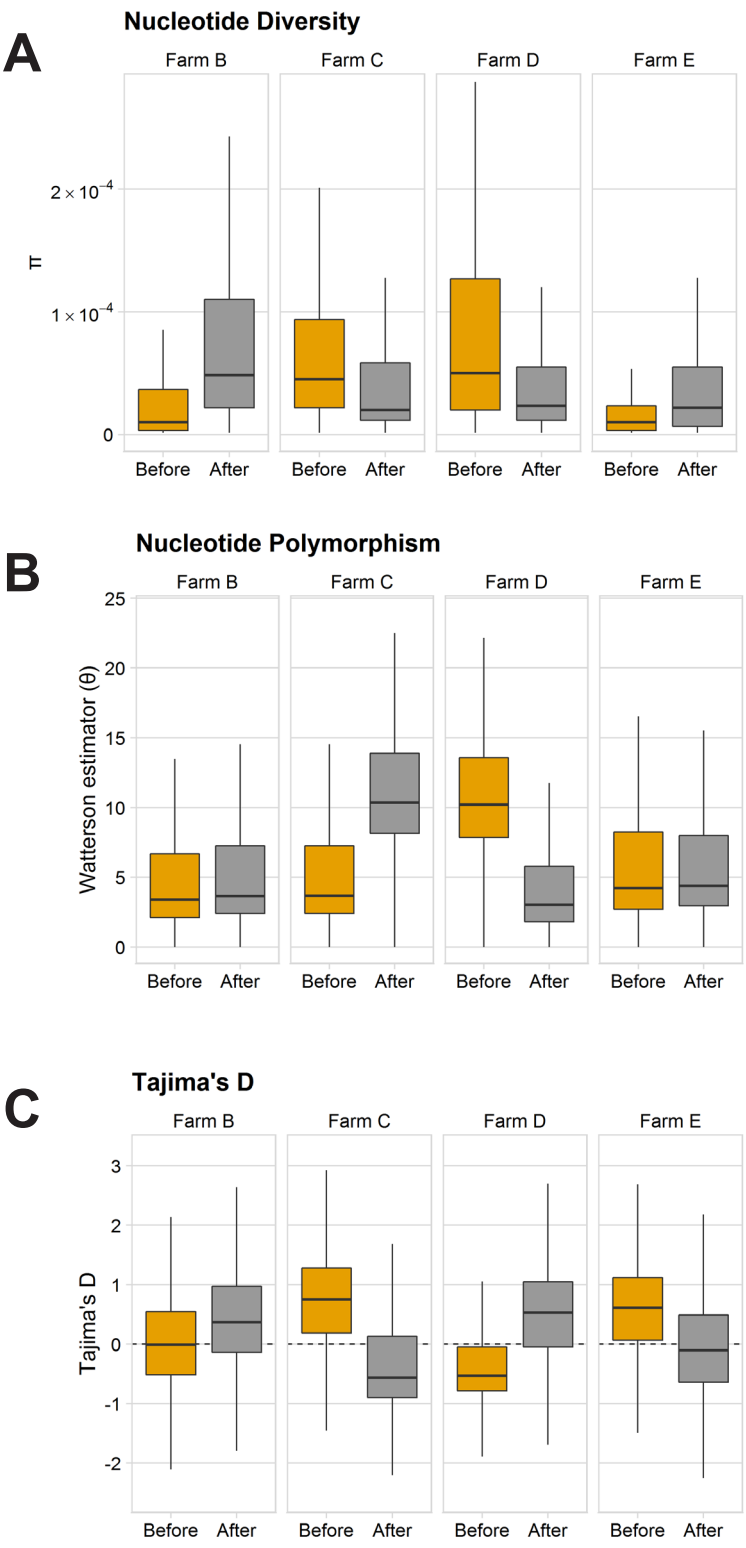
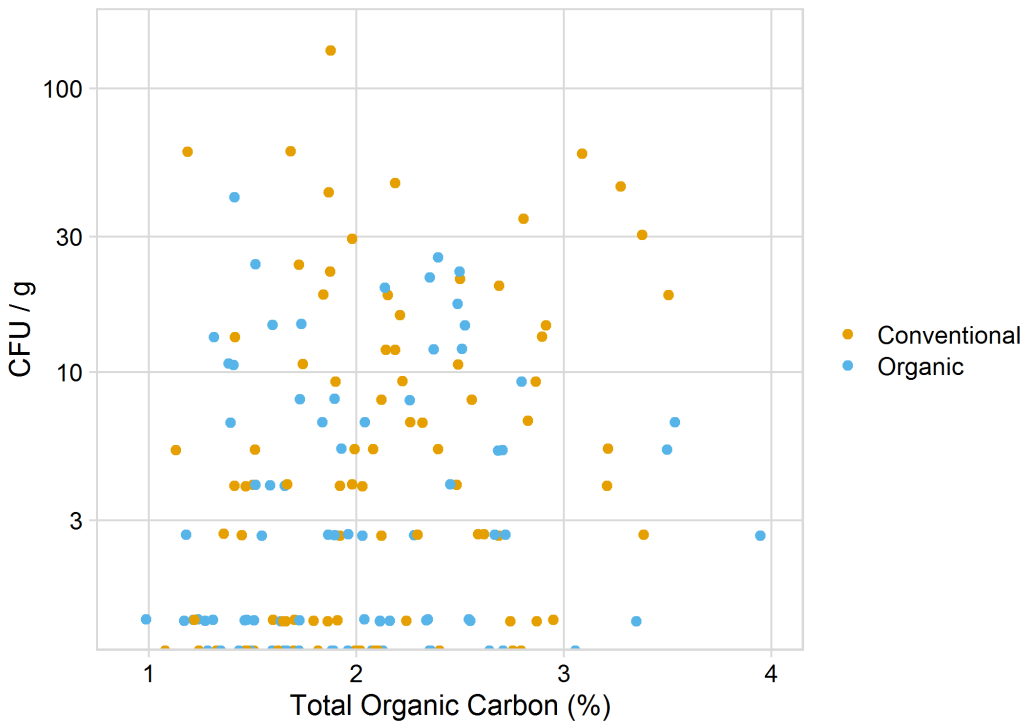
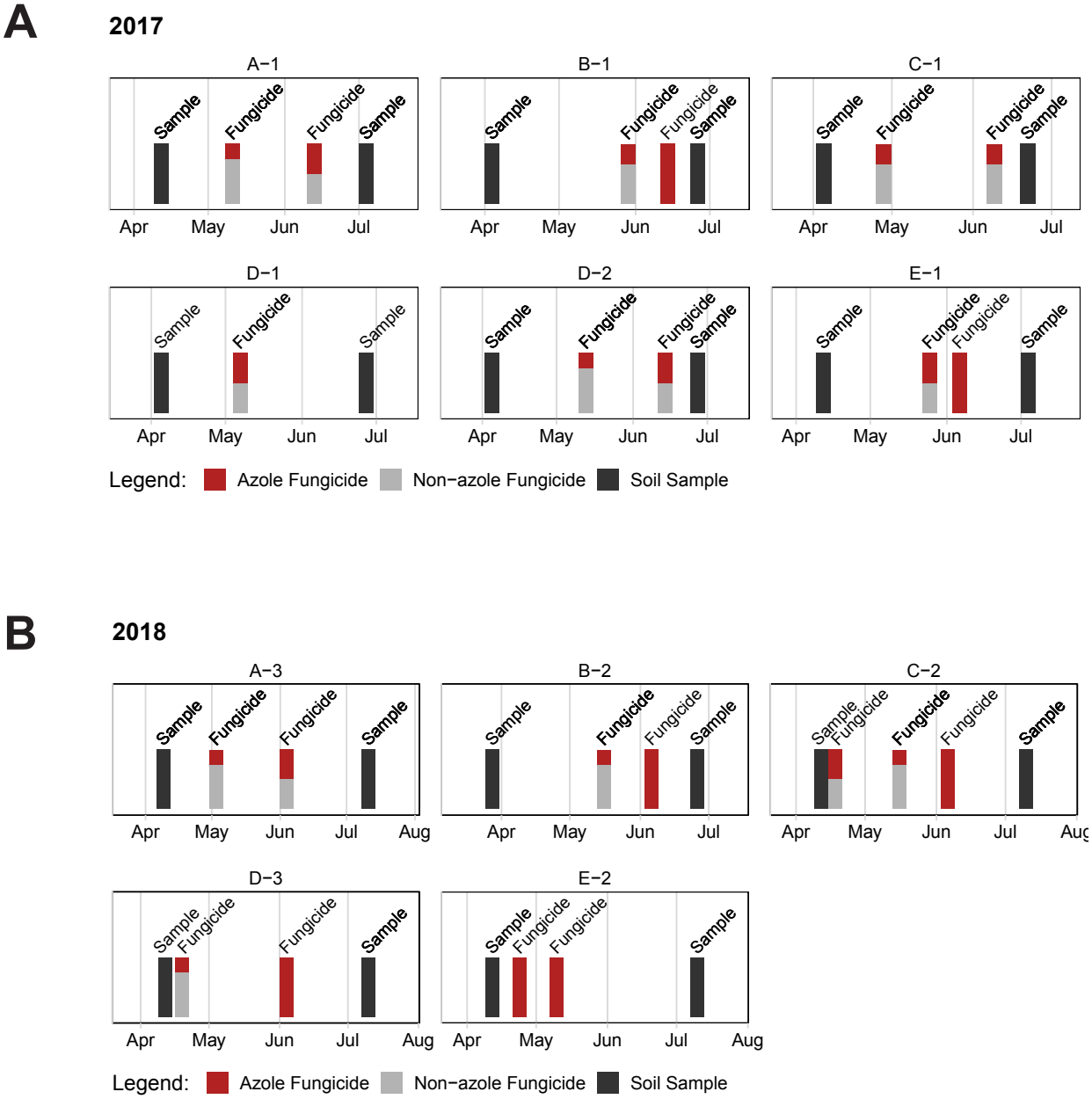


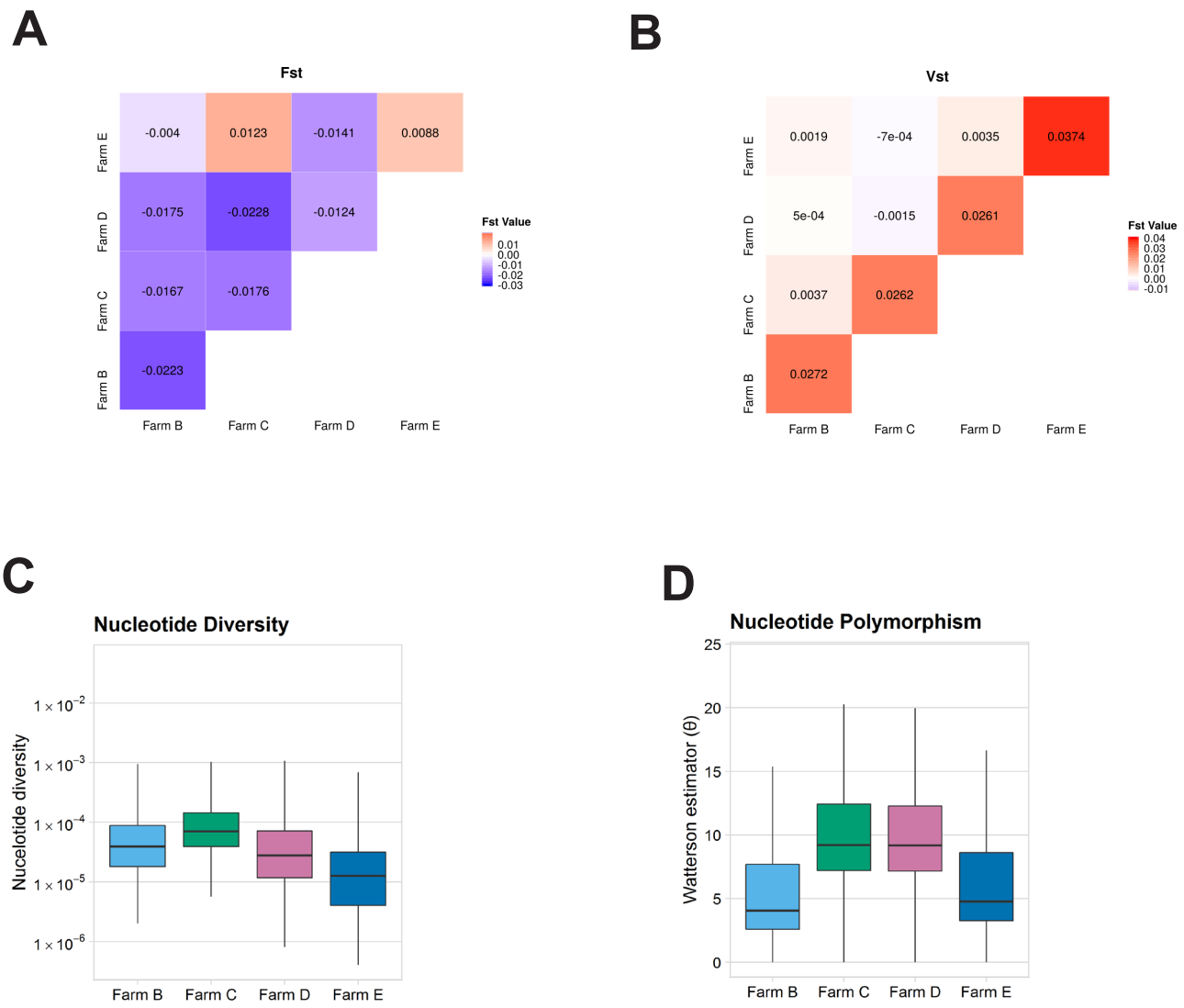
Figure 5. Genetic diversity among isolates from before and after the vegetative period and azole exposure from four farms.
(A-C) Nucleotide diversity (π) (A), nucleotide polymorphism (Watterson estimator or θ) (B), and Tajima's D (C) along 5 kb windows before and after the vegetative period and azole exposure. n = 8 isolates per farm and time point, 64 in total.



Supplemental Figure 1. *A. fumigatus* abundance does not correlate with total organic carbon content of soil. n = 170 soil samples (10 per field from 17 fields on 10 different farms).



Supplemental Figure 2. Schematic representation of the sampling time points and fungicide applications.
(A, B) Schematic representation of the sampling time points and fungicide applications for the fields sampled in 2017 (A) and 2018 (B). Soil sampling time points are marked in dark grey, while fungicide applications are noted in a combination of red and light grey indicating the relative proportion of azoles within the fungicide cocktail applied. Fungicide history was not available for fields on Farms H and L.



Supplemental Figure 3. Population genetic of farms using isolates from before and after azole exposure.
n = 16 isolates per farm. (A-B) Heat map representing weighted Weir and Cockerham F_{ST} values (A) and V_{ST} values (B) between farms. Blue colors represent more similarity between populations, while red represents greater population differentiation. (C-D) Comparison of nucleotide diversity (π) (C) and nucleotide polymorphism (Watterson estimator or θ) (D) between farms. Values calculated from 5 kb sliding windows.

SUPPLEMENTAL MATERIALS

SUPPLEMENTAL MATERIALS & METHODS

Additional information on field samling and the naming of fields

Six of the sampled farms practiced biological (organic) measures of land management without azole application, while seven practiced integrated pest management, including application of azole fungicides. To differentiate the year the sampling occurred, a corresponding suffix was added to the Field ID (*e.g.* D-2-17 identifies the second field sampled on farm D in the year 2017). Due to crop rotation, the same field could not be surveyed over subsequent years, nor was it possible to always sample the same number of fields from each site each year. To avoid confusion, when we returned to farms the following year, fields were assigned new numbers starting with where the labeling system left off the previous year. (*e.g.* on farm D, two field were sampled in 2017, so in 2018 the labeling system started with D-3). Fields that were sampled over subsequent years, which was only possible for apple orchards, maintained the same numbering system (*e.g.* L-4-17 and L-4-18 are the same fields). In the case where two fields of the same agricultural type on the same farm were sampled, the number of soil samples was divided between the two fields and 25 samples per field were collected.

Assessment of TOC content

Soil samples were air dried and sieved through a 2 mm stainless steel mesh to remove larger organic particles and stones. Samples were then manually ground to a fine powder and divided into two parts. The first fraction (approximately 1000 mg) was used to measure total carbon (TC) content by total combustion with oxygen at 1000°C using an elemental macro-analyzer (Elementar Vario Max Cube, Langenselbold, Germany). The second fraction (appr. 200 mg) was used to determine the total inorganic fraction (TIC) present by volumetrically

adding hydrochloric acid and recording the volume of CO_2 produced (also using an Elementar Vario Max Cube, Langenselbold, Germany). The final total organic carbon (TOC) content in the soil was calculated by subtracting the total carbon (TC) from the total inorganic carbon (TIC).

Supplemental Table 1. Summary of the agricultural sites surveyed 2016- 2018.

Farm	Type of Agriculture	Crop	Soil samples (n)			*A. fumigatus* isolates (n)		
			2016	2017	2018	2016	2017	2018
A	Conventional and organic	Cereal	150	150	150	232	94	81
B	Conventional	Cereal	100	100	100	114	266	335
C	Conventional	Cereal	100	100	100	29	123	37
D	Conventional	Cereal	100	100	100	65	228	287
E	Conventional	Cereal	100	100	100	61	131	165
F	Organic	Cereal	50	50	25	73	87	15
G	Organic	Cereal	50	50	50	29	87	13
H	Conventional and organic	Apple	100	150	150	158	301	421
K	Organic	Cereal	50	50	50	45	26	6
L	Conventional and organic	Apple	100	150	150	91	184	162
		Total	900	1000	975	897	1527	1522

Supplemental Table 2. Resistance summary of the fields sampled during the spring of 2017 (A) and 2018 (B).

(A)

				Proportion of isolates that grow at the concentration indicated				
Type of agriculture	Field	Crop	n tested	DIF (1 mg/L)	TEB (2 mg/L)	ITR (4 mg/L)	VOR (2 mg/L)	POS (0.5 mg/L)
Conventional	A-1-17	Cereal	20	0.15	0.00	0.00	0.00	0.00
	B-1-17	Cereal	20	0.25	0.05	0.00	0.00	0.00
	C-1-17	Cereal	20	0.10	0.00	0.00	0.00	0.00
	D-1-17	Cereal	20	0.25	0.00	0.00	0.00	0.00
	D-2-17	Cereal	20	0.40	0.05	0.00	0.00	0.00
	E-1-17	Cereal	20	0.15	0.05	0.00	0.00	0.00
	H-1-17	Apple	20	0.15	0.05	0.00	0.00	0.00
	L-1-17	Apple	20	0.15	0.00	0.00	0.00	0.00
	L-4-17	Apple	20	0.65	0.00	0.00	0.00	0.00
Organic	A-2-17	Cereal	20	0.35	0.15	0.05	0.00	0.00
	F-1-17	Cereal	20	0.25	0.00	0.00	0.00	0.00
	G-1-17	Cereal	20	0.25	0.10	0.05	0.00	0.00
	G-2-17	Cereal	20	0.15	0.00	0.00	0.00	0.00
	K-1-17	Cereal	20	0.25	0.00	0.00	0.00	0.00
	H-2-17	Apple	20	0.25	0.00	0.00	0.00	0.00
	L-2-17	Apple	20	0.10	0.05	0.00	0.00	0.00
	L-3-17	Apple	20	0.35	0.25	0.00	0.00	0.00

(B)

				Proportion of isolates that grow at the concentration indicated				
Type of agriculture	Field	Crop	n tested	DIF (1 mg/L)	TEB (2 mg/L)	ITR (4 mg/L)	VOR (2 mg/L)	POS (0.5 mg/L)
Conventional	A-3-18	Cereal	11	0.27	0.00	0.00	0.00	0.00
	B-2-18	Cereal	20	0.10	0.05	0.00	0.00	0.00
	C-2-18	Cereal	11	0.09	0.00	0.00	0.00	0.00
	D-3-18	Cereal	20	0.10	0.05	0.00	0.00	0.00
	E-2-18	Cereal	20	0.10	0.10	0.00	0.00	0.00
	H-1-18	Apple	20	0.20	0.10	0.00	0.00	0.00
	L-1-18	Apple	18	0.11	0.00	0.00	0.00	0.00
	L-4-18	Apple	12	0.17	0.00	0.00	0.00	0.00
Organic	A-4-18	Cereal	20	0.40	0.20	0.00	0.00	0.00
	F-2-18	Cereal	10	0.40	0.00	0.00	0.00	0.00
	G-3-18	Cereal	12	0.50	0.00	0.00	0.00	0.00
	K-2-18	Cereal	5	0.00	0.00	0.00	0.00	0.00
	H-2-18	Apple	20	0.20	0.05	0.00	0.00	0.00
	L-2-18	Apple	6	0.17	0.00	0.00	0.00	0.00
	L-3-18	Apple	8	0.38	0.00	0.00	0.00	0.00

Supplemental Table 3. Resistance summary of the fields sampled before and after the vegetative period and azole application in 2017 (A) and 2018 (B).

(A)

				Proportion of isolates that grow at the concentration indicated				
Field	Crop	Time Period	n tested	DIF (1 mg/L)	TEB (2 mg/L)	ITR (4 mg/L)	VOR (2 mg/L)	POS (0.5 mg/L)
B-1-17	Cereal	Before	20	0.25	0.05	0.00	0.00	0.00
		After	20	0.50	0.15	0.00	0.00	0.00
C-1-17	Cereal	Before	20	0.10	0.00	0.00	0.00	0.00
		After	20	0.45	0.15	0.10	0.00	0.00
D-1-17	Cereal	Before	20	0.25	0.00	0.00	0.00	0.00
		After	20	0.50	0.25	0.00	0.00	0.00
D-2-17	Cereal	Before	20	0.40	0.05	0.00	0.00	0.00
		After	20	0.35	0.05	0.00	0.00	0.00
E-1-17	Cereal	Before	20	0.15	0.05	0.00	0.00	0.00
		After	15	0.47	0.07	0.00	0.00	0.00
H-1-17	Apple	Before	20	0.15	0.05	0.00	0.00	0.00
		After	20	0.55	0.20	0.00	0.00	0.00
L-4-17	Apple	Before	20	0.65	0.00	0.00	0.00	0.00
		After	20	0.50	0.10	0.00	0.00	0.00

(B)

				Proportion of isolates that grow at the concentration indicated				
Field	Crop	Time Period	n tested	DIF (1 mg/L)	TEB (2 mg/L)	ITR (4 mg/L)	VOR (2 mg/L)	POS (0.5 mg/L)
A-3-18	Cereal	Before	11	0.27	0.00	0.00	0.00	0.00
		After	11	0.27	0.18	0.00	0.00	0.00
B-2-18	Cereal	Before	20	0.10	0.05	0.00	0.00	0.00
		After	20	0.25	0.10	0.05	0.00	0.00
C-2-18	Cereal	Before	11	0.09	0.00	0.00	0.00	0.00
		After	16	0.25	0.06	0.00	0.00	0.00
D-3-18	Cereal	Before	20	0.10	0.05	0.00	0.00	0.00
		After	20	0.35	0.10	0.05	0.00	0.00
H-1-18	Apple	Before	20	0.20	0.10	0.00	0.00	0.00
		After	20	0.50	0.10	0.00	0.00	0.00
L-1-18	Apple	Before	18	0.11	0.00	0.00	0.00	0.00
		After	10	0.50	0.00	0.00	0.00	0.00
L-4-18	Apple	Before	12	0.17	0.00	0.00	0.00	0.00
		After	12	0.58	0.08	0.00	0.00	0.00

Supplemental Table 4. Primers used for amplifying and sequencing *cyp51a*.

Primer	**Sequence**	**Use**
cyp51a_F	5'-CGTAGCAAGGGAGAAGGAAA	Amplification and sequencing
cyp51a_R	5'-CACCTATTCCGATCACACCA	Amplification and sequencing
cyp51a_internal	5'-ATGTCAATGCGGAAGAGGTC	Sequencing