Antifungal alternation can be beneficial for durability but at the cost of generalist resistance

Short title: Delayed but generalist resistance in alternation

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Abstract

The evolution of resistance to pesticides is a major burden in agriculture. Resistance management involves maximizing selection pressure heterogeneity, particularly by combining active ingredients with different modes of action. We tested the hypothesis that alternation may delay the build-up of resistance not only by spreading selection pressure over longer periods, but also by decreasing the rate of evolution of resistance to alternated fungicides, by applying an experimental evolution approach to the economically important crop pathogen *Zymoseptoria tritici.* Our results show that alternation is either neutral or slows the overall resistance evolution rate, relative to continuous fungicide use, but results in higher levels of generalism in evolved lines. We demonstrate that the nature of the fungicides, and therefore their relative intrinsic risk of resistance may underly this trade-off, more so than the number of fungicides and the rhythm of alternation. This trade-off is also dynamic over the course of resistance evolution. These findings open up new possibilities for tailoring resistance management effectively while optimizing interplay between alternation components.

Keywords: ecological specialization; environmental variation; experimental evolution; fungicide resistance; generalism; selection drivers; temporal heterogeneity; *Zymoseptoria tritici*

Introduction

Humanity faces a number of crucial challenges, including obtaining sufficient feed, food, fuel, and fiber. The protection of crops still relies heavily on the use of pesticides to control diverse pests. However, the efficacy of these active ingredients (AIs) has been compromised by the generalization of their use, leading to the rapid and widespread evolution of resistance[1, 2]. Resistance can be seen as phenotype optimization in response to the selection imposed by pesticides, and has provided many examples of contemporary adaptive evolution[3]. The costs engendered by the evolution of resistance in pathogens, arthropods and weeds amount to billions of US dollars each year [4, 5]and entail hidden costs, due to greater pesticide use [6] and impacts on biodiversity [7, 8]. The use of smart strategies for preventing and delaying the evolution of resistance is, thus, crucial for sustainable agriculture.

Diverse AIs, with different modes of action, are often available for controlling pests and pathogens. Strategies can therefore be developed in which these AIs are skilfully deployed over space and time so as to limit the evolution of resistance [9, 10]. More precisely, the optimization of sequences, mixtures, alternation, mosaic or dose strategies for pesticides involves maximizing the heterogeneity of selection pressures exerted on pathogen and pest populations [11, 12, 13, 14, 15]. The alternation (also known as cycling, rotation, or periodic application) of unrelated AIs was first proposed by Coyne in 1951[16] and provides multiple means of intergenerational killing, maximizing the probability of killing the offspring of resistant individuals [10]. The efficacy of alternation for preventing resistance remains a matter of debate, as ranking discrepancies between modeling and empirical data have been noted, for control strategies for insects, weeds and pathogens [10, 17, 18, 19]. The biological traits of the organisms concerned (*e.g.* the reproduction mode, the ploidy of the species, the dominance of the resistance alleles, the duration and overlapping of generations with regard to pesticide exposure…) may partly account for this divergence, as these traits were not systematically made explicit in the mathematical models, and different numbers of generations were considered in the two approaches. However, the variability of the performance of alternation, and, indeed, of other strategies, raises questions about which

62 components of the strategy are the most relevant for delaying the evolution of resistance. Indeed, the
63 heterogeneity of selection in an alternation strategy depends on how many AIs are alternated, the
64 nature of the AIs concerned, and the pattern of alternation adopted.
65 Another limitation is that strategies are mostly evaluated on the basis of their quantitative impact on
66 resistance evolution (*e.g.* resistance frequency, proportion of healthy crop, time to reach a certain
67 amount of resistance, effective life of the pesticide), and more rarely on the basis of the characteristics
68 of the resistant individuals (*e.g.* resistance intensity; pattern of cross-resistance; nature and number
69 of resistance mechanisms). This may be because the genetic basis of resistance is rarely known, in
70 empirical studies, or made explicit, or because mathematical models assume the resistance trait to be
71 specialist [20, 21]. Nevertheless, the diversity of resistance mechanisms observed in microbes, animals
72 and plants is considerable. The evolutionary origins of these mechanisms may also differ [22]. Target site
73 resistance (TSR) affects the structure and/or expression of the target gene of the pesticide by one or
74 several changes in its coding sequence and/or promoter or regulatory gene, resulting in cross-
75 resistance to AIs with the same mode of action (MoA). This type of resistance is considered to be a
76 specialist adaptation, even if levels of cross resistance for TSR may vary between mutations and MoAs.
77 By contrast, non-target site resistance (NTSR) involves the regulated expression of one or multiple
78 genes involved in AI transport, bypass, detoxification, efflux or sequestration. This generally leads to
79 cross-resistance to AIs with different MoAs and is usually considered a generalist trait [23, 24, 25, 26]. Theory,
80 with support from experimental evolution experiments, suggests that specialists evolve in
81 homogeneous environments, whereas generalists are more likely to evolve in heterogeneous habitats
82 [27]. Thus, in terms of adaptation to pesticides, we would expect generalist resistance to be more readily
83 selected with increasing heterogeneity of selection pressure, as in alternation, mixture or mosaic
84 strategies, in contrast to the continuous use of a single AI. This hypothesis has been validated for
85 herbicide mixtures, for which increasing use of different MoAs was found to be associated with lower
86 levels of TSR and higher levels of NTSR (*i.e.* detoxification) in the economically important weed
87 blackgrass, in a national survey in the UK [28]. A similar result was found in the clinical environment,

88 where generalist resistance has also been shown to be more frequently selected in situations in which
89 antibiotics are alternated [29]. Overall, these studies highlight the importance of treatment frequency
90 relative to the pathogen life cycle [30, 31]. The question of the efficacy of alternation strategies thus
91 remains unresolved for pesticides, as treatment frequency may result in the interval between
92 treatments being much longer than the generation times of pests, particularly for some pathogens.
93 Here, we use the ascomycete fungus *Zymoseptoria tritici* — the cause of septoria leaf blotch (STB), the
94 most devastating foliar disease of wheat in Europe [32, 33] — as a general model to determine how the
95 components of fungicide alternation drive the quantitative and qualitative performance of this
96 strategy. This highly adaptive pathogen, with large and diverse populations and dual reproduction
97 mode, has evolved resistance in the field to four different unisite modes of action in Western Europe,
98 resulting in contrasting decreases in field efficacy depending on AIs and their combination[34, 35, 36, 37, 38,
99 39, 40]. Resistance dynamics also differs according to MoAs, as demonstrated quantitatively for France[41,
100 42]. *Z. tritici* exhibits TSR, determined by various specific target-site mutations, that can be associated
101 to target overexpression [40, 43, 44, 45] but also to NTSR *via* enhanced efflux. This latter generalist
102 mechanism causes weak multidrug resistance (MDR) when not associated to TSR and affects at least
103 three modes of action [43, 46]. These biological features and field background make *Z. tritici* a textbook
104 model for dissecting the adaptation of populations under contrasting selection pressures and
105 reasoning about general principles. We made use of the haploid yeast-like easily-culturable form of
106 this pathogen (*i.e.* blastospores)[47] in liquid medium, in an experimental evolution approach [48] based
107 on the selection of resistance under regimes mimicking alternation. The selection patterns were
108 designed such that three components of alternation — the intrinsic risk of resistance to AIs, the
109 number of AIs alternated and the alternation rhythm — could be disentangled, to assess their
110 respective impacts on resistance dynamics and on the resistance mechanisms selected. We found that
111 the combination and interplay between alternation drivers clearly guided the evolutionary trade-off
112 between the quantitative (*i.e*. the overall and fungicide-specific resistance evolution rates) and
113 qualitative (*i.e.* the degree of generalism of evolved strains) performances of alternation, opening up

114 new perspectives for the informed tailoring of this strategy in the field, for the first time in a
115 phytopathogenic fungus.
116

117 Results

118 Fungicide alternation has a neutral-to-beneficial effect, 119 slowing the overall evolution rate of resistance

120 We observed the dynamics of *Z. tritici* resistance to benzovindiflupyr (B), carbendazim (C) and
121 prothioconazole-desthio (P), here representing three modes of action (SDHIs, benzimidazoles and
122 DMIs, respectively), after experimental evolution in 56 independent lines subjected to 14 regimes of
123 continuous or alternating B, C and P, including B, C or P at their EC_{95} selection doses (Fig **1a** and **1b**).
124 Mean normalized Malthusian growth M_{it}^{n} (referred to hereafter as the overall resistance evolution rate
125 ρ) was used as a proxy for increasing resistance. Resistance establishment was declared when ρ
126 reached at least 90% of that of the control line. Resistance evolved in all populations continuously
127 exposed to B, C or P, and was established after three, six and six cycles, respectively. Resistance also
128 evolved in lines treated with these three fungicides in alternation, regardless of the alternation rhythm
129 (*i.e.* the number of successive cycles undergoing the use of the same AI) and the number of fungicides
130 alternated, although resistance never established, even after 12 cycles, in some situations including
131 medium- to low risk AIs (Fig **1c**). For lines undergoing direct selection (with a single compound), ρ was
132 1.1 times higher with C than with B and 1.4 times higher with C than with P; these significant
133 differences reflected the hierarchy of the relative intrinsic risks of resistance associated with
134 benzimidazoles (high; C), SDHIs (medium; B) and DMIs (low; P) (Fig **S1**). The overall resistance evolution
135 rates of the lines were modified by alternation regimes, being significantly lower, by a factor of 1.1-
136 1.3, for alternation regimes containing carbendazim than for continuous exposure to this high-risk
137 fungicide. Alternation thus reduced the risk of resistance to C to a level similar to that observed for

138 medium- and low-risk fungicides in conditions of continuous exposure. The benefits of alternation, by
139 comparison to the use of single AIs, were even more pronounced, with a 25-58% decrease in resistance
140 selection (as estimated by ρ) when low- and medium-risk fungicides were alternated or when
141 fungicides of the three categories were alternated. Thus, depending on the fungicides alternated,
142 alternation had either a neutral effect or decreased the overall resistance evolution rate relative to
143 single fungicides in continuous use.
144

145 The risk of resistance inherent to the fungicide is the key
146 driver for tailoring alternation

147 Alternation may reduce the selection of resistance to a particular fungicide by limiting the total time
148 of exposure to this fungicide, but also by modifying the overall resistance evolution rate ρ, potentially
149 through the diversification of selection pressure. We investigated the potential effect of multi-
150 directional selection in alternation, separating this effect from that of the decrease in exposure time,
151 by comparing ρ only for time segments corresponding to fungicide *f* exposure (ρ_f, referred to hereafter
152 as the fungicide-specific resistance evolution rate), and by alternation component (Fig **2a**). The impact
153 of alternation on ρ_f was significantly dependent on the intrinsic risk of resistance associated with the
154 alternating partner. Indeed, the selection of resistance to the high-risk fungicide was decreased (by
155 9%) only if the fungicide concerned was alternated with the low-risk fungicide. Only alternation with
156 the low-risk fungicide was able to decrease (by 22%) the selection pressure associated with the use of
157 the medium-risk fungicide. Medium- and high-risk alternation partners had neutral or detrimental
158 (+82%) impacts, respectively, on the rate of selection of resistance to the low-risk fungicide. Overall,
159 the selection capacity of a fungicide was decreased only by alternation with a fungicide of sufficiently
160 lower risk. Thus, decreases in selection pressure in alternation were always achieved at the expense
161 of the selection capacity of the partner with the lower intrinsic risk of resistance. This conclusion also
162 extended to strategies involving an alternation of three fungicides. The duration of continuous

163 exposure to the same fungicide (alternation rhythm over the cycles) had a lesser effect, increasing
164 resistance evolution rate only for the longest sequences (three cycles) and for only two of the 18
165 possible comparisons. Finally, the fungicide-specific resistance evolution rate was driven principally by
166 the inherent resistance risk of its alternation partner relative to its own resistance risk, and secondarily,
167 by other alternation components, such as the number of fungicides alternated and the alternation
168 rhythm.
169

170 Resistance selection differs over the course of evolution, 171 reflecting contrasting shifts in the interplay between 172 fungicides and phenotype-adaptive landscapes

173 Changes in the frequency of resistance in a population typically follow sigmoid curve, with an
174 emergence phase of low resistance (<15%; here, the lowest frequency reliably detectable by
175 measuring the change in OD_{405} in our experimental conditions), a selection phase (here, resistance
176 rates between 15% and 90%, chosen as a convention) and an established-resistance phase (here, over
177 90%)[13]. Cox survival analysis was used to investigate the effect of alternation components on the
178 exposure time (number of cycles with a given fungicide) required to reach a given threshold of mean
179 normalized Malthusian growth. Fig **2b** shows the number of cycles of fungicide exposure gained or lost
180 to reach critical values of M_{it}^{n} compared to the lines continuously exposed to the same fungicide,
181 depending on alternation components. Alternation decreased the resistance evolution rate of the
182 high-risk fungicide mostly towards the end of the selection phase and during the established-resistance
183 phase, rather than during the early phases (delaying the established-resistance phase by 0.77 and 1.31
184 cycles for alternations of two and three AIs, respectively). For the medium- and low-risk fungicides,
185 this beneficial delaying of the established-resistance phase was observed only in the case of alternation
186 with the partner of lowest possible risk (delayed by 1.70 and 1.01 exposure cycles, respectively), with

187 the high-risk partner decreasing the time required to reach the established-resistance phase (by 1.22
188 and 2.42 exposure cycles, respectively). By contrast, alternation with a partner with a higher resistance
189 risk generally increased the rate of selection of resistance to the low-risk fungicide, particularly during
190 the emergence and early selection phases (by 1.52 to 2.13 exposure cycles).
191 The relative selection pressure exerted by each fungicide during alternation therefore varied not only
192 according to the selection regime and its components, but also according to the phase of resistance
193 dynamics. This contrasting pattern of selection over time can be first explained by the decreasing
194 efficacy of the alternation partner, as resistance spreads more or less rapidly depending on its
195 resistance risk. Indeed, a low-risk alternation partner may control population size longer (*i.e.* possibly
196 until the latest phases of resistance dynamics) than a high-risk one, which leads according to our
197 protocol to greater immigration from the control lines and then to greater dilution of the selected
198 resistance in the population initiating the upcoming selection cycles. By contrast, while losing its
199 efficacy more quickly, a high-risk alternation partner is expected to increase resistance selection from
200 the early phases of selection, especially since immigration of susceptible cells from the control lines is
201 rapidly unnecessary. The observed patterns of selection evolution are globally consistent with this
202 rationale. However, discrepancies between observed and expected patterns may result from the
203 exploration of adaptive landscapes[49], *i.e.* the successive selection of different mutations increasing
204 fitness over the course of the experiment, to adjust to the overall selection pressure. We therefore
205 analyzed the change in population composition over time in regimes under continuous exposure (lines
206 0B, 0C and 0P; Fig **3**), to obtain proof-of-concept. As expected, resistance profiles were generally
207 narrow and mostly focused on resistance to the fungicide used for continuous selection. However,
208 phenotypic resistance profiles differed significantly between different resistance dynamics stages. In
209 AMOVA analyses, within-selection regime diversity was always greater than between-selection regime
210 diversity, but was concentrated throughout evolution, in a manner that varied with the fungicide
211 exerting the selection pressure. The high-risk fungicide (C) ultimately drove the selection of four highly
212 specialized profiles (resistant to a mean of 1.25 AIs). By contrast, the medium- and low-risk fungicides

213 (B and P) selected multiple resistance phenotypes, differing in terms of both cross-resistance and
214 resistance intensity. The medium-risk fungicide selected patterns of resistance to a mean of 2 AIs. The
215 patterns selected by the low-risk fungicide were the most generalist (resistance to a mean of 4 AIs)
216 and often included resistance to tolnaftate, an indicator of multidrug resistance in several pathogens
217 [43]. In alternating lines, such contrasting patterns of adaptation to a single AI should recur regularly as
218 AIs succeed to each other over the generations, thereby resulting in even more complex trajectories.
219

220 Fungicide alternation preferentially selected for multiple or
221 generalist resistance mechanisms

222 The effect of the anticipated greater selection heterogeneity of alternation regimes was investigated
223 at the end of the experiment, by establishing the phenotypic resistance profile of the evolved
224 individuals in each line. Therefore, we analysed the average ability of growth under 12 different testing
225 modalities of selected isolates (Fig **4a**) and established heatmaps showing the diversity of these
226 phenotypic resistance profiles according to the origin of the tested isolates (Fig **4b**). The profiles of
227 strains evolving under direct selection regimes were generally narrower and less diverse than those of
228 strains evolving under alternation regimes. PCA confirmed this finding (Fig **4c**), with direct selection
229 clusters being more concentrated than any other and well separated from those displaying resistance
230 to fungicides not used for selection (*e.g.* boscalid, another SDHI from a different chemical class and
231 azoxystrobin and tolnaftate, with different modes of action). The diversity of the lines was further
232 explained by resistance to the low-risk fungicide (P), which structured the second axis of the PCA. The
233 width of multiple or cross-resistance increased significantly with the number of AIs used in the
234 selection regime and depended, to a lesser extent, on the alternation partner. Resistance to tolnaftate,
235 reflecting multidrug resistance, was significantly more frequent in lines treated with alternating
236 fungicide regimes with a long alternation rhythm. The intensity of resistance was significantly

237 decreased by alternations including medium- and low-risk fungicides but increased slightly with
238 increasing number of AIs in the selection regime and with long alternation rhythms.
239 The broader resistance profiles of strains subjected to alternation regimes is potentially consistent
240 with the selection of multiple resistance (*i.e.* the co-selection, in the same strain, of several mutations,
241 each conferring resistance to a single fungicide) and/or generalist resistance (*i.e.* the selection of
242 pleiotropic mutations, associated with more general resistance to fungicides). We initially tested these
243 hypotheses by sequencing the genes encoding the targets of carbendazim, prothioconazole and
244 benzovindiflupyr in 141 isolates corresponding to the various phenotypic resistance profiles. Only the
245 E198K substitution in β-tubulin was detected in a few isolates evolving under direct carbendazim
246 selection. This target site mutation has been reported to confer a high degree of resistance to
247 benzimidazoles in field isolates of 12 fungal species, and in nematodes [50, 51]. No other change was
248 recorded relative to the 330 full-gene sequences established for *tub2, cyp51, sdhB, sdhC* or *sdhD.* The
249 systematic genotyping of the promoter regions of *tub2, sdhC* and *cyp51* did not reveal any indels for
250 these strains by comparison to the ancestral isolate. The normalized expression of these genes,
251 quantified by qRT-PCR after cultivation without fungicide induction, varied roughly between 0.5 to 4
252 comparatively to that of the ancestral isolates (Fig. **S2**). As these weak variations included a susceptible
253 isolate collected from the control line, we assumed that overexpression of these three target genes
254 did not operate in the dataset or was rather weak. Enhanced efflux is a non-target site resistance
255 mechanism associated to low RFs *per se* but causing cross-resistance to unrelated compounds in *Z.*
256 *tritici* [43]. In field strains, it is determined by insertions in the promoter region of the MFS1 transporter
257 gene [52]. Systematic genotyping of 249 isolates collected in this experiment revealed no changes in the
258 length of the *mfs1* promoter, particularly in strains displaying tolnaftate resistance. However, qRT-PCR
259 revealed approximately 5-, 11- and 30- fold overexpression of *mfs1*, compared to the ancestral strain,
260 in three out of eight isolates cultivated without fungicide induction and collected from lines subjected
261 to alternation regimes (Fig. **S2**). These isolates exhibited moderate to low scores of tolnaftate
262 resistance. Overall, these findings suggest that alternation components favour generalist phenotypes,

263 with enhanced efflux due to MFS1 transporter, regulated from genomic changes differing from those
264 occurring in the field in some isolates, occurring in some but not all isolates. The low correlation
265 observed between scores of tolnaftate sensitivity and *mfs1* expression implies that unknown
266 transporters are also frequently observed in this context, possibly in addition to other unknown non-
267 target site resistance mechanisms.

268

269 Discussion

270 This study confirms the value of experimental evolution as an approach for investigating conceptual
271 issues in temporal adaptation, such as the factors affecting the adaptive differentiation of local
272 populations [48, 53, 54]. Here, we used an experimental evolution approach and the economically
273 important wheat pathogen *Z. tritici* as a model for general principles to demonstrate that temporal
274 heterogeneity can mitigate the evolution of resistance to fungicides in lines subjected to treatment
275 with fungicide alternation regimes. These experiments clearly highlight the relationships between the
276 components of the temporal heterogeneity of fungicide selection and both resistance dynamics and
277 the generalism or specialism of the evolved isolates. With this experimental design, we were able to
278 validate, in a filamentous plant pathogen *in vivo*, the theoretical assumptions that temporal
279 heterogeneity of selection can quantitatively modulate adaptation [10, 17] and mediate generalism in
280 evolved individuals [27, 55], as previously demonstrated for weeds, insects and bacteria [12, 29, 56, 57, 58, 59].
281 Specifically, using three different fungicides, we found that alternation was either neutral or beneficial
282 in terms of delaying the overall resistance evolution rate, relative to the continuous use of a single
283 fungicide, whatever its resistance risk. In all cases, alternation strategies performed at least as well as
284 the continuous use of the lower risk fungicide. Alternation may delay the build-up of resistance to a
285 particular fungicide because the selection pressure exerted by the fungicide is spread over a longer
286 period than in a sequence strategy[21]. However, this would suggest that fungicide-specific resistance

287 evolution rates (ρ_f) are similar for continuous-use and alternation strategies, and independent of
288 alternation partner and alternation rhythm. By contrast, during our dissection of the impact of
289 alternation components, we obtained variable ρ_f values and revealed the primary influence of the
290 fungicide resistance risk, determined by its mode of action, relative to alternation partners, on
291 selection and the overall performance of a strategy. Thus, increasing the number of AIs in the
292 alternation may either increase or decrease the fungicide-specific resistance evolution rate, depending
293 on the intrinsic risk of resistance for the added AI relative to those already present in the alternation.
294 This may reflect the balance between the relative fitness costs and levels of resistance of the multiple
295 genotypes selected by a given AI in a cycle, which finally determines the composition of the population
296 at the beginning of the next cycle, before contrasting selection with a different AI operates. Our
297 observation then also reflects the relative effectiveness of each partner over time in relation to the
298 current population composition. When needed to prevent line extinction, in the early cycles,
299 immigration of susceptible isolates from the control lines contributed to slow down resistance but
300 promoted the relative effectiveness of both partners. Finally, the temporal heterogeneity of
301 environments may determine the direction of evolution, with more fine-grained temporal
302 environments (in which the environment varies over time scales shorter than the generation time)
303 being more efficient at delaying adaptation while maintaining population diversity[60, 61]. In our design,
304 even the one-week alternation rhythm greatly exceeded the generation time of *Z. tritici* (estimated at
305 6-7 generations per cycle), so all the temporal environments tested were coarse-grained, particularly
306 the regimes with a three-week alternation. However, alternation rhythm had a much lesser impact
307 than other components on the resistance evolution rate of a given fungicide. This implies that some
308 flexibility is possible in the design of field strategies, as the frequency of fungicide sprays is likely to
309 create coarse-grained environments in many agrosystems. The respective relevance of these selection
310 drivers for mixture strategies, most commonly used in the field, could be interestingly compared in
311 further studies.

312 We confirm here, in a plant pathogenic fungus, that temporal variation in fungicide application may
313 be correlated with a higher degree of generalism in resistant strains. An analysis of resistance
314 phenotype profiles in the course of direct selection (continuous use of the same fungicide) revealed
315 high within-line phenotype diversity (data not shown) and the concentration of this diversity over time,
316 culminating in specialist profiles, for which the degree of specialism depended on the AI. This dynamics
317 is also consistent with the continual change in population composition, with the rise, selection, fixation
318 or extinction of beneficial mutations, depending on their relative fitness costs and the degree of
319 resistance in the local population [62]. High-risk fungicides can thus explore smooth fitness landscapes,
320 such as those generated by the selection of a unique change underlying high resistance (*e.g.* the tub2
321 E198K variant conferring high resistance to C). By contrast, medium- and low-risk fungicides can
322 explore less smooth landscapes. According to theory, the global fitness optimum is less likely to be
323 achieved in variable environments [63]. Combining the respective paths associated with AIs not
324 exhibiting cross-resistance, and considering the population dynamics as previously discussed, we
325 would then expect lines evolving under alternation regimes to explore rugged landscapes, constraining
326 resistance mutations to one fungicide with trade-offs in resistance to other fungicides or to favour
327 genotypes with a higher average fitness over time[29, 64]. This scenario is consistent with the fixation of
328 multiple discrete resistance mechanisms, together with the accumulation of relative fitness costs
329 associated with each resistant trait, as suggested by the wider patterns of cross-resistance (including
330 resistance to new AIs), lower resistance scores and higher frequency of resistance to tolnaftate
331 (associated with enhanced efflux) observed in isolates collected from lines subjected to alternating
332 treatments. The molecular analysis of a representative subsample of resistant isolates hardly detected
333 mutations in genes encoding the targets of selecting fungicides and indicated that enhanced efflux due
334 to the MFS1 transporter was at work in a few - but not all - resistant strains. Our collection therefore
335 provides a good opportunity to make use of the progress in genomics to elucidate unknown genetic
336 and non-genetic bases of adaptation mostly at work in our dataset. These unknown resistance
337 mechanisms (*i.e.* other than target alteration or overexpression), may also operate *in natura*, without

338 our suspecting it and may, therefore, have an overlooked impact on resistant traits [65, 66, 67]. Indeed, a
339 genomic analysis has shown that not only SNPs but also indels, copy number variants, transposable
340 element insertions, chromosome duplications and aneuploidy occur in fungal pathogens exposed to a
341 sublethal dose of fungicides [68]. Our observation of unknown resistance mechanisms in isolates from
342 alternation as well as sequence strategies, is consistent with this pan-genome characterization of
343 fungal adaptation to fungicides, without *a priori* on resistance mechanisms. This finding brings the
344 proof of concept that *Z. tritici* is also a relevant model to explore the various shades of non-target site
345 adaptation, in addition to inform on the general principles to manage resistance, whatever mutations
346 were selected. We also found that alternation components had different impacts on the rate of
347 resistance evolution over the course of the experiment. Alternation significantly delayed the
348 establishment phase of the dynamics of resistance to the high-risk fungicide, suggesting that lower-
349 risk fungicides perform better than high-risk ones after the emergence phase. By contrast, alternation
350 with high-risk fungicides decreased the time required for the early stages of resistance to be reached
351 for a low-risk fungicide, demonstrating that the high-risk fungicide did not counter-select mutations
352 with a low resistance intensity. Finally, experimental evolution has proposed a simplified model for
353 evolution *in natura* [54] and enriched our understanding of the general principles of resistance
354 management by highlighting the relative impact of selection components. Due to the laboratory
355 environment, our experimental design assumes a finite population size, low ancestral diversity, limited
356 time scale and asexual reproduction. However, many plant pathogens, including *Z. tritici,* have large
357 population sizes, high levels of genetic diversity and both sexual and asexual reproduction, and have
358 to adapt simultaneously to their host and abiotic environment [69]. Gene flow is also expected to occur
359 between evolving metapopulations. Because of these differences, we felt that selecting for this
360 experiment adaptive mutations distinct from those occurring in the field would not limit the relevance
361 of this study for reasoning about antifungal management strategies. Beyond these limitations, our
362 study clearly demonstrates that not all alternation strategies guarantee the same performance for
363 preventing resistance, but in no instance did these strategies increase the risk of resistance relative to

364 that associated with the continuous use of individual fungicides. It might then offer an alternative to
365 the mixture strategy to delay resistance, while reducing pesticide load, a growing socio-environmental
366 demand. Indeed, for example, commercial mixtures used on wheat to control Septoria leaf blotch
367 combine fungicide components at rates close to those recommended for their individual use, resulting
368 in the application of almost double the amount of fungicide [70]. Efforts to educate stakeholders about
369 alternation strategies should, therefore, focus on optimizing the interplay between the various
370 components of alternation. The results of this study pave the way for the effective tailoring of
371 resistance management for plant pathogens based on temporal heterogeneity, especially in perennial
372 crops where multiple sprays are often applied yearly. We would also argue that resistance
373 management *via* temporal heterogeneity is possible provided that the environmental grain is
374 appropriate [61]. The environment should generally be fine-grained, requiring alternating generations to
375 be exposed to compounds with a different mode of action, but we found that coarse-grained temporal
376 fluctuations were potentially acceptable. Furthermore, the order in which the AIs are cycled may affect
377 the trajectory of evolution. Modeling suggests that the most effective AI should be used first [21], to
378 control emerging mutations as efficiently as possible, but further explorations in experimental
379 evolution experiments would be required to confirm this. The overall performance of alternation
380 approaches including AIs with different inherent resistance risks is achieved at the expense of the AIs
381 with the lowest risk of resistance, suggesting that the use of high-risk AIs in alternation sequences
382 should be limited and/or that the alternation of AIs of similar risk should be preferred. The relative risk
383 between AIs might also be adjusted in the field by using different doses or alternation rhythms for
384 each partner, or by mixing some of the alternated AIs. The efficacy of alternation strategies, as well as
385 of mixtures, should also be confronted to the real-world case in which populations are not naïve and
386 the initial levels of resistance to each fungicide differ, not to mention multiple resistance[71]. Finally, the
387 design of rational strategies with optimized components should take into account the evolutionary
388 trade-off between the rate of resistance evolution and the degree of generalism of the evolved
389 individuals, which may call into question the efficacy of future modes of action. In this respect, the

390 future control of weeds, pests and diseases should be based not only on a diversity of modes of action,
391 but should consider integrated pest management including non-chemical strategies to mitigate overall
392 adaptation [72].
393
394

395 Methods

396 Ancestral population, culture conditions and assessment

397 We used IPO-323, as an ancestral isolate, to found all lines, *i.e.* similar ancestral populations further
398 evolving under fungicide selection. This isolate is susceptible to all fungicides and was the source of
399 the high-quality reference genome for *Z. tritici* (Goodwin et al., 2011). The stock spore suspension was
400 kept at -80°C in 20% glycerol and gently thawed at room temperature for the inoculation of solid YPD
401 plates (20 $g.L^{-1}$ dextrose, 20 $g.L^{-1}$ peptone, 10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ agar; USBiological), which were
402 then incubated at 18°C in the dark for seven days. These plates were used to prepare precultures in
403 liquid YPD (as previously described, but without agar), which were shaken at 150 rpm in similar
404 conditions for seven days before the start of the experiment.
405 The culture medium used for experimental evolution was liquid YPD supplemented with 100 $mg.L^{-1}$
406 streptomycin and penicillin to prevent contamination of this long-term experiment. The culture vessels
407 were 50 mL borosilicate Erlenmeyer flasks containing 25 mL medium, with carded cotton wool inserted
408 into the neck. Cultures were grown in the dark at 18°C, with a RH of 70% and shaking at 150 rpm during
409 the duration of the cycle. In these conditions, *Z. tritici* can be kept in its yeast-like form, making it
410 possible to obtain homogeneous liquid cultures [73].
411 Population size was routinely determined by measuring optical density at 405 nm (or OD_{405}) for two
412 200 µL replicates of the fungal culture in a 96-well microtiter plate (Sarstedt) sealed with a gas-
413 permeable membrane (Breathe Easy®; Diversified Biotech). The detection threshold of the
414 spectrophotometer (SpectraMax M2, Molecular Devices) was 3.5 x 10^5 $spores.mL^{-1}$ in the experimental

415 conditions used. The OD_{405} of the fungal cultures was normalized against that of the non-amended
416 medium. Spore concentration was estimated from the OD_{405} with a mathematical model calibrated on
417 the basis of preliminary experiments in our conditions with serial dilutions and hemocytometer-based
418 spore quantification, and cross-validated (R^2=0.91). Population size in spores.mL^{-1} was used in further
419 statistical analyses.
420

421 ## Fungicides and selection regimes

422 In the experimental evolution experiments, we selected resistance to three fungicides representative
423 of different modes of action: carbendazim (C; benzimidazole, interfering with β-tubulin assembly in
424 mitosis), benzovindiflupyr (B; pyrazole carboxamide, inhibiting complex II, succinate dehydrogenase,
425 in the mitochondrial respiratory chain) and prothioconazole-desthio (P; triazole, inhibiting the sterol
426 14α-demethylase during sterol biosynthesis), the active metabolite of prothioconazole. These modes
427 of action are associated with contrasting resistance risk. The fungicides were dissolved in 80% ethanol.
428 The concentration of this solvent never exceeded 0.5% of the final culture volumes.
429 The selection doses were chosen by examination of dose-response curves for the ancestral strain. For
430 each fungicide dose, 10^7 spores of IPO-323 were used to inoculate YPD in an Erlenmeyer flask, which
431 was then incubated for seven days before OD_{405} measurement. OD_{405} data were modelled by logistic
432 regression, to calculate, for each fungicide, the EC_{50} (concentration inhibiting 50% of growth), EC_{95}
433 (concentration inhibiting 95% of growth) and MIC (minimum inhibitory concentration) values. We
434 finally chose to use the EC_{95} as the selection dose, as 99% inhibition was not achieved for some
435 fungicides, for which the MIC would exceed fungicide solubility in the solvent. The selection doses used
436 were 0.5 mg.L^{-1} for benzovindiflupyr, 0.1 mg.L^{-1} for carbendazim and 0.005 mg.L^{-1} for prothioconazole-
437 desthio.
438 Selection regimes were organized for analysis of the respective impacts of three components of
439 alternation on resistance evolution, as described in Fig **1b**. First, in treated lines, the number of AIs in

440 the alternation regime ranged from one (direct selection) to three different fungicides alternated over
441 time. Second, the intrinsic risk of resistance differed between MoAs and was predicted to be high for
442 benzimidazoles (represented by C), moderate to high for SDHIs (represented by B) and moderate for
443 DMIs (represented by P) by agrochemical companies[74]. In practice, as we observed similar relative
444 ranking of these fungicides in our experiments, we referred to high-, medium- and low-risk fungicides,
445 respectively, throughout this work, for the sake of simplicity. Finally, the duration of exposure to the
446 same fungicide (or alternation rhythm) was continuous for direct selection (0, no alternation) and
447 ranged from 1 (fungicide changed every cycle) to 3 (fungicide changed every three cycles) cycles. All
448 combinations of these three possible components were tested, except for the three-fungicide
449 alternation, which was tested only for an alternation rhythm of one cycle.
450

451 Design of the experimental evolution experiment

452 The ancestral population was founded from the seven-day liquid YPD preculture of IPO-323. The
453 density of this spore suspension was adjusted to 2 x 10^7 spores.mL^{-1} and each Erlenmeyer flask was
454 inoculated with 10^7 spores (500 µL). The resulting starting concentration of 4 x 10^5 spores.mL^{-1} offered
455 a compromise between large populations in which mutations were likely to occur and not exceeding
456 the culture carrying capacity in the experimental conditions used, based on the findings of preliminary
457 experiments. Each of the 14 selection regimes (1 control and 13 different treatments) was repeated
458 four times (four lines per selection regime). Control solvent lines received only 0.5% solvent, whereas
459 treated lines were treated as shown in Fig **1b**, giving rise to 56 independent lines. Control lines without
460 fungicides nor solvent were grown only to provide susceptible cells for immigration (see below) and
461 were not included in statistical analysis comparing strategies.
462 Experimental evolution was conducted over 12 cycles of seven days each (about six to seven
463 generations per cycle). This cycle duration corresponds approximately to the time for which a control
464 line remained in the exponential growth phase before reaching a plateau. OD_{405} was measured at the

465 end of each cycle and transformed into population size, as described above. The OD_{405} of the treated
466 lines was normalized with the mean OD_{405} of the control solvent lines. At each transfer, 500 µL of the
467 evolving culture (*i.e.* 2% of the total volume of medium) was transferred to a new Erlenmeyer flask
468 containing fresh medium. If the number of spores in the 500 µL of culture medium was less than 10^7,
469 which would be the case before the evolution of resistance, then the appropriate number of spores
470 from one of the untreated populations was added to make the number of spores present up to 10^7.
471 The starting population size was equalized between the lines at the beginning of each new cycle, and
472 the process mimicked the immigration occurring in field situations. For each of the four replicates, the
473 same source population was used for immigration throughout the experiment. In preliminary
474 experiments, immigration was found to be useful for preventing population extinction after weekly
475 bottlenecks and to minimize genetic drift.
476 At the end of each cycle, 2 mL of each line was mixed with glycerol (25%), frozen and stored at -80°C
477 for further analyses.
478

479 **Statistical analysis of resistance evolution rate**

480 The Malthusian growth[75] of each line was calculated as

481 $$M_{it} = \ln\left(\frac{Spore\ concentration\ of\ line\ i\ at\ the\ end\ of\ cycle\ t}{Spore\ concentration\ of\ line\ i\ at\ the\ beginning\ of\ cycle\ t}\right)$$

482 and normalized against that of the solvent control line *C* such that $M_{it}^{n} = \frac{M_{it}}{M_{Ct}}$.

483 For each selection regime, the mean Malthusian growth ("overall resistance evolution rate" or ρ) is the
484 mean of M_{it}^{n} calculated over the 12 cycles, and constitutes a quantitative summary of the increase in
485 resistance of the four replicates over the entire experiment. Similarly, ρ_f ("fungicide-specific resistance
486 evolution rate") reflects the increase in resistance associated with a fungicide *f* during the alternation
487 regime, taking into account only the time segments during which this fungicide was used. This
488 approach made it possible to compare selection due to the same number of fungicide applications (6

489 applications, except for the BCP regime for which there were only 4 applications), but in the context
490 of different selection regimes. ρ and ρ_f were explained in ANOVA linear models, initially considering
491 the alternation partners as qualitative fixed factors, and then the alternation rhythm for each
492 alternation partner. The reference used was the continuous application of the fungicide.
493 The exposure time (*i.e.* the number of cycles exposed to a given fungicide) required for the normalized
494 Malthusian growth M_{it}^{n} to reach certain thresholds (15%, 25%, 50%, 75%, 90%) was investigated in a
495 Cox survival analysis taking into account the number of AIs, the alternation partner and the alternation
496 rhythm.
497 All analyses and figures were produced with R 4.0.4 and the additional packages listed in **S1** Methods.
498

499 Isolation and phenotyping of evolved individuals

500 We assessed the change in resistance phenotypes during the course of selection, by isolating pure
501 strains from direct selection regimes, at critical points in the resistance dynamics, *i.e.* when normalized
502 Malthusian growth reached 15% (early resistance), 75% (intermediate resistance frequency) and 98%
503 (established resistance) relative to that of the control line, and at the end of the experiment. We
504 isolated 12 individuals (3 per replicate) per selection regime from cycles 1, 5, 6, 12 for 0B lines, from
505 cycles 2, 3 and 12 for 0C lines, and from cycles 5, 6 and 12 for 0P lines. This resulted in the isolation of
506 120 strains in total. We also explored phenotype diversity within and between selection regimes, by
507 systematically isolating 12 strains (3 per replicate) at the end of the final cycle, for all selection regimes.
508 This resulted in the isolation of 129 additional strains. Isolates were retrieved from glycerol population
509 stocks collected at the end of each cycle. Two successive isolations were performed on YPD solid
510 medium supplemented with 100 $mg.L^{-1}$ streptomycin and penicillin. Single isolates were stored at -
511 80°C in 25% glycerol.
512 The cross-resistance patterns of the 249 isolates were established in droplet tests. We deposited 10^7,
513 10^6, 10^5, and 10^4 $spores.mL^{-1}$ suspensions as drops organized into columns, in 12 cm square Petri

514 dishes. These plates contained YPD supplemented with discriminatory doses of fungicides. The 15
515 treatments tested encompassed a control with 0.5% solvent and fungicides B, C and P at their selection
516 doses (0.1, 0.5 and 0.005 $mg.L^{-1}$, respectively) and twice their selection doses (0.2, 1 and 0.01 $mg.L^{-1}$,
517 respectively). Mixtures of pairs of the three fungicides were also included (each fungicide used at 0.8
518 times its selection dose, as this was found to be the lowest discriminatory dose in mixtures of the
519 reference resistant and susceptible strains). Finally, for the detection of generalist resistance, we also
520 included fungicides not used for experimental evolution (0.5 $mg.L^{-1}$ azoxystrobin, 2 $mg.L^{-1}$ boscalid and
521 2 $mg.L^{-1}$ tolnaftate). Tolnaftate, in particular, identified individuals displaying enhanced fungicide efflux
522 leading to non-specific multidrug resistance (MDR), as described in field isolates of *Z. tritici* [43]. The
523 ancestral strain IPO-323, and three field strains, each resistant to one or several of the fungicides used
524 for selection, were included as control strains. After seven days of incubation, drops were scored 0 or
525 1, according to the presence or absence of fungal growth. A total score was established for each strain,
526 ranging between 0 (susceptible) and 4 (resistant if >0), according to the number of dilution droplets
527 for which growth was observed. For each strain, the resistance phenotype profile was determined as
528 the concatenation of scores established for the 14 sets of fungicide conditions. In total, 132 different
529 resistance phenotype profiles were identified.
530

531 Statistical analysis of resistance phenotype profiles

532 The evolution of strains isolated from the 0C, 0B and 0P direct selection lines over the course of the
533 experiment was represented by heatmaps of 13-17 resistance phenotype profiles detected during
534 three to four critical time windows in resistance dynamics. The Euclidean pairwise distance was used
535 for the hierarchical clustering of these profiles, with dendrograms for the rows and columns. The
536 heatmap of the 102 resistance phenotype profiles detected at the end of the experiment in the 56
537 lines was produced in the same way. We also performed principal component analysis (PCA) on the
538 same data subsets.

539 Diversity within and between selection regimes, measured at the end of the experiment, was
540 quantified by an analysis of molecular variance (AMOVA) performed with the Arlequin program [76].
541 AMOVA is generally used for molecular data. Here, we assumed that different scores for a particular
542 test corresponded to different alleles or combination of alleles.
543 Finally, the number of AIs to which a line is resistant and the intensity of resistance (*i.e.* in each test
544 and for each line, the mean resistance score calculated from individual droplet scores greater than 0)
545 were calculated from the resistance phenotype profiles recorded at the end of the experiment. The
546 effects of AI number, alternation rhythm, alternation partner (and their interaction) on resistance to
547 tolnaftate, and the number of AIs to which a line was resistant and the intensity of resistance were
548 investigated with linear models (quasiPoisson GLM model, Poisson GLM and log-transformed linear
549 models, respectively).
550 All analyses and figures were produced with R 4.0.4 and the packages listed in **S1** Methods.
551

552 Sequencing, genotyping and gene expression of evolved 553 isolates

554 Target site resistance to B, C and P has been described in field strains of *Z. tritici* and is associated with
555 mutations of genes encoding some of the subunits of succinate dehydrogenase (*sdhB, sdhC* or *sdhD*),
556 β-tubulin (*tub2*) and 14α-demethylase (*cyp51*), respectively [34, 39, 40, 41]. In addition to the reference
557 isolates used in droplet tests, isolates from treated populations with scores for B, C or P resistance in
558 the droplet test was greater than one were retained for target gene sequencing. The target genes of
559 the fungicides concerned were amplified with primers, under the PCR conditions detailed in **S2**
560 Methods, and were sequenced with the Sanger protocol (by Eurofins Genomics, Ebersberg, Germany),
561 for single isolates representative of their resistance phenotype profile, chosen at random. DNA was
562 extracted from 141 individuals, and 65 *sdhB*, 69 *sdhC*, 66 *sdhD*, 83 *tub2* and 47 c*yp51* sequences were
563 obtained.

564 In order to identify possible overexpression of these target genes, their promoter regions were
565 amplified by PCR for the same isolates, searching for indels (**S3** Methods). Insertions into the promoter
566 of the gene *mfs1* have been shown to cause MDR in field isolates of *Z. tritici.* Amplicon size after PCR
567 for this gene was systematically determined for the 249 isolates, to check for any MFS1 promoter
568 alteration, with previously described protocols and reference strains [52]. At last, eight isolates exhibiting
569 high resistance scores to B, C, P and/or tolnaftate were chosen among the collection to represent
570 specialist or generalist phenotypes. Their RNA was isolated at the same time as that of the ancestral
571 isolate after a 6-day culture, as previously described, and without fungicide exposure. *sdhC, tub2,*
572 *cyp51* and *mfs1* expression was quantified for these isolates by qRT-PCR using *EF1α* and *UBC* as
573 housekeeping genes for expression normalization of *tub2, cyp51* and *mfs1* and *tub2* as a housekeeping
574 gene for the normalization of *sdhC* expression (**S3** Methods). The normalized expression of these genes
575 was shown as $2^{-\Delta\Delta Ct}$.

576

577 # Data availibity

578 Data were deposited on the Dryad repository under DOI
579 https://doi.org/10.11...01/2021.07.11.451819. Scripts were deposited on Zenodo under accession
580 https://doi.org/10.5281/zenodo.5106475.

581

582

583 # Supporting Information

584 **S1 Methods: Statistical programs used for data analysis**

585 **S2 Methods: Sequencing fungicide target genes in *Z. tritici***

586 **S3 Methods: Detecting polymorphism in promoters of target genes in *Z. tritici***

587 **S4 Methods: Quantifying the expression of *tub2*, *cyp51*, *sdhC*, *mfs1* in *Z. tritici* by qRT-PCRS1 Figure:**

588 **Variation of resistance evolution in selection regimes**

589 **S2 Figure: Expression of *tub2*, *sdhC*, *cyp51* and *mfs1* in evolved strains of *Z. tritici***

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Author contributions

ASW and FC conceived and designed the study, with contribution from AD and AB. AB performed the experimental evolution experiment. PD and CD isolated evolved strains and established their phenotypes and genotypes. AB and FC performed the statistical analysis, with contributions from ASW and AD. The paper was written by ASW and AB, with additional contributions from all authors.

Competing interests

The authors declare no competing interests.

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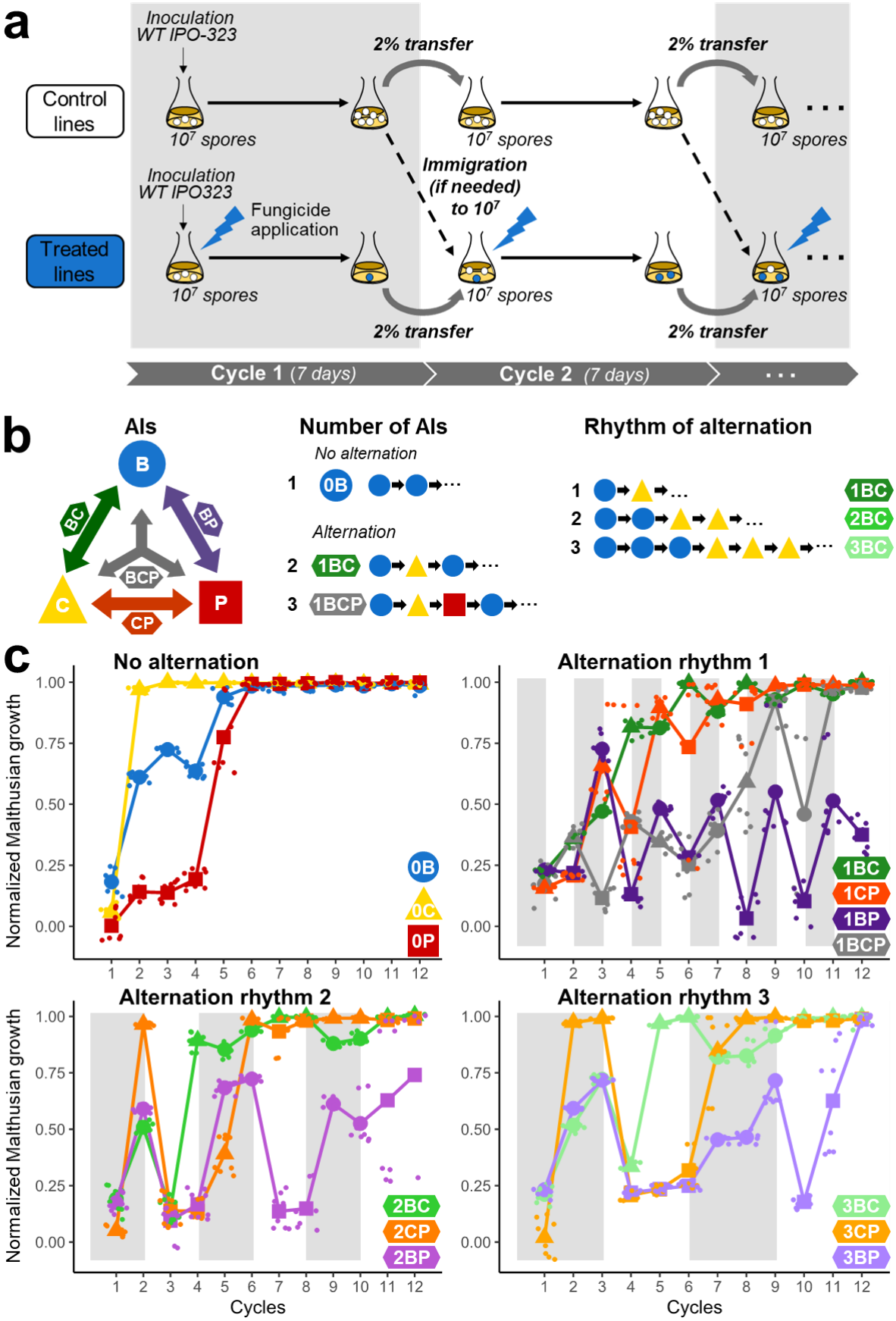

a
Inoculation
WT IPO-323
2% transfer
2% transfer
Control lines
10^7 spores
10^7 spores
10^7 spores
Immigration
(if needed)
to 10^7
Inoculation
WT IPO323
Fungicide
application
Treated lines
10^7 spores
10^7 spores
10^7 spores
2% transfer
2% transfer
Cycle 1 (7 days)
Cycle 2 (7 days)
b
AIs
B
BC
BP
BCP
C
CP
P
Number of AIs
No alternation
1 0B
Alternation
2 1BC
3 1BCP
Rhythm of alternation
1 1BC
2 2BC
3 3BC
c
No alternation
Alternation rhythm 1
Alternation rhythm 2
Alternation rhythm 3
Normalized Malthusian growth
0B
0C
0P
1BC
1CP
1BP
1BCP
2BC
2CP
2BP
3BC
3CP
3BP
Cycles

841

842 **Figure 1. Experimental evolution of fungicide resistance under treatments with contrasting temporal**
843 **heterogeneities.** (**a**) The sensitive strain IPO-323 of Z. *tritici* was used to found 56 lines, with each
844 Erlenmeyer flask inoculated with 10^7 spores. Fungicides were added to the treated lines to mimic 14
845 patterns of selection. After seven days, 2% of the population was used to inoculate fresh medium to
846 start the next cycle, supplemented, if necessary, by immigration from the untreated control line to
847 achieve a total of 10^7 spores. This procedure was repeated for 12 cycles in four replicate lines per
848 fungicide treatment. (**b**) Three fungicides and all the possible combinations (as described by the arrows
849 and their specific labels and colors) of two and three fungicides were studied: carbendazim (C; yellow;
850 high risk), benzovindiflupyr (B; blue; moderate risk), prothioconazole-desthio (P; red; low risk). For
851 each pair of fungicides, three alternation rhythms were tested (1, 2 or 3, depending on the number of
852 cycles with the same selection). (**c**) Some fungicide alternations effectively attenuated resistance
853 dynamics. For each treated line, normalized Malthusian growth, estimated at the end of the cycle, is
854 shown as a function of time. Colors indicate the treated lines and symbols (triangle, circle and square)
855 indicate the fungicide applied (C, B and P, respectively) during a given cycle. For each cycle and
856 selection regime, these large symbols represent the mean of observations, with individual
857 observations shown as light dots. Overall, resistance evolved and became established within six cycles
858 in lines with continuous exposure, whereas establishment in lines subjected to alternating exposure
859 was either delayed or had not yet been reached after 12 cycles (1BP, 2BP).

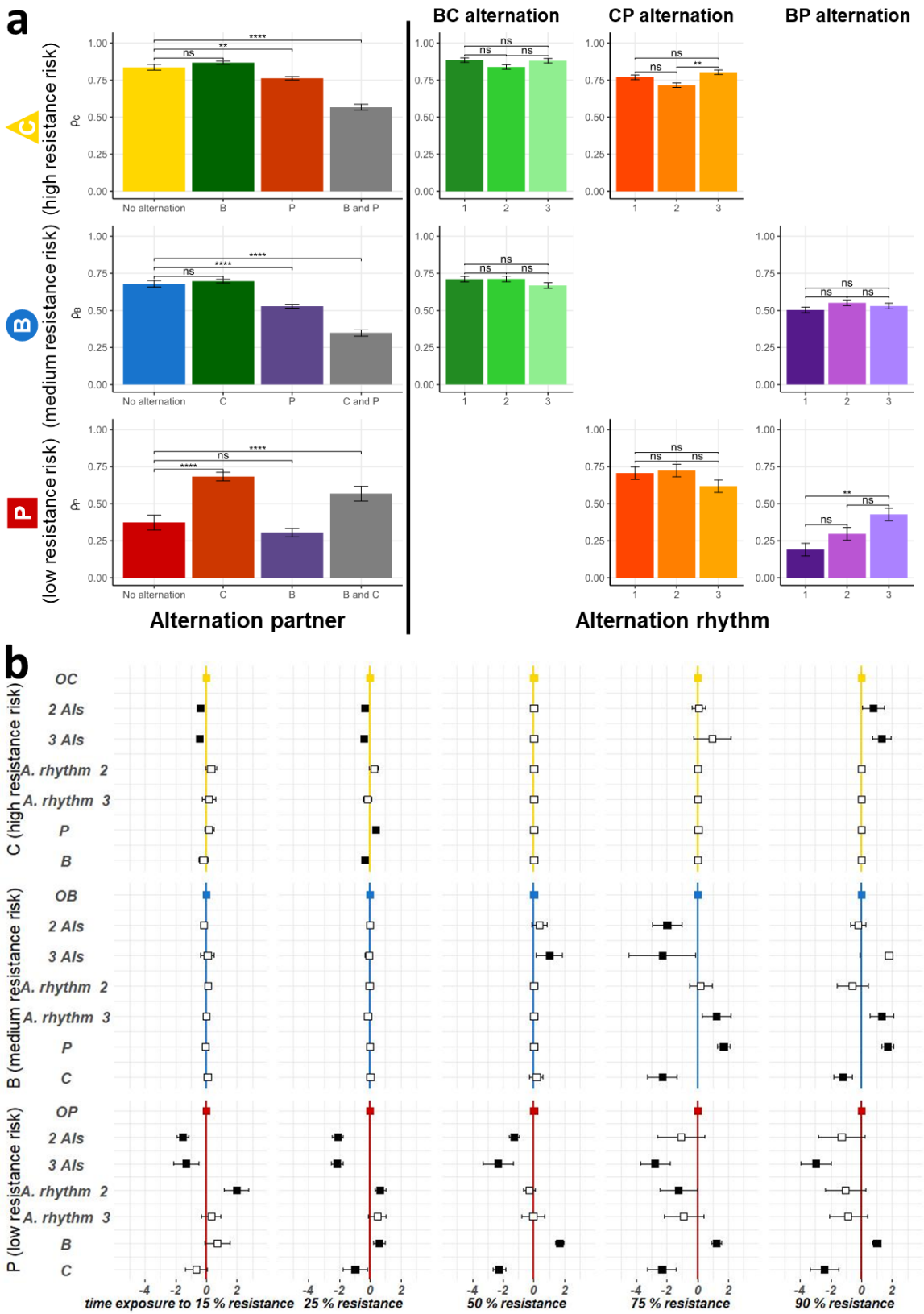


Figure 2. Effects of alternation components on the evolution of resistance. (a) ρ_f, the mean rate of resistance evolution for time of exposure to fungicide *f,* is shown as a function of alternating AIs on the

left, and for each alternation pair, as a function of alternation rhythm on the right. The error bars represent the standard error. *P*-values for pairwise comparisons were calculated with linear models (Tukey's *post-hoc* correction). "ns" not significant ($P > 0.05$), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Alternation decreased resistance evolution relative to continuous exposure, except for the low-risk fungicide (P) in alternation with the high-risk fungicide (C). Fast alternations (low rhythm) are recommended, even if the effect of rhythm alternation is often weak. (**b**) The effect of alternation components on Malthusian growth M_{it}^{n} is shown as the exposure time required to reach several thresholds. These results were estimated by Cox analysis and are expressed as cycles of fungicide *f* exposure gained or lost to reach these critical values relative to the lines continuously exposed to *f*. From top to bottom: C, B and P. Squares represent the estimated effects and errors bars their 95% interval. Black and white squares correspond to significant and non-significant effects, respectively. Alternation had a positive effect on Malthusian growth (*i.e.* the time taken to reach a particular threshold was greater in lines subjected to alternating regimes), mostly during the established-resistance phase (resistance >90%), regardless of the alternation partner for the high-risk fungicide C, but depending on alternation partner for B and P. For the low-risk fungicide P, the effect of alternation on Malthusian growth was negative (*i.e.* alternation with partners with a higher resistance risk decreased the time taken to reach thresholds) during the early phases of alternation.

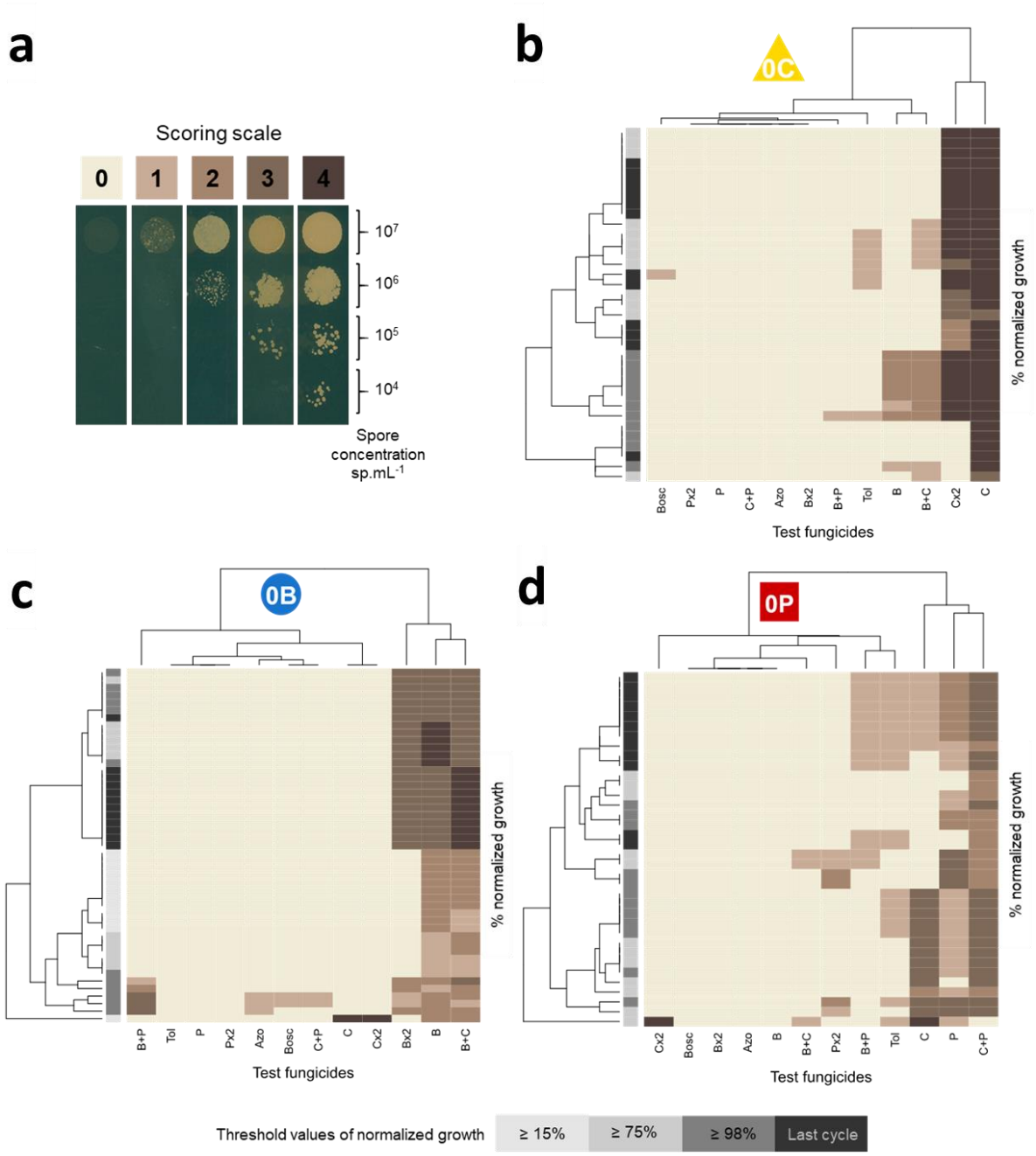
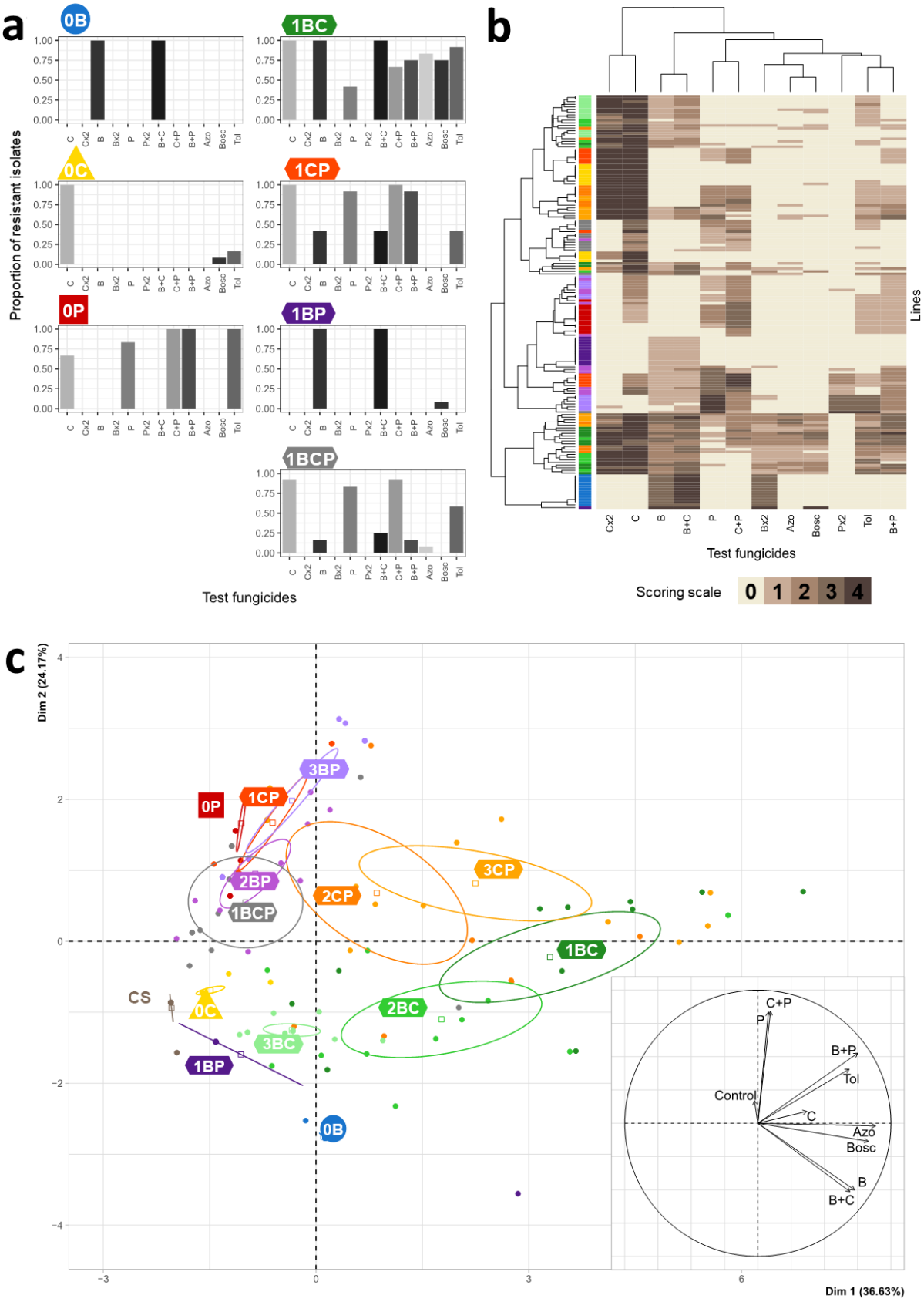


Figure 3. Evolution of phenotype resistance profiles during experimental evolution. (**a**) Scoring scale used to assess the growth of isolates on solid medium supplemented with discriminatory doses of fungicides (droplet tests). For each isolate, four spore concentrations were tested on the same plate. 0: no growth (susceptible isolate). 1-4: growth varying with spore concentration (resistant isolate). The fungicides tested included B, C and P at their selection doses, at twice this dose and in mixtures, as well as fungicides not used in the experiment (boscalid, azoxystrobin, tolnaftate). Tolnaftate was used

887 to identify isolates displaying multidrug resistance due to enhanced efflux. (**b**), (**c**) and (**d**) Heatmaps
888 of phenotype resistance profiles scored for isolates collected during experimental evolution, and for
889 lines continuously exposed to C, B and P, respectively. For each line and fungicide test, the resistance
890 rating scores (0-4; represented by the brown scale) of 12 isolates collected from the four replicates are
891 shown for three to four critical values of spore concentration in lines subjected to alternating regimes
892 relative to control lines (≥15% final frequency; represented by the gray scale). The structure of the
893 lines under continuous B, C or P selection revealed significant changes between these different stages
894 of resistance evolution (AMOVA; $0.273<F_{st}<0.453$; $P<0.001$).
895


a
0B
1BC
0C
1CP
0P
1BP
1BCP
Proportion of resistant isolates
Test fungicides
b
Lines
Test fungicides
Scoring scale
0 1 2 3 4
c
Dim 2 (24.17%)
Dim 1 (36.63%)
3BP
0P
1CP
2BP
1BCP
2CP
3CP
1BC
CS
0C
2BC
3BC
1BP
0B
C+P
P
B+P
Tol
Control
C
Azo
Bosc
B
B+C

896

897 **Figure 4. Phenotypic resistance profiles selected at the end of experimental evolution** (**a**) The average
898 phenotypic resistance profile obtained with the different selection regimes (*i.e.* after continuous or
899 alternating exposure; alternation rhythm = 1 cycle) at the end of the experiment. Diagrams show the
900 proportion of isolates resistant (rating score >0) to the test fungicides among the 12 collected from the
901 four replicate lines of each selection regime. The test and scores are described in the legend to Fig **3**.
902 (**b**) Heatmap of the phenotypic resistance profiles at the end of experimental evolution. The resistance
903 rating scores (0-4; represented by the brown scale) are shown for each of the 12 isolates collected for
904 each of the 13 selection regimes (4 replicate lines per regime; represented by the rainbow scale, as
905 described in Fig **1**) and for each fungicide tested. The structure of the lines differed significantly
906 between selection regimes (AMOVA; F_{st}=0.455, P<0.001). Alternation favored generalist phenotypes:
907 the number of AIs to which a line is resistant (excluding tolnaftate) increased with the number of AIs
908 used in the selection regime (χ^2=36.5; df=2; P=10^{-8}) and was also dependent, to a lesser extent, on
909 alternation partner. Similarly, resistance to tolnaftate, an indicator of multidrug resistance in several
910 pathogens, increased with the number of AIs used in the selection regime (χ^2=4.91; df=2; P=8 x 10^{-3})
911 and alternation rhythm (χ^2=3.85; df=2; P=0.022). The intensity of resistance, assessed with rating
912 scores, was significantly affected by all components of alternation. In particular, alternations including
913 the medium- and low-risk fungicides decreased the intensity of resistance (F=70.17; df=1,225; P=6 x
914 10^{-15} and F=57.06; df=1; P=10^{-12}, respectively). By contrast, alternation rhythm and the number of AIs
915 used in the selection regime slightly increased the intensity of resistance (F=4.91; df=2; P=0.008 and
916 F=3.85; df=2; P=0.02, respectively). (**c**) PCA on the phenotypic resistance profiles for all lines at the end
917 of the experiment showed structuring according to independent AIs (not used for selection during the
918 evolution experiment; Azo and Bosc) and according to the low-risk fungicide (P). CS: control solvent
919 lines.