Effects of agricultural fungicide use on *Aspergillus fumigatus* abundance, antifungal susceptibility, and population structure

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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 observed a reduction in the abundance of *A. fumigatus* on fields following fungicide treatment in 2017—a finding that was not observed on an organic control field applying only natural plant protection agents. However, this finding was less pronounced during our 2018 sampling, indicating that the impact of fungicides on *A. fumigatus* population size is variable and influenced by additional factors. 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 alterations in antifungal susceptibility, fungicide 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* in this context.

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. We find a low overall azole resistance rate among agricultural isolates, as well a lack of genomic and population impact following fungicide application, leading us to conclude azole use on crops does not significantly contribute to resistance in *A. fumigatus*.

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-

70 isolated *A. fumigatus* (3, 4). Regrettably, the mortality for resistant infections is upwards
71 of 90% in some patient populations (5-7). While resistance can evolve during patient
72 therapy (8, 9), the emergence of resistance in *A. fumigatus* has mainly been linked to
73 the use of azoles in agriculture, as structurally similar and mechanistically
74 indistinguishable compounds are heavily used for plant protection (10). This resistance
75 has been described as collateral damage as *A. fumigatus* is not a plant pathogen that is
76 being directly targeted by fungicide treatments (11). The triazoles were first released for
77 agriculture in 1973, well before they were first introduced to human medicine in the early
78 1990s, and are currently the most widely-utilized antifungal compound group in
79 agriculture due to their systemic distribution in treated plants, high efficiency and broad
80 spectrum of target pathogens (12, 13). Crops, particularly cereals and fruits, are sprayed
81 multiple times each growing season at a recommended dose of 100 g/hectare to control
82 powdery mildew, rust, septoria leaf blotch, and other phytopathogenic fungi (14).
83 Currently, there are 32 azoles commercially available for plant protection (15), but only
84 five in regular use in human medicine (16).

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

94 in *A. fumigatus* include other variations of the tandem repeat, such as
95 TR_{46}/Y121F/T289A and TR_{53}, as well as other point mutations in the *cyp51a* coding
96 sequence (20, 21).

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

109

110 **RESULTS**

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

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

To identify whether our sampling areas are representative of azole and fungicide usage for Germany as a whole, we calculated the fraction of each administrative district that is theoretically treated with azoles in the context of agriculture using publicly available data. Overall, 51% of Germany is designated as agricultural land (see Material & Methods for data sources). However, not all this land is sprayed with azoles. For example, meadow or pastureland is rarely treated with fungicides. Additionally, approximately 7% of agriculture in Germany utilizes organic farming and does not apply azoles, with a range of 3-15% between the different federal states. Using land use data on arable farmland and permanent crop areas for each district in Germany, we calculated that the mean fraction of potentially azole-treated area in Germany is 32% (for the districts where data is available), with a range of 1-68% (Fig 1B). The districts where we performed our soil sampling were almost all above the average for Germany, with a mean of 52% potentially azole-treated hectares and a range of 30-65%.

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

141 To examine the stability of *A. fumigatus* population sizes in agricultural soil, we
142 investigated the same farms a year later in the spring of 2018 and repeated the soil
143 sampling on eight conventional and seven organic fields. Due to crop rotation, it was not
144 possible to sample the identical fields as the previous year, except for the apple
145 orchards on Farms H and L. During the 2018 sampling period, an overall lower
146 proportion of samples were positive for *A. fumigatus* (51% with a range of 10-96%
147 between different fields) (Fig 1D), but the mean CFU/g for the 1000 samples was similar
148 to the previous year (5.18 CFU/g soil for 2017 compared to 5.74 CFU/g for 2018). As in
149 the previous year, there was a large variability in the mean CFU of *A. fumigatus* present
150 between fields. When comparing the six apple fields that were sampled over
151 consecutive years, we did not observe a consistent trend in the stability *A. fumigatus*
152 population size. Two fields showed similar levels of *A. fumigatus* between 2017 and
153 2018, while two fields showed an increased abundance between the years, and the
154 remaining two field showed a significant reduction in abundance (Supplemental Fig 1A).
155 To investigate potential factors that might support a higher abundance of *A. fumigatus*,
156 we compared the total organic carbon (TOC) content of a random selection of samples
157 with the CFU/g *A. fumigatus,* but did not detect a clear relationship between the two
158 (Supplemental Fig 1B). Altogether, we observed a non-uniform distribution of *A.*
159 *fumigatus* in soil samples taken from the same field and a large degree of heterogeneity
160 between fields.
161
162 <u>Variable effects of fungicide application on *A. fumigatus* abundance</u>
163 To examine the impact of fungicide application and the azoles on *A. fumigatus* in
164 agricultural soil, we performed additional soil sampling on the conventional fields

165 surveyed in the spring at the end of the vegetative period and after several months of
166 fungicidal crop protection. A schematic illustration of the soil sampling and fungicide
167 application timelines for 2017 and 2018 can be found in Supplemental Figures 2A and
168 B. Unfortunately, the fungicide history for Farms H and L was not available to us. Fields
169 were typically treated with fungicides twice during the growing period and azoles were
170 by far the most dominant class of fungicide applied. Every application recorded
171 contained at least one azole. However, fungicides are often applied as commercially
172 available cocktails of different chemicals, so other classes were also present in 0-55% of
173 applications in 2017 and 2018 (summarized in Supplemental Fig 2C).

174 When comparing the amount of *A. fumigatus* on fields before fungicide
175 application to after fungicide application and azole exposure, we detected a significant
176 reduction in the CFU/g soil on the majority of fields in 2017 (Fig 2A), even though it is
177 not being directly targeted as a plant pathogen. To investigate whether this reduction in
178 agricultural *A. fumigatus* populations was the result of fungicide application, and not a
179 seasonal effect from comparing April to July, monthly soil samples were taken from a
180 conventional field and an organic field not treated with azoles or other non-natural
181 fungicides as a control. Samples were taken beginning in April, before azoles were
182 applied to the conventional field, through the harvest period in July, and then additional
183 samples were taken in October and November to allow for a period without fungicide
184 application. From April to July, the conventional field was sprayed with azoles every 3-5
185 weeks. When comparing the abundance of *A. fumigatus* on the organic field, we did not
186 observe any significant differences between the abundance recorded monthly between
187 April and July (Fig 2B). However, the conventional field showed a significant reduction in
188 abundance between April and May, corresponding to the beginning of the azole

189 application period, and this reduction was maintained through the rest of the azole
190 application period (Fig 2C).

191 When comparing *A. fumigatus* density before and after fungicide treatment in
192 2018, we did not observe the same reduction in abundance and most fields did not show
193 significant changes between the time points (Fig 2D). In fact, only one of eight fields
194 sampled showed a statistically significant reduction in *A. fumigatus* abundance.
195 Altogether, the impact of fungicide application on *A. fumigatus* abundance was variable
196 between fields and more so between years, as other environmental factors also appear
197 to influence *A. fumigatus* population size in agricultural soil.

198

199 <u>Reduced susceptibility to agricultural azoles in populations isolated after the growing</u>
200 <u>season and azole exposure</u>

201 To assess the susceptibility of *A. fumigatus* to commonly-applied agricultural
202 azoles, we screened 435 isolates from 2017 and 342 isolates from 2018 for their ability
203 to grow at a set concentration of difenoconazole and tebuconazole (approximately 20
204 isolates per field and sampling point). To limit potentially clonal isolates from skewing the
205 results, a maximum of two isolates per soil sample were included for testing. As there
206 are no established breakpoints for defining resistance to these compounds in *A.*
207 *fumigatus*, we selected concentrations that mimicked MIC90 values for these azoles (1
208 mg/L for difenoconazole and 2 mg/L for tebuconazole). When examining conventional
209 and organic farms in the spring, we observed a wide range in the fraction of isolates per
210 field that were able to grow when challenged with agricultural azoles. For
211 difenoconazole, this ranged from 10-55% per field in 2017 (n = 17 fields, 320 isolates in
212 total) and 0-50% in 2018 (n = 15 fields, 261 isolates in total) (Fig 3A and Supplemental

213 Table 2). For tebuconazole, the rates ranged from 0-25% in 2017 and 0-20% in 2018
214 (Fig 3B and Supplemental Table 2). We did not detect any significant differences in the
215 rates between conventional and organic fields in the spring or between fields growing
216 different crops (cereals or apples).

217 Given the reduction in the *A. fumigatus* population size observed on most
218 conventional following fungicide application in 2017, and more variably on fields in 2018,
219 we wanted to examine the effect of fungicides on the local azole susceptibility following
220 the vegetative period and several months of fungicide application. We observed an
221 increase in the proportion of isolates that were able to grow at the test concentrations of
222 difenoconazole (1 mg/L) and tebuconazole (2mg/L) for fields sampled after azole
223 exposure compared to the same field in the spring prior to azole application (Fig 3C, D,
224 and Supplemental Table 3). We detected a 1.97-fold increase in the proportion of
225 isolates that were resistant to our test concentration of difenoconazole in 2017 with a
226 range from a -1.1 to 4.5-fold for individual fields (Fig 3C). In 2018, this increased to a
227 2.84-fold with a range of 1-4.5-fold change increase for individual fields (Fig 3D). We
228 also saw a similar increase in the fraction of isolates with reduced susceptibility to our
229 test concentration of tebuconazole after azole exposure compared to the before. In
230 2017, we observed a 5.9-fold in number of isolates that were able to grow at the test
231 concentration after azole exposure vs. before exposure with a range of 0-25.0% for
232 individual fields (Fig 3E). In 2018, we detected a more modest 1.9-fold with range of -5
233 to 18.2% for individual fields (Fig 3F).

234 For the fields growing cereals, we were not able to sample the same fields over
235 subsequent years due to crop rotation and the fields not being in use the following year.
236 However, we were able to compare the same apple fields in both 2017 and 2018. We

237 tracked the local susceptibility to agricultural azoles in these fields over two consecutive
238 years at two time points: in the spring prior to fungicide application and just prior to
239 harvest after ≈ 3 months of fungicide application. The fraction of isolates that were able
240 to grow in the presence of 1 mg/L difenoconazole or 2 mg/L tebuconazole was, in
241 general, low in the spring, and increased after fungicide application (Fig 3G,
242 Supplemental Table 3). Interestingly, the spring of 2018, the proportion had returned to a
243 level comparable to what we observed in the spring of 2017—indicating that the reduced
244 susceptibility is transient and recedes when the selective pressure imposed by fungicide
245 is removed. In summary, we found a wide range of susceptibilities to agricultural azoles
246 between different fields, but a consistent decrease in susceptibility following the growing
247 season, fungicide application, and azole exposure. However, this change is seemingly
248 transient or reversible, and the *A. fumigatus* populations from fungicide-treated fields
249 typically returned to what they were prior to fungicide application by the following spring.
250

251 Resistance to medical azoles in agricultural *A. fumigatus* isolates

252 We next examined our isolate collection for resistance to medical azoles and
253 determined the proportion that would be considered clinically resistant. Using the
254 VIPcheck™ agar-based screening method followed by broth microdilution for isolates
255 showing growth on agar-containing wells (22, 23), we determined that only a very small
256 fraction of *A. fumigatus* isolated from agriculture showed resistance to itraconazole,
257 voriconazole, or posaconazole in 2016-2018 (Table 1A). The overall resistance rate to
258 itraconazole among all isolates collected was highest, with 3.0% (11/333) of isolates
259 being resistant in 2016, 0.7% in 2017 (4/460), and 0.6% (2/322) in 2018. We observed
260 lower resistance rates for posaconazole and voriconazole, with only 2.1% (7/333) being

261 resistant in 2016, 0.7% (4/460) in 2017, and 0.0% (0/322) in 2018. As there are no
262 clinical breakpoints established for agricultural azoles, we calculated epidemiological
263 cutoff values (ECOFFs) for these difenoconazole and tebuconazole using MICs from
264 160 randomly selected isolates from 2017 and 2018. Using these values, we found that
265 1.3% of the 160 isolates had MICs above the ECOFF for difenoconazole (2 mg/L) and
266 4.4% of isolates had MICs above the ECOFF for tebuconazole (2 mg/L) (Table 1B). As
267 has been described previously (24), isolates resistant to one or more medical azoles,
268 often displayed elevated MICs to agricultural azoles, indicating cross resistance
269 (Supplemental Table 4).

270 To quantify what mutations were responsible for azole resistance in the
271 population analyzed, we genotyped the *cyp51a* locus of all isolates resistant to one more
272 medical azole using Sanger sequencing (n = 18). This gene is the target of the azoles
273 and the major source of resistance among both environmental and clinical *A. fumigatus*.
274 We found that the most dominant DNA alteration observed was the well-characterized
275 TR_{34}/L98H mutation (Fig 3H, Supplemental Table 4). This *cyp51a* genotype accounted
276 for 6/12 (50%) of resistant isolates in 2016 and 3/4 (75%) in 2017. In 2018, we identified
277 only two resistant isolates among the 322 screened and both had wild type *cyp51a* loci.
278 However, both of these isolates were only weakly resistant to itraconazole, but not other
279 azoles, with itraconazole MIC values right at the breakpoint of 2-4 mg/L. For
280 comparison, resistant isolates from other years had MIC values of >8 mg/L. In total, the
281 genetic cause of resistance remained unknown for 8 isolates from 2016-18.

282 Since the majority of fields had no resistant isolates, it was not possible to
283 effectively compare resistance rates among agricultural *A. fumigatus* for medical azoles
284 before and after fungicide treatment. In lieu of this, we examined the MIC distribution for

285 isolates collected before and after the growing season and fungicide application.
286 Examining 79 isolates from the before period and 79 isolates from after azole exposure,
287 we observed a shift in the MIC distribution towards higher MICs for all azoles examined,
288 both medical and agricultural (Fig 3G). However, the median MICs remained
289 unchanged, indicating that the majority of the population following azole exposure does
290 not exhibit a change in MIC. Taken together, these results indicate that the rate of
291 resistance to clinical azoles among environmental *A. fumigatus* isolated from agricultural
292 environments is overall low and that exposure to agricultural azoles causes a minor
293 increase in MIC values for some of the population, but the majority of isolates are left
294 unchanged.
295
296 No population structure observed for *A. fumigatus* in agricultural fields
297 To better understand the population structure of *A. fumigatus* in the environment
298 and how it is impacted by the azoles, we performed whole genome sequencing on
299 isolates from four conventional farms collected before and after azole exposure. 64
300 isolates were sequenced by Illumina paired-end sequencing, representing eight isolates
301 per farm and time point. Isolates from each field and time point were randomly selected,
302 with a maximum of two per soil sample to avoid sequencing of clonal isolates. Raw
303 reads were checked for quality and then aligned to the Af293 reference genome,
304 resulting in a median depth of coverage after mapping of 31.5X (range 10-90X) and a
305 median genome coverage median of 94% (range 92.8-96.7%). Consistent with what has
306 been observed among sequenced clinical strains (25), isolates differed from the Af293
307 reference by a median of 84,690 single nucleotide variants (SNVs) or 2.88 SNVs/kb,
308 with a range of 65,854-146,055 SNVs. The analysis of copy-number variations (CNVs)

309 identified 8,277 unique CNVs in total, with a median of 3,115 CNVs per isolate (range
310 1,666-4,532 CNVs). These CNVs were further delimited into a median of 2,589 deletions
311 (range 1,247-4,405) and 5,687 insertions (range 3,872-7,030).

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

324 Comparative analysis of molecular variance (AMOVA) indicated that the majority
325 of the variation seen among the 64 isolates came from the population as a whole (within
326 sample) (94.8%) and between samples (5.0%) (Table 2). There was no significant
327 molecular variance between farms (0.2%), with the exception of modest variation
328 between Farm B and Farm C (1.2% of variation observed). Weighted Weir and
329 Cockerham's fixation indexes (F_{ST}) for each farm were essentially zero, indicating an
330 interbreeding, panmimetic population with no separation between farms (Supplemental
331 Fig 3A). V_{ST} values estimating population differentiation based on copy number variation
332 also indicated no subdivision among the farms (Supplemental Fig 3B). To examine the

genetic diversity within farms, nucleotide diversity (the average number of nucleotide differences per site for all possible pairs in the population or *π)*, and the number of polymorphic sites (Watterson estimator or θ) were calculated along 5 kb windows with a 500 bp step size for each farm population. Farm C showed the greatest intra-farm diversity, while Farm E showed the smallest (Supplemental Fig 3C-D). Taken together, there was no population differentiation between *A. fumigatus* isolates from different farms in Germany, a finding in line with the fungus' capacity for aerosol dispersal.

Changes in population genetics following azole exposure vary by field

Given the observed reduction in overall *A. fumigatus* abundance after azole treatment, we examined the populations for changes in genetic diversity and evidence of selective sweeps in *A. fumigatus* field populations. Using 5 kb sliding windows with a 500 bp step size, nucleotide diversity (*π*) was calculated for the individual farms before and after azole treatment. No clear trend was seen regarding changes in nucleotide diversity between the time points. The isolates from Farms B and E showed increased diversity following azole application, while nucleotide diversity decreased on Farms C and D (Fig 5A). We also calculated the number of segregating sites (*θ*) for the same 5 kb windows and observed the same lack of consensus. Farms B and C showed similar values of *θ* before as compared to after azole application, while Farm D showed a dramatic decrease in *θ* following azole application (Fig 5B). Molecular variance analysis (AMOVA) on isolates from before and after the vegetative period and azole exposure did not indicate any significant molecular variation between the time periods, with the majority of the variation being between and within samples (Table 3). Finally, we measured Tajima's D to test neutrality along 5 kb windows, where negative values indicate less

357 variation than expected and are indicative of a selective sweep. Positive values denote a
358 population that is more heterogenous than would be expected and suggest either a
359 sudden population contraction or balancing selection. Overall, the bulk of the Tamija's D
360 values were close to neutral and there was no clear trend between farms, indicating that
361 there was no genomic signature of a population bottleneck or selective sweep following
362 azole exposure (Fig 5C). The median Tajima's D was roughly zero for Farm B and
363 increased slightly to 0.37 following azole exposure prior to azole application, and the
364 same direction shift was seen for Farm D, but the starting Tajima's D was negative at
365 the time point before azole exposure (-0.53 to 0.53) (Fig 5C). Conversely, the median
366 Tajima's D for farms C and E shifted from positive to negative after the growing season
367 and azole exposure (0.75 to -0.57 for field C and 0.61 to -0.10 for Field E). Taken
368 together, these results indicate that despite the reduction in abundance of *A. fumigatus*
369 on agricultural fields following azole application, we were unable to detect any marked
370 changes at the population level.
371

372 **DISCUSSION**

373 The use of azole fungicides for plant protection has been previously suggested as
374 a driver of clinical resistance in the environmental saprobe and human pathogen
375 *A. fumigatus*, as well as the emergence of new fungal pathogens such as *Candida auris*
376 (11, 26, 27). However, direct evidence linking azole use in agriculture and clinical
377 resistance is missing. Additionally, delineation of specific roles for the use of azoles in
378 crops vs. ornamental plants such as flower bulbs has not been defined and is important
379 to make, as limiting azole fungicide use would significantly impact disease control and
380 yield in many crops. In this study, we provide the first systematic investigation of the

381 impact of fungicide use on the ecology and azole resistance status of a human
382 pathogen, *A. fumigatus*. Through analysis of 2,875 soil samples over a three-year period
383 in central Germany, we found an overall low incidence of *A. fumigatus* isolates that
384 would be considered clinically resistant (1-3%). However, we observed a modest, but
385 consistent decrease in azole susceptibility following the growing season and azole
386 exposure, as well as a more variable reduction in fungal abundance following fungicide
387 application. Interestingly, this change in susceptibility was transient and reset by the
388 following spring. We also assessed the influence of fungicide application on *A. fumigatus*
389 population dynamics by WGS and were unable to find a clear impact on the population
390 structure or genetic diversity.

391 Despite sampling on fields that were actively treated with azole fungicides, and in
392 regions of Germany with above average fungicide exposure, only 1-3% of *A. fumigatus*
393 isolates collected were resistant to medical azoles. This incidence is in agreement with
394 clinically-reported frequencies in Germany and other European countries, where the rate
395 of triazole resistance ranged from 0.6% to 4.2% (3.2% in Germany) (28, 29). The
396 environmental resistance rates reported for Europe, however, have been much broader.
397 Some studies have found environmental resistance frequencies approaching 20%, while
398 others have reported virtually no incidence of resistant environmental isolates (30-35).
399 Part of these differences could be attributed to differences in methodology, such as the
400 usage of azole selection during the isolation procedure or inclusion of potentially clonal
401 isolates from a given sample to skew the data. In the current study, we avoided azole
402 selection during the isolation procedure, as well as only allowed two isolates per soil
403 sample, to avoid potentially clonal isolates that influence the results. Another potentially
404 contributing factor could be that while all technically “environmental” isolates, rural or

405 agricultural settings could be a completely different niche than an urban flower garden—
406 a supposition supported by a recent study where rural areas in the UK had much lower
407 resistance rates (1.1%) than urban locations (13.8%) (34).

408 Another important and novel finding from this study is that isolates from after the
409 vegetative period and azole exposure consistently showed decreased azole
410 susceptibilities to difenoconazole and tebuconazole, as well as the subtle MIC shift to
411 both agricultural and medical azoles. However, one shortcoming to our study is that we
412 did not collect isolates from organic fields at a matching time point for susceptibility
413 testing, so we cannot exclude that the changes observed in azole susceptibility are not
414 also influenced by seasonal changes. The observation that changes in susceptibility are
415 transient and reset in the period between the end of the growth period and the following
416 spring on the two field is intriguing and worthy of further study. This transformation could
417 either be the result of a naïve population coming in via aerosol dispersal, or a
418 consequence of isolates acquiring unstable, epigenetic-mediated resistance—a
419 phenomena previously observed in the environmental saprobe and human pathogen
420 *Mucor circinelloides* as well as the plant-pathogenic fungi *Monilinia fructicola* following
421 azole exposure (36, 37). Either scenario would be in agreement with our finding that
422 fungicide application does not alter the population structure of genetic diversity of *A.*
423 *fumigatus* in agricultural fields.

424 We observed a wide range in the CFU/g soil within and between farms during
425 annual spring sampling. Our mean *A. fumigatus* CFU/g soil was in line with what was
426 reported recently for abundance in wheat grain, maize silage, and fruit waste (38).
427 However, this abundance is several magnitudes lower than that reported for *A.*

428 *fumigatus* in flower bulb waste and green material waste in this same study, where it
429 was not uncommon to isolate 10^4 CFU/g. We demonstrated a reduction in the *A.*
430 *fumigatus* population size on most fields sampled in 2017 following the vegetative period
431 and fungicide application. However, this finding was not strongly observed in 2018,
432 indicating that other environmental factors also influence the abundance of *A. fumigatus*
433 in agricultural soil. One example of such a potential factor is that Germany experienced
434 extreme heat and drought during the 2018 growing season and, in fact, one field had to
435 be removed from analysis this year because it caught fire during the vegetative period.

436 Our study also provides the first WGS-based study focused on *A. fumigatus* in its
437 natural niche, the environment. Previous studies have primarily concentrated on clinical
438 isolates, with particular priority given to resistant strains (25, 39). Even while sampling
439 within the same field, we found a large degree of genetic diversity, where the majority of
440 diversity came from within samples. We also did not observe a defined population
441 structure or separation between farms or regions. The degree to which this
442 environmental diversity is recapitulated in clinical isolates, and whether there are
443 enrichments for particular subgroups in the transition from environment to clinic is an
444 interesting question for further study.

445 Given our low observed resistance rate among agricultural isolates, as well the
446 lack of genomic impact of azole application on the fields surveyed, our study does not
447 find evidence that azole fungicide use in crop agriculture significantly contributes to
448 resistance in *A. fumigatus,* nor should be necessarily removed from use in this context
449 due to their crucial role in global food production. Instead, we propose a refined model,
450 where azole resistance in *A. fumigatus* is being driven by the use of these compounds in

451 the cultivation of flowers and other ornamentals. It is common practice to dip flower
452 bulbs directly in fungicide stocks prior to storage and shipment, and their use in this
453 setting should be more carefully considered and managed (40, 41). This revised model
454 is supported by the recent identification of flower bulb and green waste as environmental
455 "hotspots" for resistance selection, which possess both high overall fungal colony counts
456 as well as increased incidence of resistant *A. fumigatus* isolates compared to soil (38).
457 Unfortunately, due to massive aerial dispersal of *A. fumigatus* conidia, the use of azoles
458 in any hotspot can lead to the worldwide distribution of resistant strains.

459

460 **MATERIALS AND METHODS**

461 Site selection and soil sampling

462 During 2016 to 2018, soil sampling was conducted on agricultural sites in the federal
463 states of Thuringia, Saxony-Anhalt, and Saxony with the approval of the land owner
464 and/or relevant ministries. The majority of fields sampled were growing cereals such as
465 wheat or barley, but some apple orchards were sampled as well (see Supplemental
466 Table 1 for full details). Farms were arbitrarily assigned an alphabetic identifier (A-L) and
467 specific fields a numeric identifier as described in the supplementary Materials &
468 Methods. Due to crop rotation, the same field could not be surveyed over subsequent
469 years, with the exception of the apple orchards. Soil samples were collected at
470 beginning of the vegetation period on the conventional and organic cultivated sites and
471 after azole application an additional sampling was carried out on the conventional sites.
472 In general, 50 soil samples per site and type of farming (conventional or organic) were
473 collected, with a total of approximately 1000 soil samples per year. Soil samples were

474 selected to best cover the field with a minimum distance of 1 m between samples. For
475 each sample, the top layer of soil was collected by a metal spatula into a sterile sample
476 cup and refrigerated until processing.
477
478 Soil processing and isolation of *A. fumigatus*
479 3 g of soil from each sample cup was weighed out and resuspended in 8 mL 0.2M NaCl
480 containing 1% Tween20. Samples were vortexed vigorously and then left to settle until a
481 phase separation became apparent. 2 mL of the upper phase was transferred to a new
482 tube for plating onto Sabouraud Glucose Agar (SGA) containing 50 µg/mL
483 Chloramphenicol (Sigma-Aldrich, Taufkirchen, Germany). Of this 2 mL, 150 µL was
484 plated onto one plate and the remaining volume was plated onto a second plate in order
485 to adjust for variable fungal concentrations in samples. Plates were then incubated at
486 50°C for 5 days to select for *A. fumigatus,* which is unique among *Aspergillus* spp. in its
487 ability to grow at this temperature. On day 5, the incubator temperature was reduced to
488 42°C to allow for sporulation and plates grown for another 2 days. The number of *A.*
489 *fumigatus* colonies was counted and up to two colonies per soil sample transferred to
490 new plates for isolation.
491
492 Antifungal susceptibility testing
493 Quick screening of susceptibility to itraconazole, voriconazole, and posaconazole were
494 assessed using the agar-based VIPcheck™ Assay (Mediaproducts BV, Groningen,
495 Netherlands) following the manufacturer's directions. For testing, isolates were grown on
496 SGA for 2-4 days at 37°C. Plates were then swabbed with a damp, sterile cotton swab
497 to prepare a conidial suspension of 0.5-2 McFarland. 25 µL of this suspension was then

498 plated onto the 4 wells of the VIPcheck plate containing 4 mg/L itraconazole, 2 mg/L
499 voriconazole, 0.5 mg/L posaconazole, or a control well containing no drug. To assess
500 susceptibility to agricultural azoles during isolates collected during 2017 and 2018,
501 RPMI+2% glucose agar plates containing either 1 mg/L difenoconazole or 2 mg/L
502 tebuconazole were prepared as in (42). Resistance was defined as significant inhibition
503 of germination and hyphal growth compared to the no drug control. Isolates that showed
504 resistance to any of the medical azoles in the VIPcheck assay were subject to broth
505 microdilution following EUCAST methodology (protocol E.DEF 9.3). ECOFFs were
506 calculated using the ECOFFinder program available from EUCAST.

507

508 DNA extraction

509 For WGS and PCR-based amplification, isolates were grown shaking in SG broth at
510 37°C and genomic DNA isolated using the Quick-DNA Fungal/Bacterial miniprep kit
511 (Zymo Research, Irvine, CA) according to the manufacturer's suggested protocol.

512

513 *cyp51a* genotyping

514 The *cyp51a* coding sequence and upstream region containing the tandem repeat was
515 amplified using the primers described in Supplemental Table 5. Cleaned up PCR
516 products were sequenced and the *cyp51a* genotype determined using FunResDB
517 (https://elbe.hki-jena.de/FunResDb/index.php).

518

519 Genome sequencing, quality assessment, and alignment

520 Library preparation and 2x150bp paired-end sequencing was performed on a NextSeq
521 500 v2 by LGC Genomics (Berlin, Germany) following the manufacturer's recommended

522 protocols. Sequence data quality control and filtering were performed using an in-house
523 script and FastQC (v0.11.5). Quality reads were mapped to the *A. fumigatus* Af293
524 reference genome (version 2015-09-27; retrieved from FungiDB (43)) using BWA-MEM
525 (version 0.7.8-r779-dirty) (44). PCR duplicates were marked using MarkDuplicate from
526 Picard version 2.18.25 embedded in the Genome Analysis Toolkit (GATK; version
527 4.1.0.0). All WGS samples included for analysis possessed greater than 10 fold genome
528 coverage after mapping and more than 90% of reads mapped to the reference genome.
529

530 Variant identification and SNV-based phylogeny

531 Short variants, including single nucleotide variants (SNVs) and short insertions and
532 deletions (InDels) were detected using GATK Haplotype Caller following their
533 recommended best practices for single calling (45). Copy number variants (CNVs) were
534 identified using Control-FREEC (46). For the phylogenetic analysis, nucleotide
535 consensus sequences were extracted from vcf files using VCFtools (47) and an in-house
536 script used to translate nucleotide to protein-coding sequences. Multiple sequence
537 alignment was performed using MUSCLE v3.8.31 (48) using 7,771 conserved core
538 genes and an approximately-maximum-likelihood phylogeny constructed using
539 FastTree2 (version 2.1.10) (49). Interactive Tree of Life (iTOL) v4 was used for
540 visualization (50).
541

542 Genetic diversity analyses

543 Analysis of molecular variance (AMOVA) was determined using the R package ade4
544 (nrep = 999). Nucleotide diversity (π) was calculated by VCFtools (version 0.1.6) using
545 5kbp windows with a step size of 500bp. Nucleotide polymorphism (θ) and Tajima's D

546 were calculated using ANGSD (51) using a window size of 5 kbp and a step size of 500
547 bp. Weighted Weir and Cockerham's F_{ST} values were calculated using VCFtools, while
548 V_{ST} values were calculated as in (52). A Mantel test correlating geographic distance
549 matrices with pairwise FST matrices was performed using the R package ade4 (nrep =
550 9999).

551

552 Estimation of fungicide treatment areas and rates in Germany

553 The fraction of each district theoretically treated with fungicides was calculated using
554 publicly reported data from 2016 available from the German Statistical Offices
555 (statistikportal.de) using the following equation:

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

556

557 The total area per district was obtained from Table 33111-01-02-4: Ground area by
558 actual use. The sum of arable farmland and permeant crop areas was calculated for
559 each administrative district using Table 41141-01-01-4: Farms and their agricultural
560 used area by crop type. To accommodate that some percentage of this area is cultivated
561 under organic agriculture methods, the hectares of crop land was then multiplied by the
562 fraction of non-organic agriculture for the federal state in which the district is located to
563 estimate the number of hectares potentially treated with fungicides (as calculated using
564 data on "Farms, agricultural areas, and workers"; accessed on November 1, 2019 on
565 https://www.statistikportal.de/node/254). Unfortunately, no data was available on the
566 breakdown of agricultural methods at the district level to allow for more exact estimation.
567 Finally, this value of estimated treated area per district was divided by the total area of
568 the district for visualization as a choropleth map.

569

570 Box and whisker plots

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

576

577 Data and isolate availability

578 Isolates generated within this study were submitted to and are publicly available in the
579 Jena Microbial Resource Collection. Raw FASTQ files were uploaded to the NCBI
580 Sequence Read Archive and are publicly available under BioProject PRJNA595552.

581

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590

591 **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.

AUTHOR ORDER

For cases of equal authorship contribution, order was determined by alphabetical order.

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Table 1. Azole resistance rates among agriculturally isolated *A. fumigatus* isolates. (A) Isolates were screened for potential azole resistance to itraconazole (ITR), posaconazole (POS), and voriconazole (VOR) using the VIPcheck agar-based screening. Resistance was confirmed and MICs determined via EUCAST broth microdilution testing. (B) MIC_{50} for medical and agricultural azoles calculated from 160 randomly selected isolates, as well as $ECOFF_{95}$ and the fraction of isolates with MICs above this value.

(A)

		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

(B)

Azole	MIC_{50}[+]	ECOFF*	Fraction of isolates above ECOFF (%)
Itraconazole	0.5	2	1.3
Voriconazole	0.5	2	0.6
Difenoconazole	2	8	1.3
Tebuconazole	2	8	4.4

[+]Calculated from 160 randomly selected isolates
*Rounded up to next dilution

791 **Table 2.** Analysis of molecular variance (AMOVA) between farms. Calculated using
792 isolates from before and after azole application. n = 16 isolates per farm.
793

	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

794

795 **Table 3.** Analysis of molecular variance between isolates collected before and after
796 azole exposure. n = 16 isolates per farm field (eight pre-azole and eight post-azole).

797

	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

798

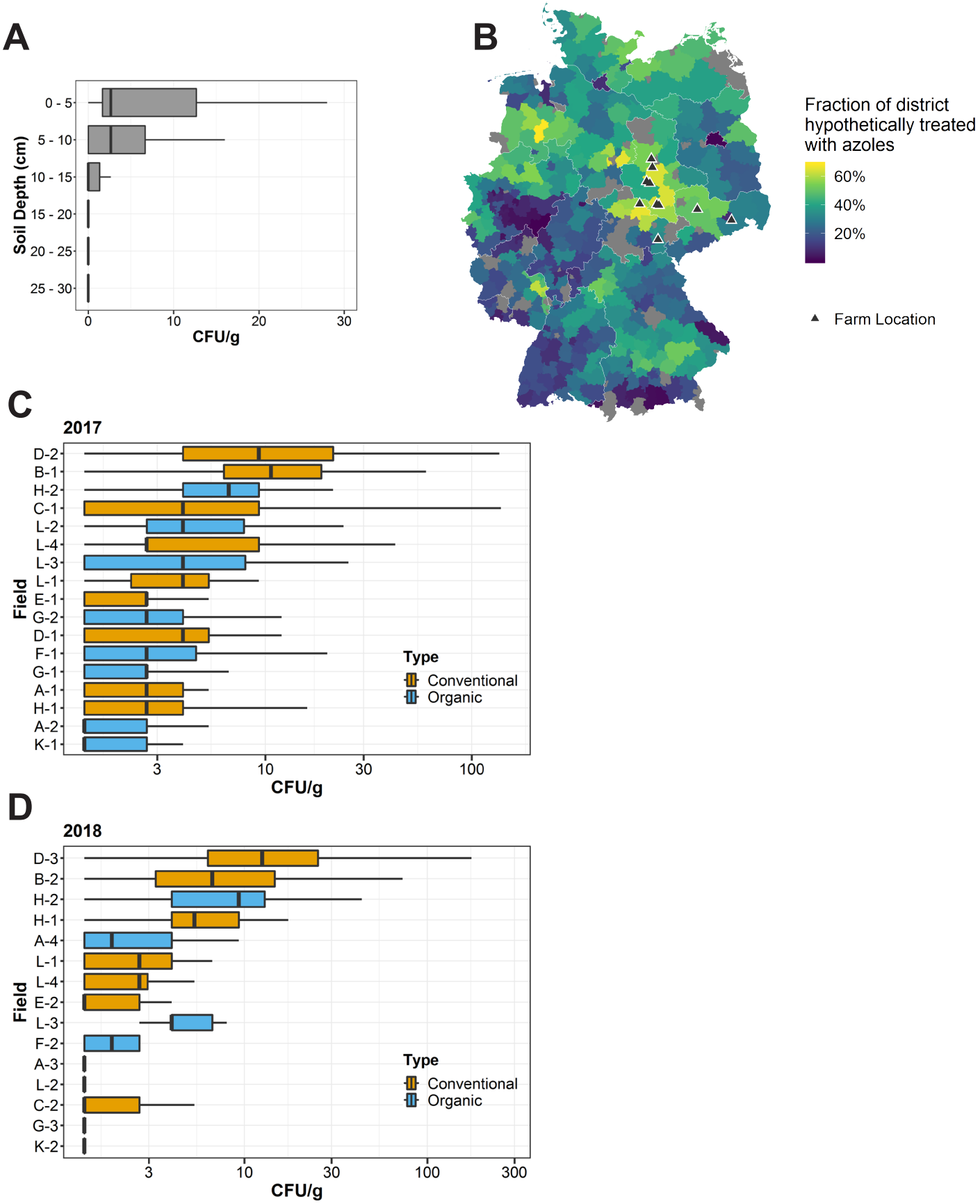


Figure 1. Abundance of *A. fumigatus* in the soil of conventional and organic farms.
(A) CFU/g *A. fumigatus* at varying soil depths. n = 10 samples per depth. (B) Estimated fungicide treatment rates and areas in Germany. The fraction of each district that is theoretically treated with fungicides was 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.

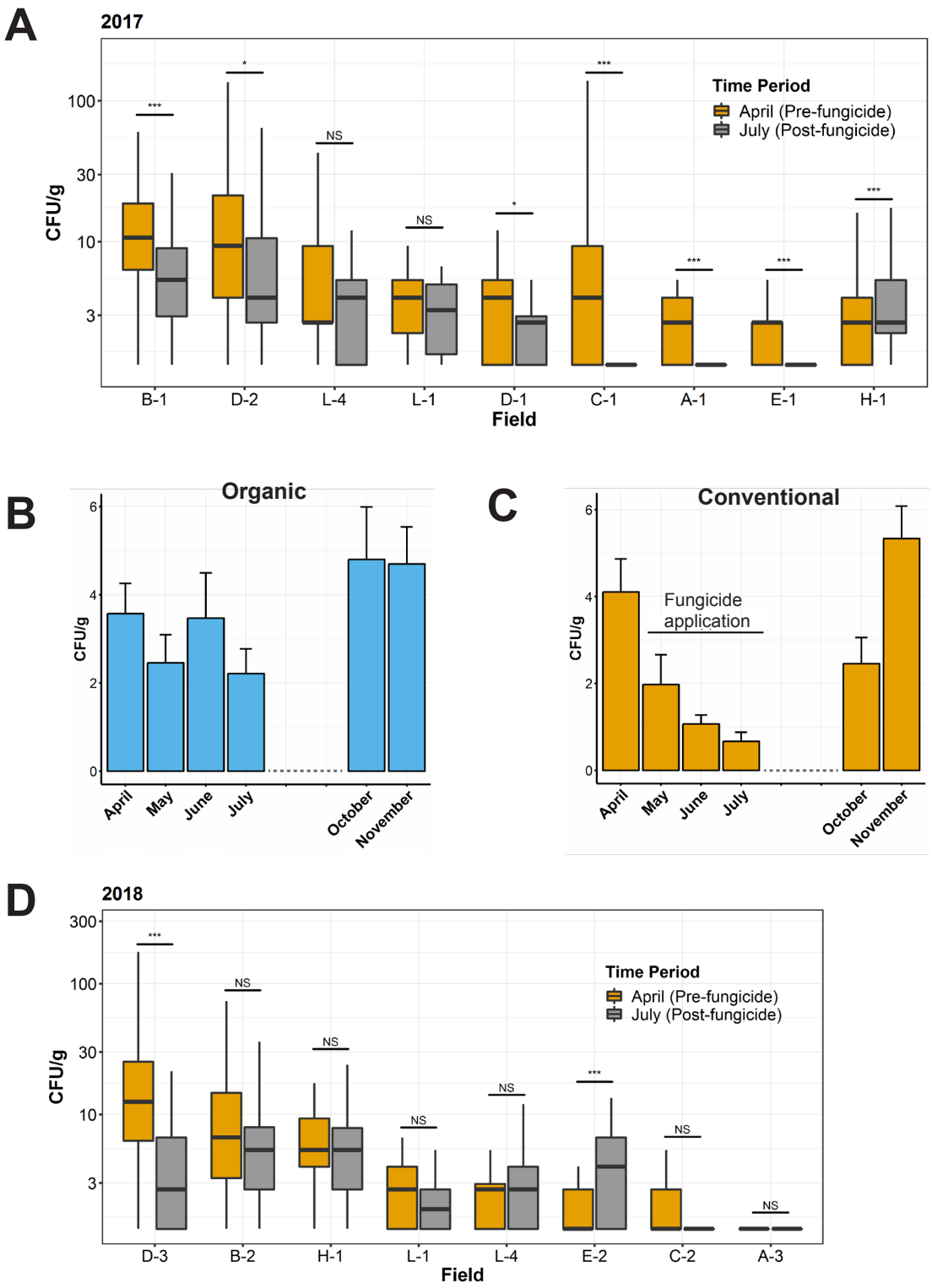


Figure 2. Abundance of *A. fumigatus* in the soil of conventional farms before and after the vegetative period and fungicide application.
(A, D) CFU/g *A. fumigatus* on conventional fields sampled in April, prior to the vegetative period and fungicide application (orange), and in July, after the vegetative period and three months of fungicide application, including azole fungicides (gray) in 2017 (A) and 2018 (D). *, $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ 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. (D) n = 50 samples per field and time point for farms A, B, C, D, E, H and n = 25 samples for farm L. (B-C) CFU/g *A. fumigatus* during the months of April, May, June, July, October and November of a conventional field applying fungicides from May-July (C) and an organic field not applying non-natural fungicides (B). 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 fungicides.

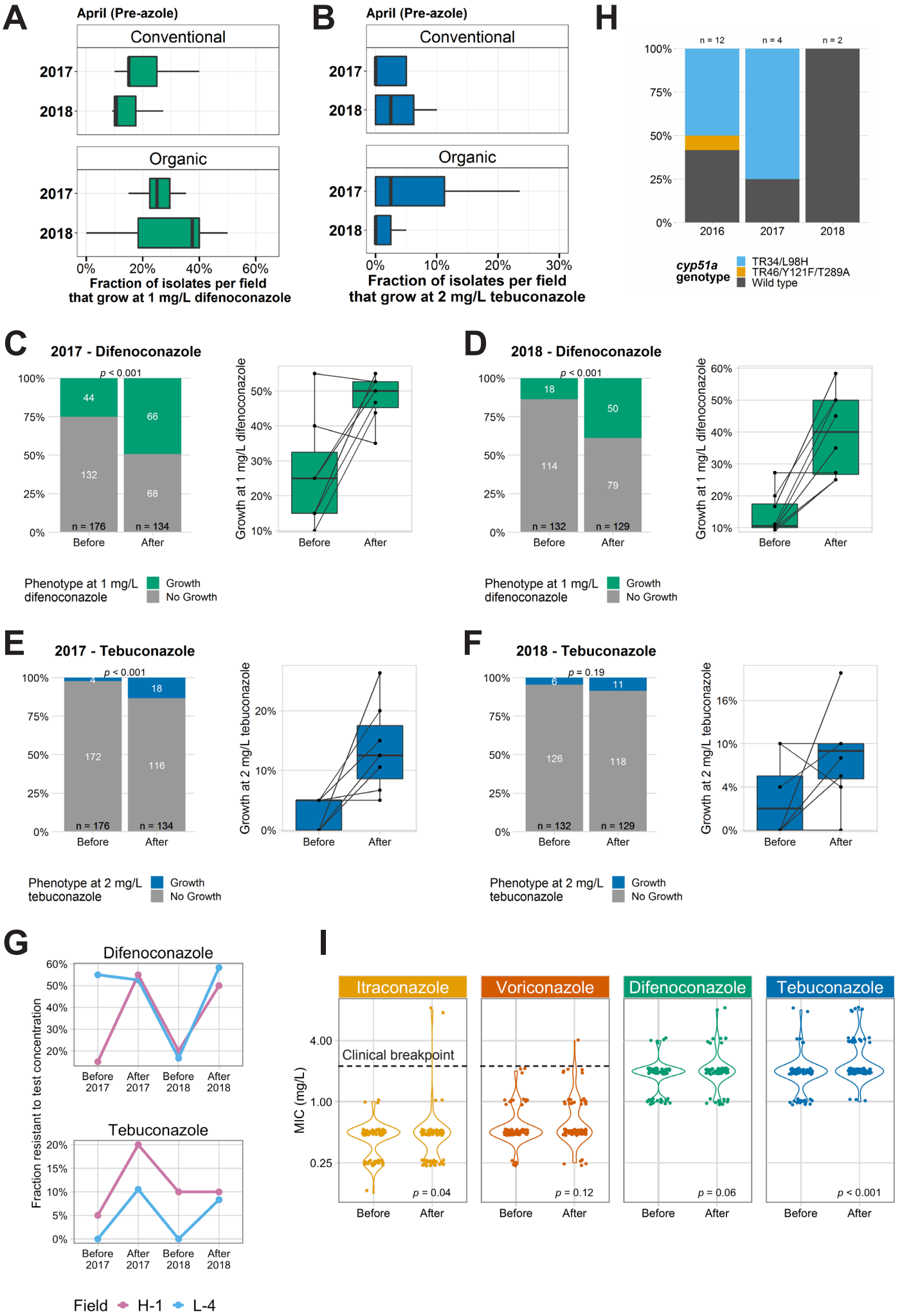

A
April (Pre-azole)
Conventional
2017
2018
Organic
2017
2018
0%
20%
40%
60%
Fraction of isolates per field
that grow at 1 mg/L difenoconazole
B
April (Pre-azole)
Conventional
2017
2018
Organic
2017
2018
0%
10%
20%
30%
Fraction of isolates per field
that grow at 2 mg/L tebuconazole
H
n = 12
n = 4
n = 2
100%
75%
50%
25%
0%
2016
2017
2018
cyp51a genotype
TR34/L98H
TR46/Y121F/T289A
Wild type
C
2017 - Difenoconazole
p < 0.001
44
66
132
68
n = 176
n = 134
Before
After
Growth at 1 mg/L difenoconazole
Phenotype at 1 mg/L difenoconazole
Growth
No Growth
D
2018 - Difenoconazole
p < 0.001
18
50
114
79
n = 132
n = 129
Before
After
Growth at 1 mg/L difenoconazole
Phenotype at 1 mg/L difenoconazole
Growth
No Growth
E
2017 - Tebuconazole
p < 0.001
4
18
172
116
n = 176
n = 134
Before
After
Growth at 2 mg/L tebuconazole
Phenotype at 2 mg/L tebuconazole
Growth
No Growth
F
2018 - Tebuconazole
p = 0.19
6
11
126
118
n = 132
n = 129
Before
After
Growth at 2 mg/L tebuconazole
Phenotype at 2 mg/L tebuconazole
Growth
No Growth
G
Difenoconazole
Tebuconazole
Fraction resistant to test concentration
Before 2017
After 2017
Before 2018
After 2018
Field
H-1
L-4
I
Itraconazole
Voriconazole
Difenoconazole
Tebuconazole
Clinical breakpoint
MIC (mg/L)
4.00
1.00
0.25
p = 0.04
p = 0.12
p = 0.06
p < 0.001
Before
After

Figure 3. Azole resistance among agricultural *A. fumigatus.*

(A-B) Fraction of the isolates 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, ≈ 20 isolates per field. For 2018, n = 213 isolates from 8 conventional and 7 organic fields, ≈ 20 isolates per field (full summary in Supplemental Tables 2 & 3). (C-F) Comparison of the proportion of isolates that grow at 1 mg/L difenoconazole (C-D) and 2 mg/L tebuconazole (E-F) before and after the vegetative period and fungicide application. For 2017 (C, E), n = 275 isolates from 7 fields and for 2018, for 2018 n = 261 isolates from 8 fields (full summary in Supplemental Tables 4 & 5). Left panels show overall summary of all isolates tested that year. Right panels show within field changes (n ≈ 40 isolates per field; 20 before, 20 after). *P* values calculated by Wilcoxon signed-rank test between before and after values. (G) Temporal changes in antifungal susceptibility of A. fumigatus on apple fields sampled before and after fungicide exposure over a two year period. 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. (H) cyp51a genotypes of isolates resistant to one or more medical azole. (I) MICs of agricultural *A. fumigatus* isolated before and after azole exposure. n = 159 randomly selected isolates from 2017 and 2018; n = 80 before and n = 79 after. P values calculated by Wilcoxon signed-rank test between before and after values.

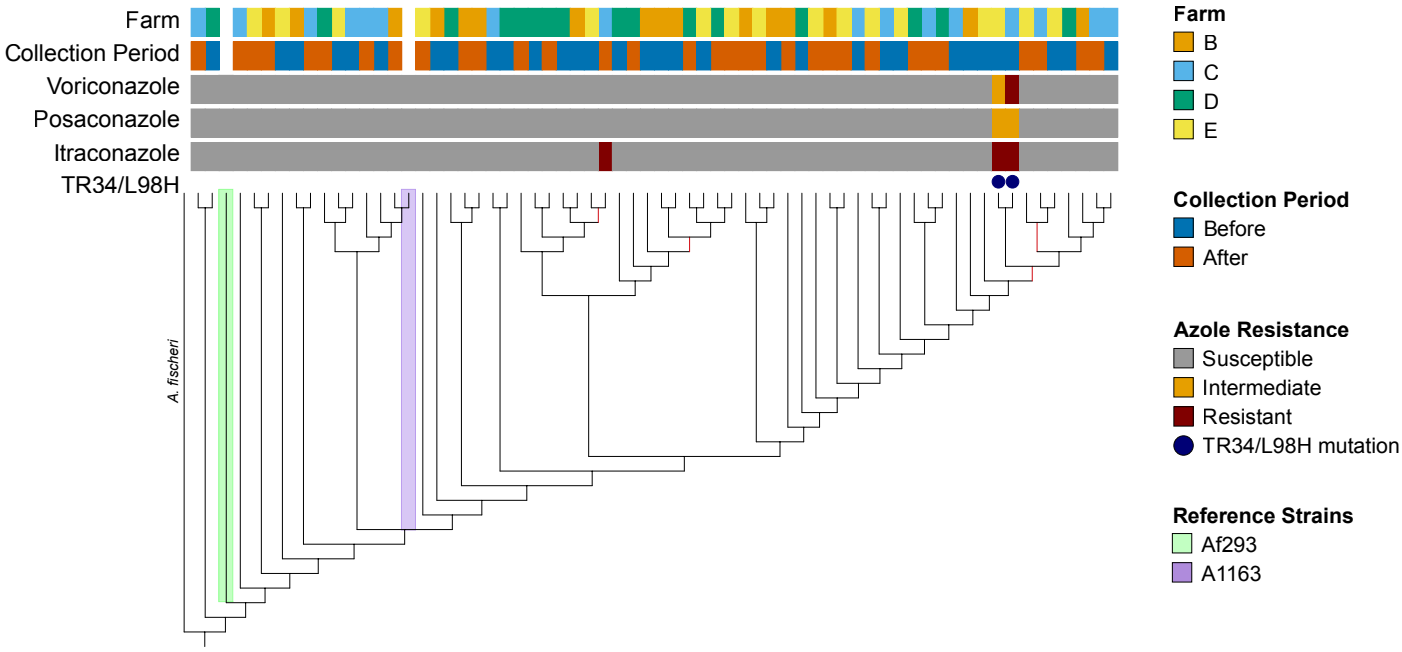


Figure 4. Phylogeny of agricultural *A. fumigatus* isolates from before and after the vegetative period and fungicide application.

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.

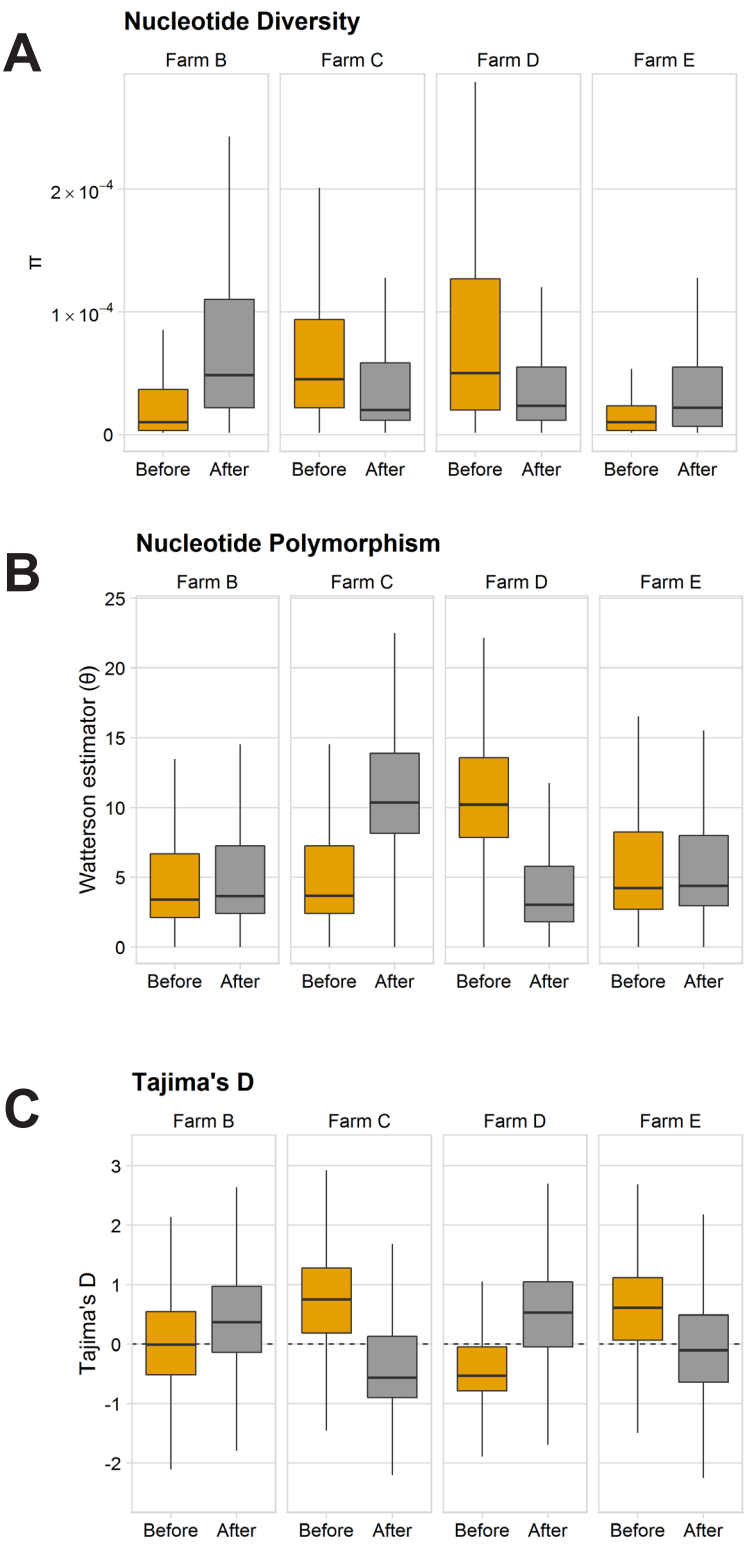
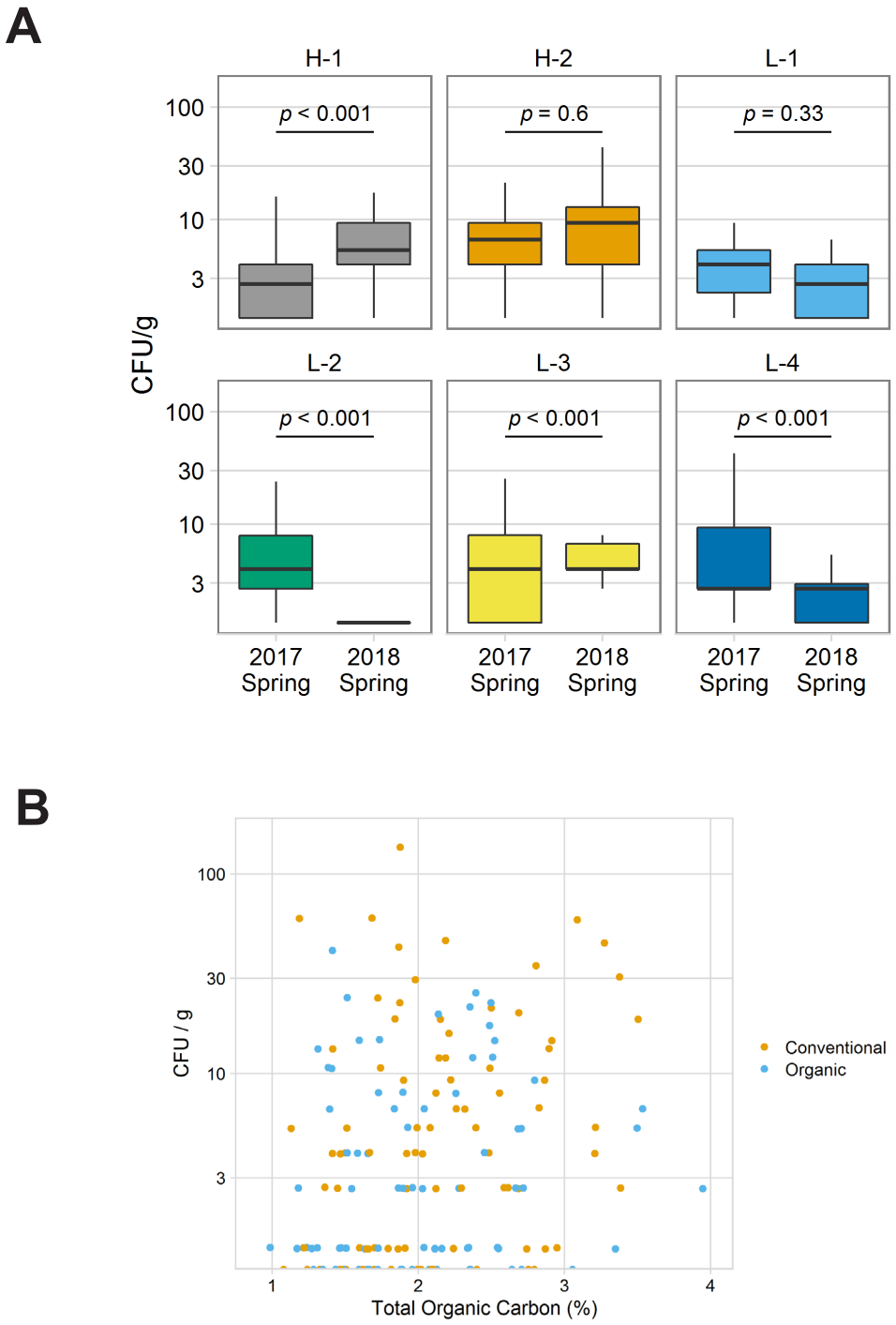


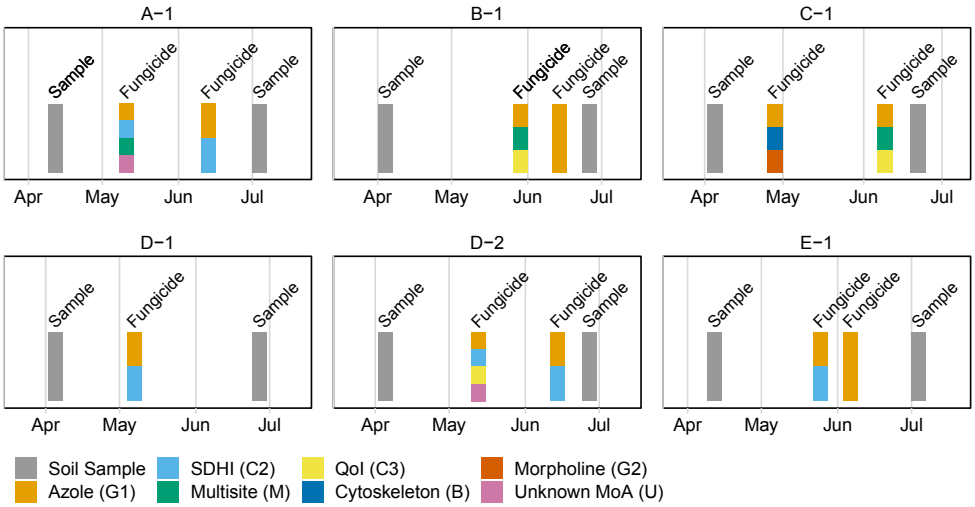
Figure 5. Genetic diversity among isolates from before and after the vegetative period and fungicide application, including azole exposure from four farms.
(A-C) Nucleotide diversity (π) (A), nucleotide polymorphism (Watterson estimator or θ) (B), and Tajima's D (C) along 5 kb windows before and after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



Supplemental Figure 1. (A) Comparison of *A. fumigatus* abundance on apple fields sampled in spring 2017 and spring 2018. n = 50 soil samples per time point for fields H-1 and H-2; n = 25 soil samples per time point for fields L-1, L-2, L-3, L-4. *P* values calculated by Wilcoxon signed-rank test between the time points. (B) *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).

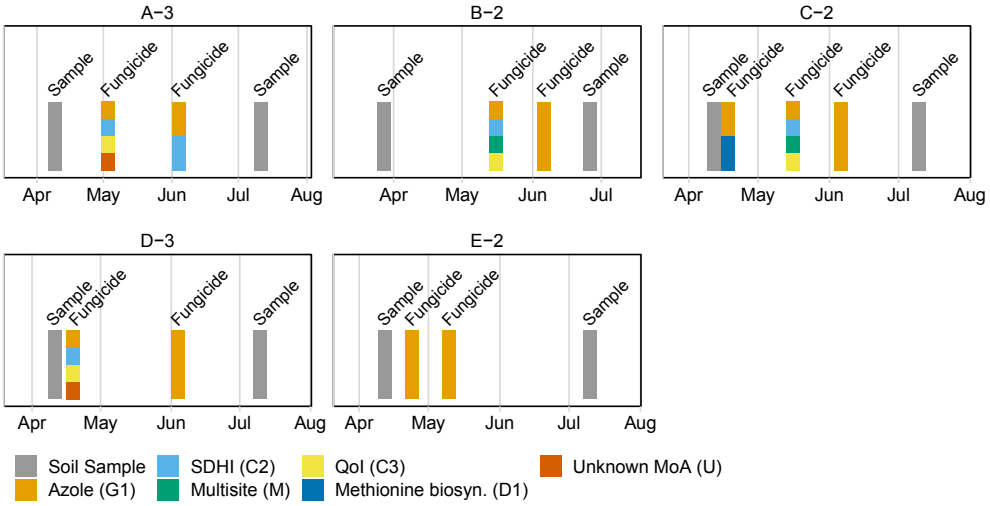
A

2017



B

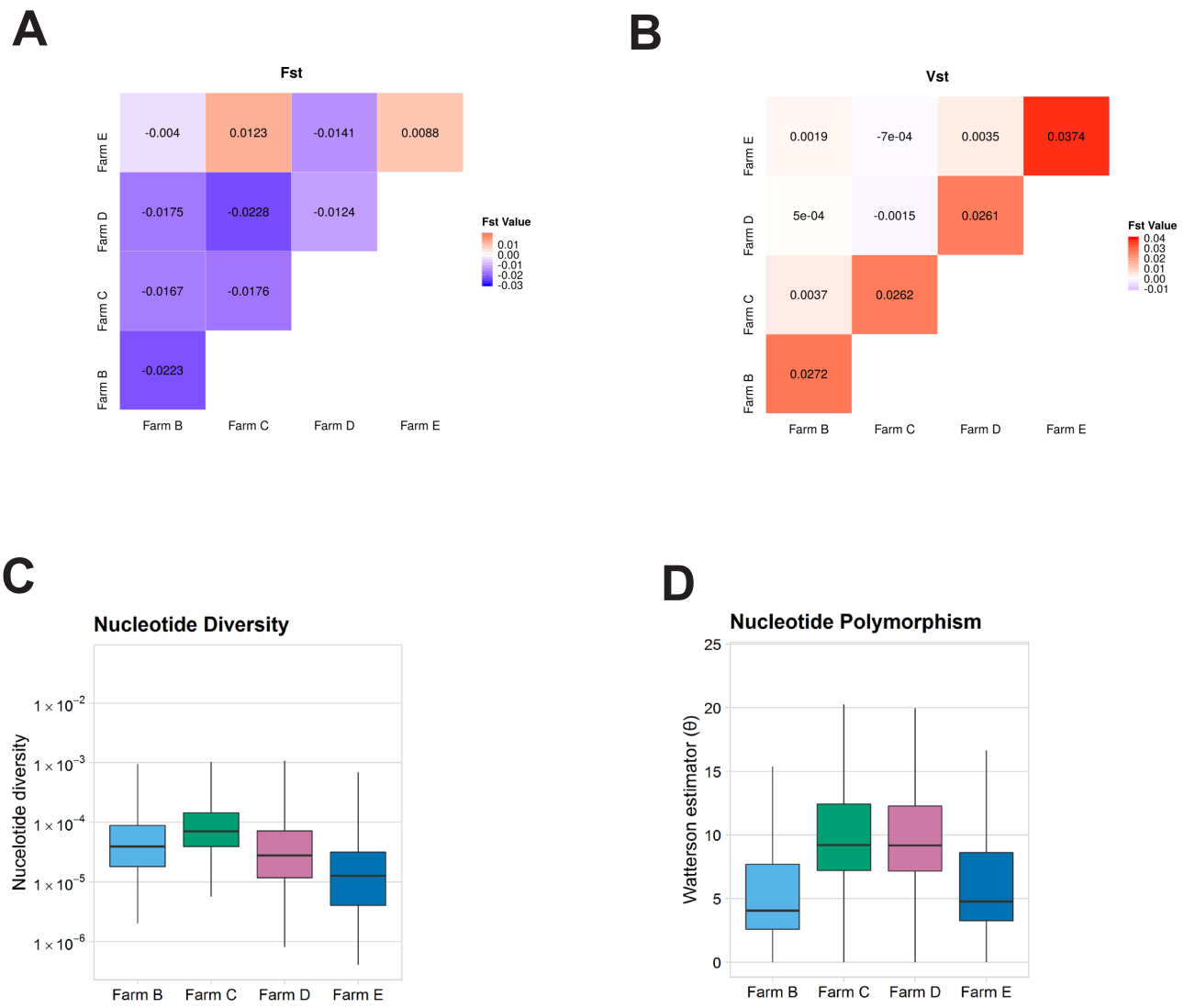
2018



C

Mechanism of Action (MoA)	2017	2018
Azoles (G1)	100%	100%
Succinate dehydrogenase inhibitors (C2)	55%	45%
Multisite inhibitors (M)	27%	18%
Quinone outside inhibitors (C3)	27%	36%
Unknown MoA (U)	18%	0%
Morpholines (G2)	9%	0%
Cytoskeleton inhibitors (B)	9%	0%
Methionine biosynthesis inhibitors (D1)	0%	9%

Supplemental Figure 2. Fungicide history and schematic representation of the sampling time points and fungicide applications for the fields analyzed in this study. (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. (C) Overall frequency of fungicide classifications applied to the fields sampled during 2017 and 2018. Fungicides are categoried by their Fungicide Resistance Action Committee (FRAC) mechanism of action and a frequency of 100% means that a category was present in every fungicide application on every field analyzed that year.



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.