Delayed but generalist resistance in alternation: a trade-off shaped by the mode of action of antifungals, as revealed by experimental evolution in a plant pathogen.

Short title: Delayed but generalist resistance in alternation

Agathe Ballu[1], Philomène Despréaux[1], Clémentine Duplaix[1], Anne Dérédec[1], Florence Carpentier[2,3¶], Anne-Sophie Walker[1*¶]

[1]Université Paris-Saclay, INRAE, AgroParisTech, UMR BIOGER, 78850 Thiverval-Grignon, France

[2]Université Paris-Saclay, INRAE, UR MalAGE, 78350 Jouy-en-Josas, France

[3]AgroParisTech, 75005 Paris, France

[¶]These authors contributed equally to this work

*Materials and correspondence requests should be addressed to Dr Anne-Sophie Walker ✉ anne-sophie.walker@inrae.fr

ORCID numbers:

Florence Carpentier: https://orcid.org/0000-0001-6204-2220

Anne-Sophie Walker: https://orcid.org/0000-0002-1246-246X

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 the temporal alternation of active ingredients 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. Here, we applied an original experimental evolution approach to the economically important crop pathogen *Zymoseptoria tritici.* We observed the dynamics of *Z. tritici* resistance in 56 independent lines subjected to 14 continuous or alternation regimes of 3 fungicides contrasting for their mode of action, at their EC_{95} selection doses. For the first time in a plant pathogen, our results show that alternation is either neutral or slows the evolution of resistance, relative to continuous fungicide use, but results in higher levels of generalism in evolved lines. We demonstrate that the mode of action of resistance of fungicides drivingly underlies this trade-off, more so than the number of fungicides and the frequency of alternation. This trade-off is also dynamic over the course of resistance evolution, as shown by the change in population phenotype structure and the relative impact of selection drivers. These findings open up new possibilities for tailoring resistance management effectively while optimizing smart interplay between alternation components. They also confirm experimental evolution as an untapped but promising approach to dissect adaptation in phytopathogenic fungi.

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

Author summary

The efficacy of pesticides has been compromised by the generalization of their use, leading to the rapid and widespread evolution of resistance. This constitutes a major economic and environmental burden in agriculture. The temporal alternation (or cycling) of active ingredients is a management strategy that induces temporal variation of selection of pathogens. Here, we dissected how it can be optimized according to the number and nature of alternated modes of action and to the rhythm of their application and sought to understand how these drivers determine the performance of alternation strategies. We used an approach original in plant pathology, experimental evolution, applied on an economically important fungus, *Zymoseptoria tritici*. We concluded that alternation can delay the rate of resistance selection with performance depending most likely on the mode of action of alternated antifungals, more so than on other drivers. But we also highlighted that alternation regimes select generalist resistance, that is the ability to resist a large diversity of antifungals. This trade-off was dynamic over time. Our study provides new insights for the informed management of pesticides and the reduction of their side effects.

Introduction

Human societies face a number of crucial challenges, including obtaining sufficient feed, food, fuel, and fiber, and treating infectious diseases. The protection of crops, and of animal and human health still relies heavily on the use of pesticides and drugs to control diverse microbes and pests. However, the efficacy of these agents has been compromised by the generalization of their use, leading to the rapid and widespread evolution of resistance [1-4]. Resistance can be seen as phenotype optimization in response to the selection imposed by pesticides and drugs, and has provided many examples of contemporary adaptive evolution [5]. The costs engendered by the evolution of resistance in pathogens, arthropods and weeds amount to billions of US dollars each year [6, 7], and the development of resistance may lead to the loss of millions of lives [8, 9], and entail hidden costs, due to greater pesticide use [10] and impacts on biodiversity [11, 12]. The use of smart strategies for preventing and delaying the evolution of resistance is, thus, crucial.

Diverse agents, with different modes of action, are often available for controlling pests and pathogens. Strategies can therefore be developed in which these agents are skilfully deployed over space and time so as to limit the evolution of resistance [13, 14]. More precisely, the optimization of sequences, mixtures, alternation, mosaic or dose strategies for pesticides (and, indeed, for drugs in the medical context) involves maximizing the heterogeneity of the selection pressure exerted on pathogen and pest populations [15-19]. The alternation (also known as cycling, rotation, or periodic application) of unrelated active ingredients (AIs) was first proposed by Coyne in 1951[20] and provides multiple means of intergenerational killing, maximizing the probability of killing the offspring of resistant individuals [14]. The efficacy of alternation for preventing resistance remains a matter of debate, as discrepancies between modeling and empirical data have been noted, for control strategies for insects, weeds and pathogens [14, 21-23]. The biological traits of the organisms concerned 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

82 two approaches. However, the variability of the performance of alternation, and, indeed, of other
83 strategies, raises questions about which components of the strategy are the most relevant for
84 delaying the evolution of resistance. Indeed, the heterogeneity of selection in an alternation strategy
85 depends on how many AIs are alternated, the identity of the AIs concerned, and the pattern of
86 alternation adopted.
87 Another limitation is that strategies are mostly evaluated on the basis of their quantitative impact on
88 resistance evolution (*e.g.* resistance frequency, proportion of healthy crop or patients, time to reach
89 a certain amount of resistance, effective life of the pesticide or drug). This may be because the
90 genetic basis of resistance is rarely known, in empirical studies, or made explicit, or because
91 mathematical models assume the resistance trait to be specialist [24, 25]. Nevertheless, the diversity
92 of resistance mechanisms observed in microbes, animals and plants is considerable. The evolutionary
93 origins of these mechanisms may also differ [26]. Target site resistance (TSR) affects the structure
94 and/or expression of the target of the pesticide or drug, resulting in cross-resistance to AIs with the
95 same mode of action. This type of resistance is considered to be a specialist adaptation. By contrast,
96 non-target site resistance (NTSR) involves the regulated expression of one or multiple genes involved
97 in AI transport, bypass, detoxification, efflux or sequestration. This generally leads to cross-resistance
98 to AIs with different modes of action and is usually considered a generalist trait [27-30]. Theory, with
99 support from experimental evolution experiments, suggests that specialists evolve in homogeneous
100 environments, whereas generalists are more likely to evolve in heterogeneous habitats [31]. Thus, in
101 terms of adaptation to pesticides and drugs, we would expect generalist resistance to be more
102 readily selected with increasing heterogeneity of selection pressure, for any given strategy. This
103 hypothesis has been validated for herbicide mixtures, for which increasing use was found to be
104 associated with lower levels of TSR and higher levels of NTSR (*i.e.* detoxification) in the economically
105 important weed blackgrass, in a national survey in the UK [32]. In the clinical environment, generalist
106 resistance has also been shown to be more frequently selected in situations in which antibiotics are
107 alternated [33]. Overall, these studies highlight the importance of treatment frequency relative to

108 the pathogen life cycle [34, 35]. The question of the efficacy of alternation strategies thus remains
109 unresolved for pesticides, as treatment frequency may result in the interval between treatments
110 being much longer than the generation times of pests, particularly for some pathogens.
111 Here, we use the ascomycete fungus *Zymoseptoria tritici* — the cause of septoria leaf blotch (STB),
112 the most devastating foliar disease of wheat in Europe [36, 37] — to determine how the components
113 of fungicide alternation drive the quantitative and qualitative performance of this strategy. *Z. tritici*
114 has evolved resistance in the field to four different unisite modes of action in Western Europe,
115 resulting in a decrease in field efficacy depending on the strategy deployed, with contrasting
116 resistance dynamics in France [38-43]. The resistance of *Z. tritici* is determined by various specific
117 target-site mutations, but also by overexpression [44-47] or enhanced efflux, a generalist mechanism
118 causing weak multidrug resistance (MDR) when not associated to TSR and affecting at least three
119 modes of action [45, 48]. We made use of the haploid yeast-like easily-culturable form of this
120 pathogen [49], in an experimental evolution approach [50] based on the selection of resistance
121 under regimes mimicking alternation. The selection patterns were designed such that three
122 components of alternation — the intrinsic risk of resistance to AIs, the number of AIs alternated and
123 the alternation rhythm — could be disentangled, to assess their respective impacts on resistance
124 dynamics and on the resistance mechanisms selected. We found that the interplay between
125 alternation components clearly guided the evolutionary trade-off between the qualitative and
126 quantitative performances of alternation, opening up new perspectives for the informed tailoring of
127 this strategy in the field, for the first time in a phytopathogenic fungus.
128

129 Results

130 Fungicide alternation has a neutral-to-beneficial effect, 131 slowing the evolution of resistance

132 We observed the dynamics of *Z. tritici* resistance to benzovindiflupyr (B), carbendazim (C) and
133 prothioconazole-desthio (P), here representing three modes of action (SDHIs, benzimidazoles and
134 DMIs, respectively), after experimental evolution in 56 independent lines subjected to 14 regimes of
135 continuous or alternating B, C and P, including B, C or P at their EC_{95} selection doses (Fig **1a** and **1b**).
136 Mean normalized Malthusian growth M_{it}^{n} (referred to hereafter as the global resistance evolution
137 rate ρ) was used as a proxy for increasing resistance. Resistance evolved in all populations
138 continuously exposed to B, C or P, and was generalized after three, six and six cycles, respectively.
139 Resistance also evolved in lines treated with these three fungicides in alternation, regardless of the
140 alternation rhythm and the number of fungicides alternated, although resistance never generalized,
141 even after 12 cycles, in some situations (Fig **1c**). For lines undergoing direct selection (with a single
142 compound), ρ was 1.1 times higher with C than with B and 1.4 times higher with C than with P, these
143 significant differences reflecting the hierarchy of the relative intrinsic risks of resistance associated
144 with benzimidazoles (high; C), SDHIs (medium; B) and DMIs (low; P) (**S1** Fig). The global resistance
145 evolution rates of the lines were modified by alternation regimes, being significantly lower, by a
146 factor of 1.1-1.3, for alternation regimes containing carbendazim than for continuous exposure to
147 this high-risk fungicide. Alternation thus reduced the risk of resistance to carbendazim to a level
148 similar to that observed for medium- and low-risk fungicides in conditions of continuous exposure.
149 The benefits of alternation were even more pronounced, with a 25-58% decrease in resistance
150 selection (as estimated by ρ) when low- and medium-risk fungicides were alternated or when
151 fungicides of the three categories were alternated. Thus, depending on the fungicides alternated,
152 alternation had either a neutral effect or decrease the evolution of resistance relative to single
153 fungicides in continuous use.
154

155 The risk of resistance inherent to the fungicide is the key
156 driver for tailoring alternation

157 Alternation may reduce the selection of resistance to a particular fungicide by limiting the total time
158 of exposure to this fungicide, but also by modifying the global resistance evolution rate ρ, potentially
159 through the diversification of selection pressure. We investigated the potential effect of multi-
160 directional selection in alternation, separating this effect from that of the decrease in exposure time,
161 by comparing ρ only for time segments corresponding to fungicide *f* exposure (ρ_f), and by alternation
162 component (Fig **2a**). The impact of alternation on the rate of selection of resistance to a given
163 fungicide was significantly dependent on the intrinsic risk of resistance associated with the
164 alternating partner. Indeed, the selection of resistance to the high-risk fungicide was decreased (by
165 9%) only if the fungicide concerned was alternated with the low-risk fungicide. Only alternation with
166 the low-risk fungicide was able to decrease (by 22%) the selection pressure associated with the use
167 of the medium-risk fungicide. Medium- and high-risk alternation partners had neutral or detrimental
168 (+82%) impacts, respectively, on the rate of selection of resistance to the low-risk fungicide. Overall,
169 the selection capacity of a fungicide was decreased only by alternation with a fungicide of sufficiently
170 lower risk. Thus, decreases in selection pressure in alternation were always achieved at the expense
171 of the selection capacity of the partner with the lower intrinsic risk of resistance. This conclusion also
172 extended to strategies involving an alternation of three fungicides. The duration of continuous
173 exposure to the same fungicide (alternation rhythm over the cycles) had a lesser effect, increasing
174 resistance evolution rate only for the longest sequences (three cycles) and for only two of the 18
175 possible comparisons. Finally, the rate of evolution of resistance to a particular fungicide was driven
176 principally by the inherent resistance risk of its alternation partner relative to its own resistance risk,
177 and secondarily, by other alternation components, such as the number of fungicides alternated and
178 the alternation rhythm.
179

180 Resistance selection differs over the course of evolution,
181 reflecting contrasting phenotype-adaptive landscapes

182 Changes in the frequency of resistance in a population typically follow sigmoid dynamics, with an
183 emergence phase of low resistance (<15%; here, the lowest frequency reliably detectable by
184 measuring the change in OD_{405} in our experimental conditions), a selection phase (here, resistance
185 rates between 15% and 90%) and a generalization phase (here, over 90%)[17]. Cox survival analysis
186 was used to investigate the effect of alternation components on the exposure time (number of cycles
187 with a given fungicide) required to reach a given threshold of Malthusian growth (Fig **2c**). Alternation
188 decreased the resistance evolution rate of the high-risk fungicide mostly towards the end of the
189 selection phase and during the generalization phase, rather than during the early phases (delaying
190 the generalization phase by 0.77 and 1.31 cycles for alternations of two and three AIs, respectively).
191 For the medium- and low-risk fungicides, this beneficial delaying of the generalization phase was
192 observed only in the case of alternation with the partner of lowest possible risk (delayed by 1.70 and
193 1.01 exposure cycles, respectively), with the high-risk partner decreasing the time required to reach
194 the generalization phase (by 1.22 and 2.42 exposure cycles, respectively). By contrast, alternation
195 with a partner with a higher resistance risk generally increased to the rate of selection of resistance
196 to the low-risk fungicide, particularly during the emergence and early selection phases (by 1.52 to
197 2.13 exposure cycles).
198 The relative selection pressure exerted by each fungicide during alternation therefore varied not only
199 according to the selection regime and its components, but also according to the phase of resistance
200 dynamics. This contrasting pattern of selection over time was consistent with the exploration of
201 adaptive landscapes [51], *i.e.* the successive selection of different mutations increasing fitness over
202 the course of the experiment, to adjust to the overall selection pressure. We therefore analyzed the
203 change in population structure over time in regimes under continuous exposure (lines 0B, 0C and 0P;
204 Fig **3**), to obtain proof-of-concept. As expected, resistance profiles were generally narrow and mostly
205 focused on resistance to the fungicide used for continuous selection. However, phenotypic resistance
206 profiles differed significantly between different resistance dynamics stages. In AMOVA analyses,
207 within-selection regime diversity was always greater than between-selection regime diversity, but

208 was concentrated throughout evolution, in a manner that varied with the fungicide exerting the
209 selection pressure. The high-risk fungicide (C) ultimately drove the selection of four highly specialized
210 profiles (resistant to a mean of 1.25 AIs). By contrast, the medium- and low-risk fungicides (B and P)
211 selected multiple resistance phenotypes, differing in terms of both cross-resistance and resistance
212 intensity. The medium-risk fungicide selected patterns of resistance to a mean of 2 AIs. The patterns
213 selected by the low-risk fungicide were the most generalist (resistance to a mean of 4 AIs) and often
214 included resistance to tolnaftate, an indicator of multidrug resistance in several pathogens [45].
215

216 Fungicide alternation preferentially selected for multiple or 217 generalist resistance mechanisms

218 The effect of the anticipated greater selection heterogeneity of alternation regimes was investigated
219 at the end of the experiment, by establishing the phenotypic resistance profile of the evolved
220 individuals in each line. The profiles of strains evolving under direct selection regimes were generally
221 narrower and less diverse than those of strains evolving under alternation regimes (Fig **4a** and **4b**).
222 PCA confirmed this finding (Fig **4c**), with direct selection clusters being more concentrated than any
223 other and well separated from those displaying resistance to fungicides not used for selection (*e.g.*
224 boscalid, another SDHI from a different chemical class and azoxystrobin and tolnaftate, with different
225 modes of action). The diversity of the lines was further explained by resistance to the low-risk
226 fungicide (P), which structured the second axis of the PCA. The width of multiple or cross-resistance
227 increased significantly with the number of AIs used in the selection regime and depended, to a lesser
228 extent, on the alternation partner. Resistance to tolnaftate, reflecting multidrug resistance, was
229 significantly more frequent in lines treated with alternating fungicide regimes with a long alternation
230 rhythm. The intensity of resistance was significantly decreased by alternations including medium-
231 and low-risk fungicides but increased slightly with increasing number of AIs in the selection regime
232 and with long alternation rhythms.

233 The broader resistance profiles of strains subjected to alternation regimes is potentially consistent
234 with the selection of multiple resistance (*i.e.* the co-selection, in the same strain, of several
235 mutations, each conferring resistance to a single fungicide) and/or generalist resistance (*i.e.* the
236 selection of pleiotropic mutations, associated with more general resistance to fungicides). We
237 initially tested these hypotheses by sequencing the genes encoding the targets of carbendazim,
238 prothioconazole and benzovindiflupyr in 141 isolates corresponding to the various phenotypic
239 resistance profiles. Only the E198K substitution in β-tubulin was detected in a few isolates evolving
240 under direct carbendazim selection. This target site mutation has been reported to confer a high
241 degree of resistance to benzimidazoles in field isolates of 12 fungal species, and in nematodes [52,
242 53]. No other change was recorded relative to the 330 full-gene sequences established for *tub2,*
243 *cyp51, sdhB, sdhC* or *sdhD.* Enhanced efflux is a non-target site resistance mechanism associated to
244 low RFs *per se* but causing cross-resistance to unrelated compounds in *Z. tritici* [45]. In field strains, it
245 is determined by insertions in the promoter region of the MFS1 transporter gene [54]. Systematic
246 genotyping of the 249 isolates collected in this experiment revealed no changes in the length of the
247 *mfs1* promoter, particularly in strains displaying tolnaftate resistance. Overall, these findings suggest
248 that alternation components favor generalist phenotypes, with enhanced efflux due to unknown
249 transporters and/or mutations more frequently observed in this context, possibly in addition to other
250 unknown non-target site resistance mechanisms.

251

252 # Discussion

253 This study confirms the value of experimental evolution as an approach for investigating conceptual
254 issues in temporal adaptation, such as the factors affecting the adaptive differentiation of local
255 populations [50, 55, 56]. Here, we used an experimental evolution approach and the economically
256 important wheat pathogen *Z. tritici* to demonstrate that temporal heterogeneity can mitigate the

257 evolution of resistance to fungicides in lines subjected to treatment with fungicide alternation
258 regimes. These experiments clearly highlight the relationships between the components of the
259 temporal heterogeneity of fungicide selection and both resistance dynamics and the generalism or
260 specialism of the evolved isolates. With this experimental design, we were able to validate, in a
261 filamentous plant pathogen *in vivo*, the theoretical assumptions that temporal heterogeneity of
262 selection can quantitatively modulate adaptation [14, 21] and mediate generalism in evolved
263 individuals [31, 57], as previously demonstrated for weeds, insects and bacteria [16, 33, 58-61].
264 Specifically, using three different fungicides, we found that alternation was either neutral or
265 beneficial in terms of delaying global resistance, relative to the continuous use of a single fungicide,
266 whatever its resistance risk. In all cases, alternation strategies performed at least as well as the
267 continuous use of the lower risk fungicide. Alternation may delay the build-up of resistance to a
268 particular fungicide because the selection pressure exerted by the fungicide is spread over a longer
269 period than in a sequence strategy, and fitness cost may help to decrease the frequency of resistance
270 during the time window in which the fungicide is not used [25]. However, this would suggest that
271 resistance evolution rate due to exposure to a given fungicide (ρ_f) is similar for continuous-use and
272 alternation strategies, and independent of alternation partner and alternation rhythm. By contrast,
273 during our dissection of the impact of alternation components, we obtained variable ρ_f values and
274 revealed the primary influence of the fungicide resistance risk, determined by its mode of action,
275 relative to alternation partners, on selection and the overall performance of a strategy. Thus,
276 increasing the number of AIs in the alternation may either increase or decrease the resistance
277 evolution rate of a fungicide, depending on the intrinsic risk of resistance for the added AI relative to
278 those already present in the alternation. Finally, the temporal heterogeneity of environments may
279 determine the direction of evolution, with more fine-grained temporal environments (in which the
280 environment varies over time scales shorter than the generation time) being more efficient at
281 delaying adaptation while maintaining population diversity [62, 63]. In our design, even the one-
282 week alternation rhythm greatly exceeded the generation time of *Z. tritici* (estimated at 6-7

283 generations per cycle), so all the temporal environments tested were coarse-grained, particularly the
284 regimes with a three-week alternation. However, alternation rhythm had a much lesser impact than
285 other components on the resistance evolution rate of a given fungicide. This implies that some
286 flexibility is possible in the design of field strategies, as the frequency of fungicide sprays is likely to
287 create coarse-grained environments in many agrosystems.
288 We confirm here, for the first time in a plant pathogenic fungus, that temporal variation in fungicide
289 application may be correlated with a higher degree of generalism in resistant strains. An analysis of
290 resistance phenotype profiles in the course of direct selection (continuous use of the same fungicide)
291 revealed high within-line phenotype diversity (data not shown) and the concentration of this
292 diversity over time, culminating in specialist profiles, for which the degree of specialism depended on
293 the AI. This dynamics is consistent with the continual change in population structure, with the rise,
294 selection, fixation or extinction of beneficial mutations, depending on their relative fitness costs and
295 the degree of resistance in the local population [64]. High-risk fungicides can thus explore smooth
296 fitness landscapes, such as those generated by the selection of a unique change underlying high
297 resistance (*e.g.* the tub2 E198K variant conferring high resistance to C). By contrast, medium- and
298 low-risk fungicides can explore less smooth landscapes. According to theory, the global fitness
299 optimum is less likely to be achieved in variable environments [65]. Combining the respective paths
300 associated with each AI, we would then expect lines evolving under alternation regimes to explore
301 rugged landscapes, constraining resistance mutations to one fungicide with trade-offs in resistance
302 to other fungicides [33, 66]. This scenario is consistent with the fixation of multiple discrete
303 resistance mechanisms, together with the accumulation of relative fitness costs associated with each
304 resistant trait, with possibly additivity or synergy, as suggested by the wider patterns of cross-
305 resistance (including resistance to new AIs), lower resistance scores and higher frequency of
306 resistance to tolnaftate (associated with enhanced efflux) observed in isolates collected from lines
307 subjected to alternating treatments. Our collection therefore provides a good opportunity to make
308 use of the progress in genomics to elucidate unknown genetic and non-genetic bases of adaptation,

309 which may also operate *in natura*, without our suspecting it and which may, therefore, have an
310 overlooked impact on resistant traits [67-69]. Indeed, a genomic analysis has shown that not only
311 SNPs but also indels, copy number variants, transposable element insertions, chromosome
312 duplications and aneuploidy occur in fungal pathogens exposed to a sublethal dose of fungicides [70].
313 We also found that alternation components had different impacts on the rate of resistance evolution
314 over the course of the experiment. Alternation significantly delayed the stabilization phase of the
315 dynamics of resistance to the high-risk fungicide, suggesting a higher relative fitness cost in the final
316 stages of adaptation than for the other fungicides. By contrast, alternation with high-risk fungicides
317 decreased the time required for the early stages of resistance to be reached for a low-risk fungicide,
318 possibly because the high-risk fungicide was more efficient at controlling mutations with a low
319 resistance intensity.
320 Finally, experimental evolution has proposed a simplified model for evolution *in natura* [56]. Due to
321 the laboratory environment, our experimental design assumes a finite population size, low ancestral
322 diversity, limited time scale and asexual reproduction. However, many plant pathogens, including *Z.*
323 *tritici,* have large population sizes, high levels of genetic diversity and both sexual and asexual
324 reproduction [71]. Gene flow is also expected to occur between evolving metapopulations. Beyond
325 these limitations, our study clearly demonstrates that not all alternation strategies guarantee the
326 same performance for preventing resistance, but in no instance did these strategies increase the risk
327 of resistance relative to that associated with the continuous use of individual fungicides. Efforts to
328 educate stakeholders about alternation strategies should, therefore, focus on optimizing the
329 interplay between the various components of alternation. The results of this study pave the way for
330 the effective tailoring of resistance management for plant pathogens based on temporal
331 heterogeneity. We would also argue that resistance management *via* temporal heterogeneity is
332 possible provided that the environmental grain is appropriate [63]. The environment should
333 generally be fine-grained, requiring alternating generations to be exposed to compounds with a
334 different mode of action, but we found that coarse-grained temporal fluctuations were potentially

335 acceptable. Furthermore, the order in which the AIs are cycled may affect the trajectory of evolution.
336 Modeling suggests that the most effective AI should be used first [25], to control emerging mutations
337 as efficiently as possibly, but further explorations in experimental evolution experiments would be
338 required to confirm this. The overall performance of alternation approaches including AIs with
339 different inherent resistance risks is achieved at the expense of the Ais with the lowest risk of
340 resistance, suggesting that the use of high-risk AIs in alternation sequences should be limited and/or
341 that the alternation of AIs of similar risk should be preferred. Finally, the design of rational strategies
342 with optimized components should take into account the evolutionary trade-off between the rate of
343 resistance evolution and the degree of generalism of the evolved individuals, which may call into
344 question the efficacy of future modes of action. In this respect, the future control of weeds, pests
345 and diseases should be based not only on a diversity of modes of action, but should consider
346 integrated pest management including non-chemical strategies to mitigate overall adaptation [72].
347
348

349 Methods

350 Ancestral population, culture conditions and assessment

351 We used IPO-323, as an ancestral isolate, to found all lines. This isolate is susceptible to all fungicides
352 and was the source of the high-quality reference genome for *Z. tritici* (Goodwin et al., 2011). The
353 stock spore suspension was kept at -80°C in 20% glycerol and gently defrosted at room temperature
354 for the inoculation of solid YPD plates (20 $g.L^{-1}$ dextrose, 20 $g.L^{-1}$ peptone, 10 $g.L^{-1}$ yeast extract, 20
355 $g.L^{-1}$ agar; USBiological), which were then incubated at 18°C in the dark for seven days. These plates
356 were used to prepare precultures in liquid YPD (as previously described, but without agar), which
357 were shaken at 150 rpm in similar conditions for seven days before the start of the experiment.
358 The culture medium used for experimental evolution was liquid YPD supplemented with 100 $mg.L^{-1}$
359 streptomycin and penicillin. The culture vessels were 50 mL borosilicate Erlenmeyer flasks containing

360 25 mL medium, with carded cotton wool inserted into the neck. Cultures were grown in the dark at
361 18°C, with a RH of 70% RH and shaking at 150 rpm during the duration of the cycle. In these
362 conditions, *Z. tritici* can be kept in its yeast-like form, making it possible to obtain homogeneous
363 liquid cultures [73].
364 Population size was routinely determined by measuring optical density at 405 nm (or OD_{405}) for two
365 200 µL replicates of the fungal culture in a 96-well microtiter plate (Sarstedt) sealed with a gas-
366 permeable membrane (Breathe Easy®; Diversified Biotech). The detection threshold of the
367 spectrophotometer (SpectraMax M2, Molecular Devices) was 3.5 x 10^5 sp.mL^{-1} in the experimental
368 conditions used. The OD_{405} of the fungal cultures was normalized against that of the non-amended
369 medium. Spore concentration was estimated from the OD_{405} with a mathematical model calibrated
370 on the basis of preliminary experiments in our conditions with serial dilutions and hemocytometer-
371 based spore quantification, and cross-validated (R^2=0.91). Population size in sp.mL^{-1} was used in
372 further statistical analyses.
373

374 Fungicides and selection regimes

375 In the experimental evolution experiments, we selected resistance to three fungicides representative
376 of different modes of action: carbendazim (C; benzimidazole, interfering with β-tubulin assembly in
377 mitosis), benzovindiflupyr (B; pyrazole carboxamide, inhibiting complex II, succinate dehydrogenase,
378 in the mitochondrial respiratory chain) and prothioconazole-desthio (P; triazole, inhibiting the sterol
379 14α-demethylase during sterol biosynthesis), the active metabolite of prothioconazole. These modes
380 of action are associated to contrasting resistance risk. The fungicides were dissolved in 80% ethanol.
381 The concentration of this solvent never exceeded 0.5% of the final culture volumes.
382 The selection doses were chosen by examination of dose-response curves for the ancestral strain. For
383 each fungicide dose, 10^7 spores of IPO-323 were used to inoculate YPD in an Erlenmeyer flask, which
384 was then incubated for seven days before OD_{450} measurement. OD_{405} data were modelled by logistic

385 regression, to calculate, for each fungicide, the EC_{50} (concentration inhibiting 50% of growth), EC_{95}
386 (concentration inhibiting 95% of growth) and MIC (minimum inhibitory concentration) values. We
387 finally chose to use the EC_{95} as the selection dose, as 99% inhibition was not achieved for some
388 fungicides, for which the MIC would exceed fungicide solubility in the solvent. The selection doses
389 used were 0.5 $mg.L^{-1}$ for benzovindiflupyr, 0.1 $mg.L^{-1}$ for carbendazim and 0.005 $mg.L^{-1}$ for
390 prothioconazole-desthio.
391 Selection regimes were organized for analysis of the respective impacts of three components of
392 alternation on resistance evolution, as described in Fig **1b**. First, in treated lines, the number of active
393 ingredients (AIs) in the alternation regime ranged from one (direct selection) to three different
394 fungicides alternated over time. Second, the intrinsic risk of resistance differed between MoAs and
395 was predicted to be high for benzimidazoles (represented by C), moderate to high for SDHIs
396 (represented by B) and moderate for DMIs (represented by P) by agrochemical companies[74]. In
397 practice, as we observed similar relative ranking of these fungicides in our experiments, we referred
398 to high-, medium- and low-risk fungicides, respectively, throughout this work, for the sake of
399 simplicity. Finally, the duration of exposure to the same fungicide (or alternation rhythm) was
400 continuous for direct selection (0, no alternation) and ranged from 1 (fungicide changed every cycle)
401 to 3 (fungicide changed every three cycles) cycles. All combinations of these three possible
402 components were tested, except for the three-fungicide alternation, which was tested only for an
403 alternation rhythm of one cycle.
404

405 Design of the experimental evolution experiment

406 The ancestral population was founded from the seven-day liquid YPD preculture of IPO-323. The
407 density of this spore suspension was adjusted to 2 x 10^7 $sp.mL^{-1}$ and each Erlenmeyer flask was
408 inoculated with 10^7 spores (500 µL). The resulting starting concentration of 4 x $10^5$$spores.mL^{-1}$
409 offered a compromise between large populations in which mutations were likely to occur and not

410 exceeding the culture carrying capacity in the experimental conditions used, based on the findings of
411 preliminary experiments. Each of the 14 selection regimes (1 control and 13 different treatments)
412 was repeated four times (four lines per selection regime). Control lines were not treated with
413 fungicides. Control solvent lines received 0.5% solvent, whereas treated lines were treated as shown
414 in Fig **1b**, giving rise to 56 independent lines.
415 Experimental evolution was conducted over 12 cycles of seven days each (about six to seven
416 generations per cycle). This cycle duration corresponds approximately to the time for which a control
417 line remained in the exponential growth phase before reaching a plateau. OD_{405} was measured at the
418 end of each cycle and transformed into population size, as described above. The OD_{405} of the treated
419 lines was normalized with the mean OD_{405} of the control solvent lines. At each transfer, 500 µL of the
420 evolving culture (*i.e.* 2% of the total volume of medium) was transferred to a new Erlenmeyer flask
421 containing fresh medium. If the number of spores in the 500 µL of culture medium was less than 10^7,
422 which would be the case before the evolution of resistance, then the appropriate number of spores
423 from one of the untreated populations was added to make the number of spores present up to 10^7.
424 The starting population size was equalized between the lines at the beginning of each new cycle, and
425 the process mimicked the immigration occurring in field situations. For each of the four replicates,
426 the same source population was used for immigration throughout the experiment. In preliminary
427 experiments, immigration was found to be useful for preventing population extinction after weekly
428 bottlenecks and to minimize genetic drift.
429 At the end of each cycle, 2 mL of each line was mixed with glycerol (25%), frozen and stored at -80°C
430 for further analyses.
431

432 Statistical analysis of resistance evolution rate

433 The Malthusian growth of each line was calculated as

434 $$M_{it} = \ln\left(\frac{Spore\ concentration\ of\ line\ i\ at\ the\ end\ of\ cycle\ t}{Spore\ concentration\ of\ line\ i\ at\ the\ beginning\ of\ cycle\ t}\right)$$

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

436 For each selection regime, the mean Malthusian growth ("global resistance evolution rate" or ρ) is
437 the mean of M_{it}^{n} calculated over the 12 cycles, and constitutes a quantitative summary of the
438 increase in resistance of the four replicates over the entire experiment. Similarly, ρ_f reflects the
439 increase in resistance associated with a fungicide *f* during the alternation regime, taking into account
440 only the time segments during which this fungicide was used. This approach made it possible to
441 compare selection due to the same number of fungicide applications (6 applications, except for the
442 BCP regime for which there were only 4 applications), but in the context of different selection
443 regimes. ρ and ρ_f were explained in ANOVA linear models, initially considering the alternation
444 partners as qualitative fixed factors, and then the alternation rhythm for each alternation partner.
445 The reference used was the continuous application of the fungicide.
446 The exposure time (*i.e.* the number of cycles exposed to a given fungicide) required for the
447 normalized Malthusian growth M_{it}^{n} to reach certain thresholds (15%, 25%, 50%, 75%, 90%) was
448 investigated in a Cox survival analysis taking into account the number of AIs, the alternation partner
449 and the alternation rhythm.
450 All analyses and figures were produced with R 4.0.4 and the additional packages listed in **S1**
451 Methods.
452

453 Isolation and phenotyping of evolved individuals

454 We assessed the change in resistance phenotypes during the course of selection, by isolating pure
455 strains from direct selection regimes, at critical points in the resistance dynamics, *i.e.* when
456 normalized Malthusian growth reached 15% (early resistance), 75% (intermediate resistance
457 frequency) and 98% (generalization of resistance) relative to that of the control line, and at the end
458 of the experiment. We isolated 12 individuals (3 per replicate) per selection regime from cycles 1, 5,
459 6, 12 for 0B lines, from cycles 2, 3 and 12 for 0C lines, and from cycles 5, 6 and 12 for 0P lines. This

460 resulted in the isolation of 120 strains in total. We also explored phenotype diversity within and
461 between selection regimes, by systematically isolating 12 strains (3 per replicate) at the end of the
462 final cycle, for all selection regimes. This resulted in the isolation of 129 additional strains. Isolates
463 were retrieved from glycerol population stocks collected at the end of each cycle. Two successive
464 isolations were performed on YPD solid medium supplemented with 100 $mg.L^{-1}$ streptomycin and
465 penicillin. Single isolates were stored at -80°C in 25% glycerol.
466 The cross-resistance patterns of the 249 isolates were established in droplet tests. We deposited 10^7,
467 10^6, 10^5 and 10^4 $spores.mL^{-1}$ suspensions as drops organized into columns, in 12 cm square Petri
468 dishes. These plates contained YPD supplemented with discriminatory doses of fungicides. The 15
469 treatments tested encompassed a control with 0.5% solvent and fungicides B, C and P at their
470 selection doses (0.1, 0.5 and 0.005 $mg.L^{-1}$, respectively) and twice their selection doses (0.2, 1 and
471 0.01 $mg.L^{-1}$, respectively). Mixtures of pairs of the three fungicides were also included (each fungicide
472 used at 0.8 times its selection dose, as this was found to be the lowest discriminatory dose in
473 mixtures of the reference resistant and susceptible strains). Finally, for the detection of generalist
474 resistance, we also included fungicides not used for experimental evolution (0.5 $mg.L^{-1}$ azoxystrobin,
475 2 $mg.L^{-1}$ boscalid and 2 $mg.L^{-1}$ tolnaftate). Tolnaftate, in particular, identified individuals displaying
476 enhanced fungicide efflux leading to non-specific multidrug resistance (MDR), as described in field
477 isolates of *Z. tritici* [45]. The ancestral strain IPO-323, and three field strains, each resistant to one or
478 several of the fungicides used for selection, were included as control strains. After seven days of
479 incubation, drops were scored 0 or 1, according to the presence or absence of fungal growth. A total
480 score was established for each strain, ranging between 0 (susceptible) and 4 (resistant if >0),
481 according to the number of dilution droplets for which growth was observed. For each strain, the
482 resistance phenotype profile was determined as the concatenation of scores established for the 14
483 sets of fungicide conditions. In total, 132 different resistance phenotype profiles were identified.
484

485 **Statistical analysis of resistance phenotype profiles**

486 The evolution of strains isolated from the 0C, 0B and 0P direct selection lines over the course of the
487 experiment was represented by heatmaps of 13-17 resistance phenotype profiles detected during
488 three to four critical time windows in resistance dynamics. The Euclidean pairwise distance was used
489 for the hierarchical clustering of these profiles, with dendrograms for the rows and columns. The
490 heatmap of the 102 resistance phenotype profiles detected at the end of the experiment in the 56
491 lines was produced in the same way. We also performed principal component analysis (PCA) on the
492 same data subsets.
493 Diversity within and between selection regimes, measured at the end of the experiment, was
494 quantified by an analysis of molecular variance (AMOVA) performed with the Arlequin program [75].
495 AMOVA is generally used for molecular data. Here, we assumed that different scores for a particular
496 test corresponded to different alleles.
497 Finally, the number of AIs to which a line is resistant and the intensity of resistance (*i.e.* in each test
498 and for each line, the mean resistance score calculated from individual droplet scores greater than 0)
499 were calculated from the resistance phenotype profiles recorded at the end of the experiment. The
500 effects of AI number, alternation rhythm, alternation partner (and their interaction) on resistance to
501 tolnaftate, and the number of AIs to which a line was resistant and the intensity of resistance were
502 investigated with linear models (quasiPoisson GLM model, Poisson GLM and log-transformed linear
503 models, respectively).
504 All analyses and figures were produced with R 4.0.4 and the packages listed in **S1** Methods.
505

506 ## Genotyping of evolved isolates

507 One strain for each resistance phenotype profile was selected at random and kept for MFS1
508 genotyping. Insertions into the promoter of this gene have been shown to cause MDR in field isolates
509 of *Z. tritici.* DNA was isolated and amplicon size after PCR for this gene was systematically

510 determined for these 249 isolates, to check for any MFS1 promoter alteration, with previously
511 described protocols and reference strains [54].
512 Target site resistance to B, C and P has been described in field strains of *Z. tritici* and is associated
513 with mutations of genes encoding some of the subunits of succinate dehydrogenase (*sdhB, sdhC* or
514 *sdhD*), β-tubulin (*tub2*) and 14α-demethylase (*cyp51*), respectively [38, 40, 43, 44]. In addition to the
515 reference isolates used in droplet tests, isolates from treated populations with scores for B, C or P
516 resistance in the droplet test was greater than one were retained for target gene sequencing. The
517 target genes of the fungicides concerned were amplified with primers, under the PCR conditions
518 detailed in **S2** Methods, and were sequenced with the Sanger protocol (by Eurofins Genomics,
519 Ebersberg, Germany), for single isolates representative of their resistance phenotype profile, chosen
520 at random. DNA was extracted from 141 individuals, and 65 *sdhB*, 69 *sdhC*, 66 *sdhD*, 83 *tub2* and 47
521 c*yp51* sequences were obtained.

522

523 # Data availibity

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

Acknowledgments

We thank Fabrice Blanc for facilitating the administrative organization of this PhD studentship. We would also like to thank Dr. Stefano Torriani, Dr. Stephanie Bedhomme and Dr. Mato Lagator for their sound comments about our findings.

Funding

AB acknowledges PhD studentship funding from the French Ministry of Higher Education, Research and Innovation, and Syngenta France, through the CIFRE program, supervised by the National Association for Research and Technology (ANRT). This work was supported by the Plant Health division of INRAE through the STRATAGEME project.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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.

References

1. Gould F, Brown ZS, Kuzma J. Wicked evolution: Can we address the sociobiological dilemma of pesticide resistance? Science. 2018;360(6390):728-32. doi: 10.1126/science.aar3780.
2. McKenna M. Antibiotic resistance: the last resort. Nature. 2013;499(7459):394-6. Epub 2013/07/28. doi: 10.1038/499394a. PubMed PMID: 23887414.
3. Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. Science. 2018;360(6390):739-42. Epub 2018/05/19. doi: 10.1126/science.aap7999. PubMed PMID: 29773744.
4. Witzany C, Bonhoeffer S, Rolff J. Is antimicrobial resistance evolution accelerating? PLOS Pathogens. 2020;16(10):e1008905. doi: 10.1371/journal.ppat.1008905.
5. Tabashnik BE, Mota-Sanchez D, Whalon ME, Hollingworth RM, Carrière Y. Defining terms for proactive management of resistance to Bt crops and pesticides. Journal of Economic Entomology. 2014;107(2):496-507. doi: 10.1603/ec13458.
6. Frisvold GB, Bagavathiannan MV, Norsworthy JK. Positive and normative modeling for Palmer amaranth control and herbicide resistance management. Pest Management Science. 2017;73(6):1110-20. doi: https://doi.org/10.1002/ps.4537.
7. Palumbi SR. Humans as the world's greatest evolutionary force. Science. 2001;293(5536):1786-90. doi: 10.1126/science.293.5536.1786.
8. Ranson H, Lissenden N. Insecticide resistance in African *Anopheles* mosquitoes: A worsening situation that needs urgent action to maintain malaria control. Trends Parasitol. 2016;32(3):187-96. Epub 2016/02/02. doi: 10.1016/j.pt.2015.11.010. PubMed PMID: 26826784.
9. Price R. O'Neill report on antimicrobial resistance: funding for antimicrobial specialists should be improved. Eur J Hosp Pharm. 2016;23(4):245-7. doi: 10.1136/ejhpharm-2016-001013. PubMed PMID: 31156859.
10. Bourguet D, Guillemaud T. The hidden and external costs of pesticide use. In: Lichtfouse E, editor. Sustainable Agriculture Reviews: Volume 19. Cham: Springer International Publishing; 2016. p. 35-120.
11. Goulson D, Nicholls E, Botías C, Rotheray EL. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science. 2015;347(6229):1255957. doi: 10.1126/science.1255957.
12. Jepson PC, Murray K, Bach O, Bonilla MA, Neumeister L. Selection of pesticides to reduce human and environmental health risks: a global guideline and minimum pesticides list. The Lancet Planetary Health. 2020;4(2):e56-e63. doi: https://doi.org/10.1016/S2542-5196(19)30266-9.
13. Raymond B. Five rules for resistance management in the antibiotic apocalypse, a road map for integrated microbial management. Evol Appl. 2019;12(6):1079-91. doi: 10.1111/eva.12808.
14. REX_Consortium. Heterogeneity of selection and the evolution of resistance. Trends in Ecology & Evolution. 2013;28(2):110-8.
15. Vacher C, Bourguet D, Rousset F, Chevillon C, Hochberg ME. Modelling the spatial configuration of refuges for a sustainable control of pests: a case study of Bt cotton. Journal of Evolutionary Biology. 2003;16(3):378-87. doi: 10.1046/j.1420-9101.2003.00553.x. PubMed PMID: WOS:000182322200002.
16. Lagator M, Vogwill T, Colegrave N, Neve P. Herbicide cycling has diverse effects on evolution of resistance in *Chlamydomonas reinhardtii*. Evol Appl. 2013;6(2):197-206. doi: 10.1111/j.1752-4571.2012.00276.x.
17. van den Bosch F, Paveley N, Shaw M, Hobbelen P, Oliver R. The dose rate debate: does the risk of fungicide resistance increase or decrease with dose? Plant Pathology. 2011;60(4):597-606. doi: 10.1111/j.1365-3059.2011.02439.x. PubMed PMID: WOS:000292310900003.
18. van den Bosch F, Paveley N, van den Berg F, Hobbelen P, Oliver R. Mixtures as a fungicide resistance management tactic. Phytopathology. 2014;104(12):1264-73. doi: 10.1094/phyto-04-14-0121-rvw. PubMed PMID: WOS:000345149900001.
19. Baym M, Stone LK, Kishony R. Multidrug evolutionary strategies to reverse antibiotic resistance. Science. 2016;351(6268):aad3292. doi: 10.1126/science.aad3292.
20. Coyne FP. Proper use of insecticides. Br Med J. 1951;2(4736):911-2. PubMed PMID: PMC2070232.
21. Hobbelen PHF, Paveley ND, Oliver RP, van den Bosch F. The usefulness of fungicide mixtures and alternation for delaying the selection for resistance in populations of *Mycosphaerella graminicola* on winter wheat: A modeling analysis. Phytopathology. 2013;103(7):690-707. doi: 10.1094/phyto-06-12-0142-r.
22. van den Bosch F, Oliver R, van den Berg F, Paveley N. Governing principles can guide fungicide-resistance management tactics. Annual Review of Phytopathology. 2014;52(1):175-95. doi: doi:10.1146/annurev-phyto-102313-050158. PubMed PMID: 24848413.
23. Blanquart F. Evolutionary epidemiology models to predict the dynamics of antibiotic resistance. Evol Appl. 2019;12(3):365-83. doi: 10.1111/eva.12753. PubMed PMID: 30828361.
24. Birkegård AC, Halasa T, Toft N, Folkesson A, Græsbøll K. Send more data: a systematic review of mathematical models of antimicrobial resistance. Antimicrobial Resistance & Infection Control. 2018;7(1):117. doi: 10.1186/s13756-018-0406-1.

25. van den Bosch F, Gilligan CA. Models of fungicide resistance dynamics. Annual Review of Phytopathology. Annual Review of Phytopathology. 462008. p. 123-47.
26. Hawkins NJ, Bass C, Dixon A, Neve P. The evolutionary origins of pesticide resistance. Biological Reviews. 2019;94(1):135-55. doi: doi:10.1111/brv.12440.
27. R4P_Network. Trends and challenges in pesticide resistance detection. Trends in Plant Science. 2016;21(10):834-53. doi: 10.1016/j.tplants.2016.06.006.
28. Berman J, Krysan DJ. Drug resistance and tolerance in fungi. Nature Reviews Microbiology. 2020;18(6):319-31. doi: 10.1038/s41579-019-0322-2.
29. Corbel V, N'Guessan R. Distribution, mechanisms, impact and management of insecticide resistance in malaria vectors: pragmatic reviews. In: Manguin S, editor. Anopheles, Mosquitoes - new insights into malaria vectors: In Tech; 2013. p. 579-633.
30. Délye C. Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. Pest Management Science. 2013;69(2):176-87. doi: 10.1002/ps.3318.
31. Kassen R. The experimental evolution of specialists, generalists, and the maintenance of diversity. Journal of Evolutionary Biology. 2002;15(2):173-90. doi: 10.1046/j.1420-9101.2002.00377.x.
32. Comont D, Lowe C, Hull R, Crook L, Hicks HL, Onkokesung N, et al. Evolution of generalist resistance to herbicide mixtures reveals a trade-off in resistance management. Nature Communications. 2020;11(1):3086. doi: 10.1038/s41467-020-16896-0.
33. Kim S, Lieberman TD, Kishony R. Alternating antibiotic treatments constrain evolutionary paths to multidrug resistance. Proceedings of the National Academy of Sciences. 2014;111(40):14494-9. doi: 10.1073/pnas.1409800111.
34. Abel zur Wiesch P, Kouyos R, Abel S, Viechtbauer W, Bonhoeffer S. Cycling empirical antibiotic therapy in hospitals: Meta-analysis and models. PLoS Pathogens. 2014;10(6):e1004225. doi: 10.1371/journal.ppat.1004225.
35. Roemhild R, Barbosa C, Beardmore RE, Jansen G, Schulenburg H. Temporal variation in antibiotic environments slows down resistance evolution in pathogenic *Pseudomonas aeruginosa*. Evol Appl. 2015;8(10):945-55. doi: https://doi.org/10.1111/eva.12330.
36. Torriani SFF, Melichar JPE, Mills C, Pain N, Sierotzki H, Courbot M. *Zymoseptoria tritici*: A major threat to wheat production, integrated approaches to control. Fungal Genetics and Biology. 2015;79:8-12. doi: http://dx.doi.org/10.1016/j.fgb.2015.04.010.
37. Fones H, Gurr S. The impact of Septoria tritici blotch disease on wheat: An EU perspective. Fungal Genetics and Biology. 2015;79:3-7. doi: http://dx.doi.org/10.1016/j.fgb.2015.04.004.
38. Garnault M, Duplaix C, Leroux P, Couleaud G, Carpentier F, David O, et al. Spatiotemporal dynamics of fungicide resistance in the wheat pathogen *Zymoseptoria tritici* in France. Pest Management Science. 2019;75(7):1794-807. doi: 10.1002/ps.5360.
39. Garnault M, Duplaix C, Leroux P, Couleaud G, David O, Walker A-S, et al. Large-scale study validates that regional fungicide applications are major determinants of resistance evolution in the wheat pathogen *Zymoseptoria tritici* in France. New Phytologist. 2021;229(6):3508-21. doi: https://doi.org/10.1111/nph.17107.
40. Huf A, Rehfus A, Lorenz KH, Bryson R, Voegele RT, Stammler G. Proposal for a new nomenclature for CYP51 haplotypes in *Zymoseptoria tritici* and analysis of their distribution in Europe. Plant Pathology. 2018;67(8):1706-12. doi: 10.1111/ppa.12891.
41. Heick TM, Justesen AF, Jorgensen LN. Resistance of wheat pathogen *Zymoseptoria tritici* to DMI and QoI fungicides in the Nordic-Baltic region - a status. European Journal of Plant Pathology. 2017;149(3):669-82. doi: 10.1007/s10658-017-1216-7. PubMed PMID: WOS:000412127400012.
42. Jørgensen LN, Matzen N, Heick TM, Havis N, Holdgate S, Clark B, et al. Decreasing azole sensitivity of *Z. tritici* in Europe contributes to reduced and varying field efficacy. Journal of Plant Diseases and Protection. 2021;(128):287-301. doi: 10.1007/s41348-020-00372-4.
43. Dooley H, Shaw MW, Mehenni-Ciz J, Spink J, Kildea S. Detection of *Zymoseptoria tritici* SDHI-insensitive field isolates carrying the SdhC-H152R and SdhD-R47W substitutions. Pest Management Science. 2016;72(12):2203-7. doi: 10.1002/ps.4269.
44. Rehfus A, Strobel D, Bryson R, Stammler G. Mutations in *sdh* genes in field isolates of *Zymoseptoria tritici* and impact on the sensitivity to various succinate dehydrogenase inhibitors. Plant Pathology. 2018;67(1):175-80. doi: doi:10.1111/ppa.12715.
45. Leroux P, Walker A. Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. Pest Management Science. 2011;67(1):44-59. doi: 10.1002/ps.2028.
46. Cools HJ, Bayon C, Atkins S, Lucas JA, Fraaije BA. Overexpression of the sterol 14α-demethylase gene (MgCYP51) in *Mycosphaerella graminicola* isolates confers a novel azole fungicide sensitivity phenotype. Pest Management Science. 2012;68(7):1034-40. doi: 10.1002/ps.3263.
47. Cools HJ, Hawkins NJ, Fraaije BA. Constraints on the evolution of azole resistance in plant pathogenic fungi. Plant Pathology. 2013;62:36-42. doi: 10.1111/ppa.12128. PubMed PMID: WOS:000328446500006.
48. Omrane S, Sghyer H, Audéon C, Lanen C, Duplaix C, Walker A-S, et al. Fungicide efflux and the MgMFS1 transporter contribute to the multidrug resistance phenotype in *Zymoseptoria tritici* field isolates. Environmental Microbiology. 2015;17(8):2805-23. doi: 10.1111/1462-2920.12781.

49. Quaedvlieg W, Kema GHJ, Groenewald JZ, Verkley GJM, Seifbarghi S, Razavi M, et al. *Zymoseptoria* gen. nov.: a new genus to accommodate *Septoria*-like species occurring on graminicolous hosts. Persoonia. 2011;26:57-69. doi: 10.3767/003158511x571841. PubMed PMID: WOS:000292593800005.
50. Van den Bergh B, Swings T, Fauvart M, Michiels J. Experimental design, population dynamics, and diversity in microbial experimental evolution. Microbiology and Molecular Biology Reviews. 2018;82(3). doi: 10.1128/mmbr.00008-18.
51. Poelwijk FJ, Kiviet DJ, Weinreich DM, Tans SJ. Empirical fitness landscapes reveal accessible evolutionary paths. Nature. 2007;445(7126):383-6. doi: 10.1038/nature05451.
52. Dilks CM, Hahnel SR, Sheng QC, Long LJ, McGrath PT, Andersen EC. Quantitative benzimidazole resistance and fitness effects of parasitic nematode beta-tubulin alleles. International Journal for Parasitology-Drugs and Drug Resistance. 2020;14:28-36. doi: 10.1016/j.ijpddr.2020.08.003. PubMed PMID: WOS:000600604300004.
53. Hawkins NJ, Fraaije BA. Predicting resistance by mutagenesis: Lessons from 45 years of MBC resistance. Frontiers in Microbiology. 2016;7(1814):e1814. doi: 10.3389/fmicb.2016.01814.
54. Omrane S, Audéon C, Ignace A, Duplaix C, Aouini L, Kema G, et al. Plasticity of the *MFS1* promoter leads to multidrug resistance in the wheat pathogen *Zymoseptoria tritici*. mSphere. 2017;2(5). doi: 10.1128/mSphere.00393-17.
55. Kawecki TJ, Ebert D. Conceptual issues in local adaptation. Ecology Letters. 2004;7(12):1225-41. doi: 10.1111/j.1461-0248.2004.00684.x.
56. Kawecki TJ, Lenski RE, Ebert D, Hollis B, Olivieri I, Whitlock MC. Experimental evolution. Trends in Ecology & Evolution. 2012;27(10):547-60. doi: https://doi.org/10.1016/j.tree.2012.06.001.
57. Levins R. Evolution in changing environments. press PU, editor. Princeton, NJ1968.
58. Fardisi M, Gondhalekar AD, Ashbrook AR, Scharf ME. Rapid evolutionary responses to insecticide resistance management interventions by the German cockroach (*Blattella germanica* L.). Scientific Reports. 2019;9(1):8292. doi: 10.1038/s41598-019-44296-y.
59. Wang A, Singh A, Huang YH, Agrawal AF. Ecological specialization in populations adapted to constant versus heterogeneous environments. Evolution. 2019;73(6):1309-17. doi: 10.1111/evo.13725. PubMed PMID: WOS:000485479600018.
60. Santos-Lopez A, Marshall CW, Scribner MR, Snyder DJ, Cooper VS. Evolutionary pathways to antibiotic resistance are dependent upon environmental structure and bacterial lifestyle. eLife. 2019;8:e47612. doi: 10.7554/eLife.47612.
61. Fitzgerald DM. The road to resistance. eLife. 2019;8:e52092. doi: 10.7554/eLife.52092.
62. Roux F, Paris M, Reboud X. Delaying weed adaptation to herbicide by environmental heterogeneity: a simulation approach. Pest Management Science. 2008;64(1):16-29. doi: 10.1002/ps.1440.
63. Venail PA, Kaltz O, Olivieri I, Pommier T, Mouquet N. Diversification in temporally heterogeneous environments: effect of the grain in experimental bacterial populations. Journal of Evolutionary Biology. 2011;24(11):2485-95. doi: https://doi.org/10.1111/j.1420-9101.2011.02376.x.
64. Hawkins N, Fraaije B. Fitness penalties in the evolution of fungicide resistance. Annual Review of Phytopathology. 2018;56(1):339-60. doi: 10.1146/annurev-phyto-080417-050012. PubMed PMID: 29958074.
65. Collins S. Many Possible Worlds: Expanding the Ecological Scenarios in Experimental Evolution. Evolutionary Biology. 2011;38(1):3-14. doi: 10.1007/s11692-010-9106-3.
66. Obolski U, Ram Y, Hadany L. Key issues review: evolution on rugged adaptive landscapes. Reports on Progress in Physics. 2017;81(1):012602. doi: 10.1088/1361-6633/aa94d4.
67. Bailey SF, Bataillon T. Can the experimental evolution programme help us elucidate the genetic basis of adaptation in nature? Mol Ecol. 2016;25(1):203-18. Epub 2015/10/14. doi: 10.1111/mec.13378. PubMed PMID: 26346808.
68. Hu M, Chen S. Non-target site mechanisms of fungicide resistance in crop pathogens: A review. Microorganisms. 2021;9(3):502. PubMed PMID: doi:10.3390/microorganisms9030502.
69. Remigi P, Masson-Boivin C, Rocha EPC. Experimental evolution as a tool to investigate natural processes and molecular functions. Trends Microbiol. 2019;27(7):623-34. doi: 10.1016/j.tim.2019.02.003. PubMed PMID: WOS:000470969400008.
70. Gambhir N, Kamvar ZN, Higgins R, Amaradasa BS, Everhart SE. Spontaneous and fungicide-induced genomic variation in *Sclerotinia sclerotiorum*. Phytopathology. 2021;111(1):160-9. doi: 10.1094/phyto-10-20-0471-fi. PubMed PMID: 33320026.
71. Stukenbrock EH, McDonald BA. The origins of plant pathogens in agro-ecosystems. Annual Review of Phytopathology. 2008;46:75-100. doi: 10.1146/annurev.phyto.010708.154114. PubMed PMID: ISI:000259199300005.
72. REX_Consortium, (Bourguet D, Delmotte F, Franck P, Guillemaud T, Reboud X, et al. Combining selective pressures to enhance the durability of disease resistance genes. Frontiers in Plant Science. 2016;7(1916). doi: 10.3389/fpls.2016.01916.
73. Francisco CS, Ma X, Zwyssig MM, McDonald BA, Palma-Guerrero J. Morphological changes in response to environmental stresses in the fungal plant pathogen *Zymoseptoria tritici*. Scientific Reports. 2019;9(1):9642. doi: 10.1038/s41598-019-45994-3.
74. Brent JK, Hollomon DW, editors. Fungicide resistance: the assessment of risk. Brussels, Belgium: Croplife; 2007.
75. Excoffier L, Lischer HEL. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources. 2010;10(3):564-7. doi: 10.1111/j.1755-0998.2010.02847.x. PubMed PMID: WOS:000276407300020.

727 # Supporting Information

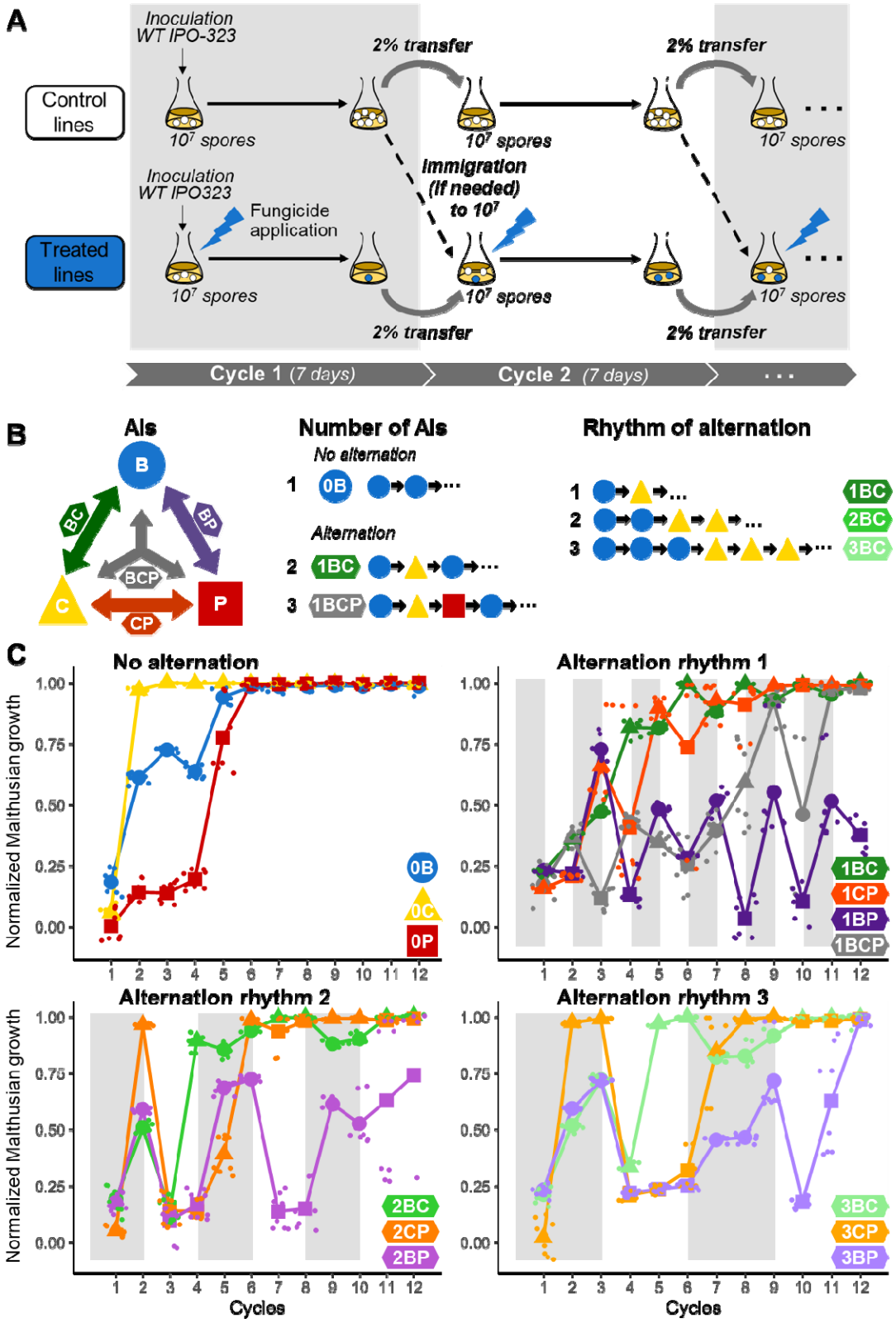
728 **S1 Methods: Statistical programs used for data analysis**

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

730 **S1 Figure: Variation of resistance evolution in selection regimes**

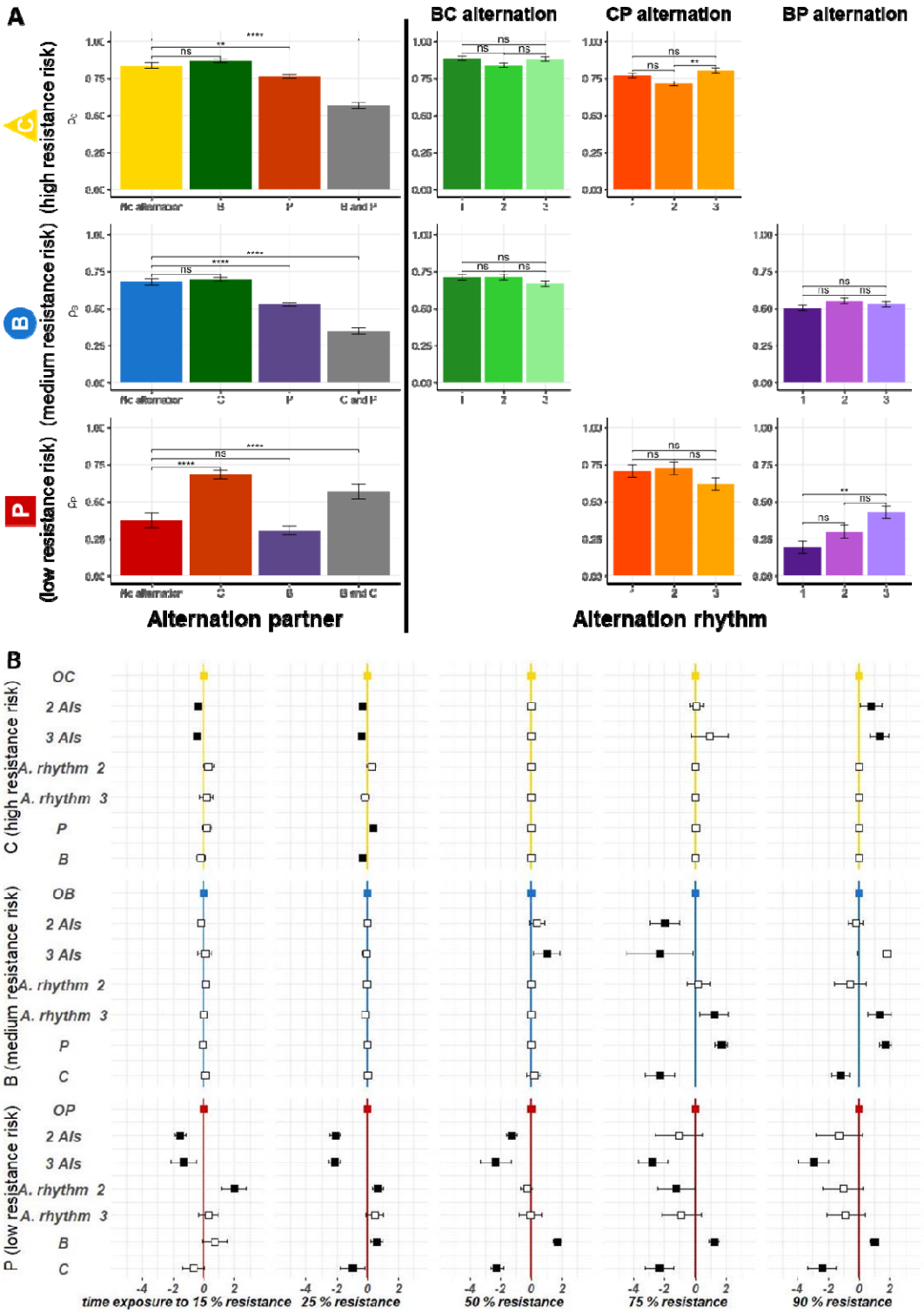
731

732 **Figure 1**

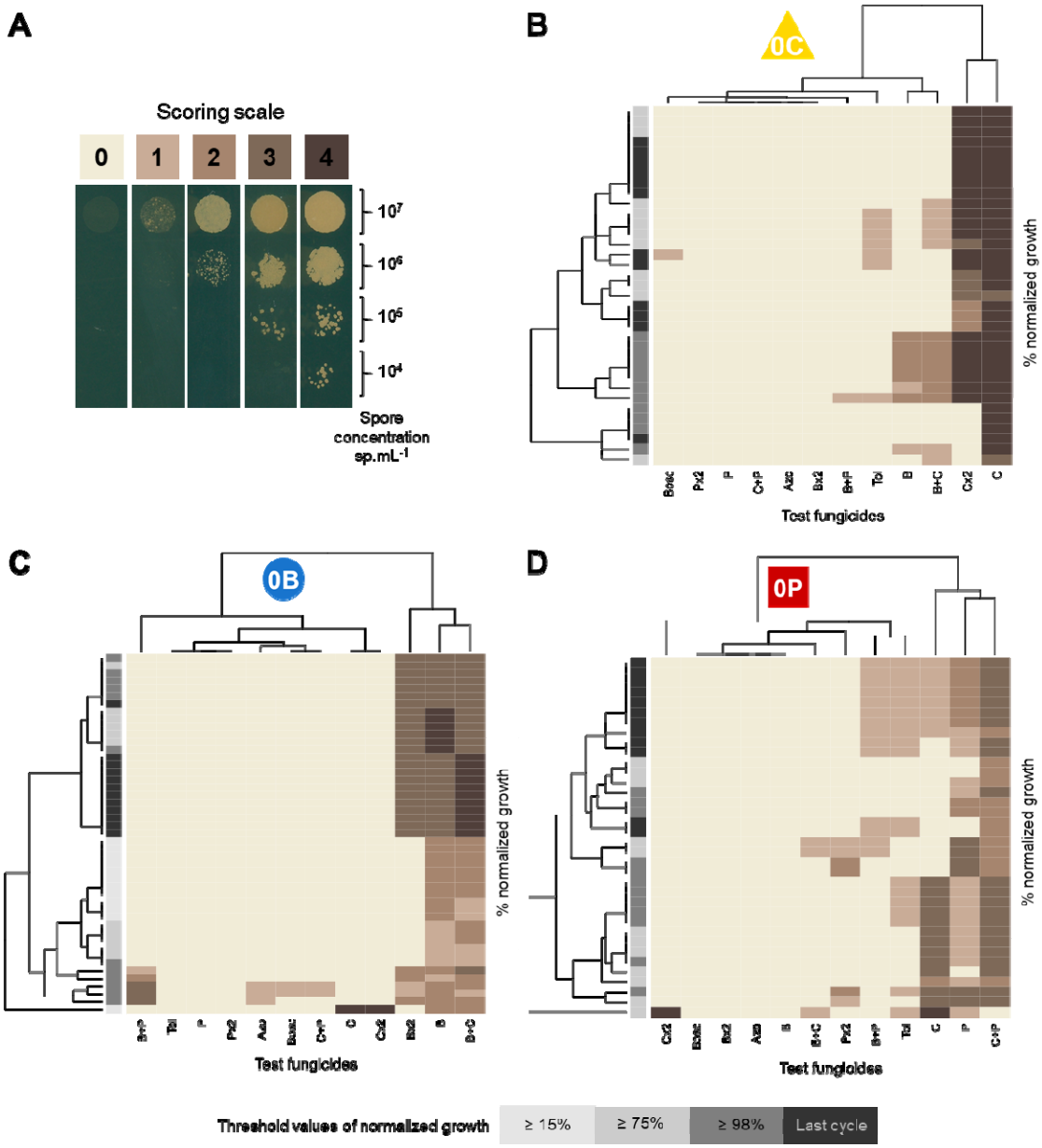


733

Figure 2


A
BC alternation
CP alternation
BP alternation
C (high resistance risk)
B (medium resistance risk)
P (low resistance risk)
Alternation partner
Alternation rhythm
B
OC
2 AIs
3 AIs
A. rhythm 2
A. rhythm 3
P
B
OB
C
OP
time exposure to 15 % resistance
25 % resistance
50 % resistance
75 % resistance
90 % resistance

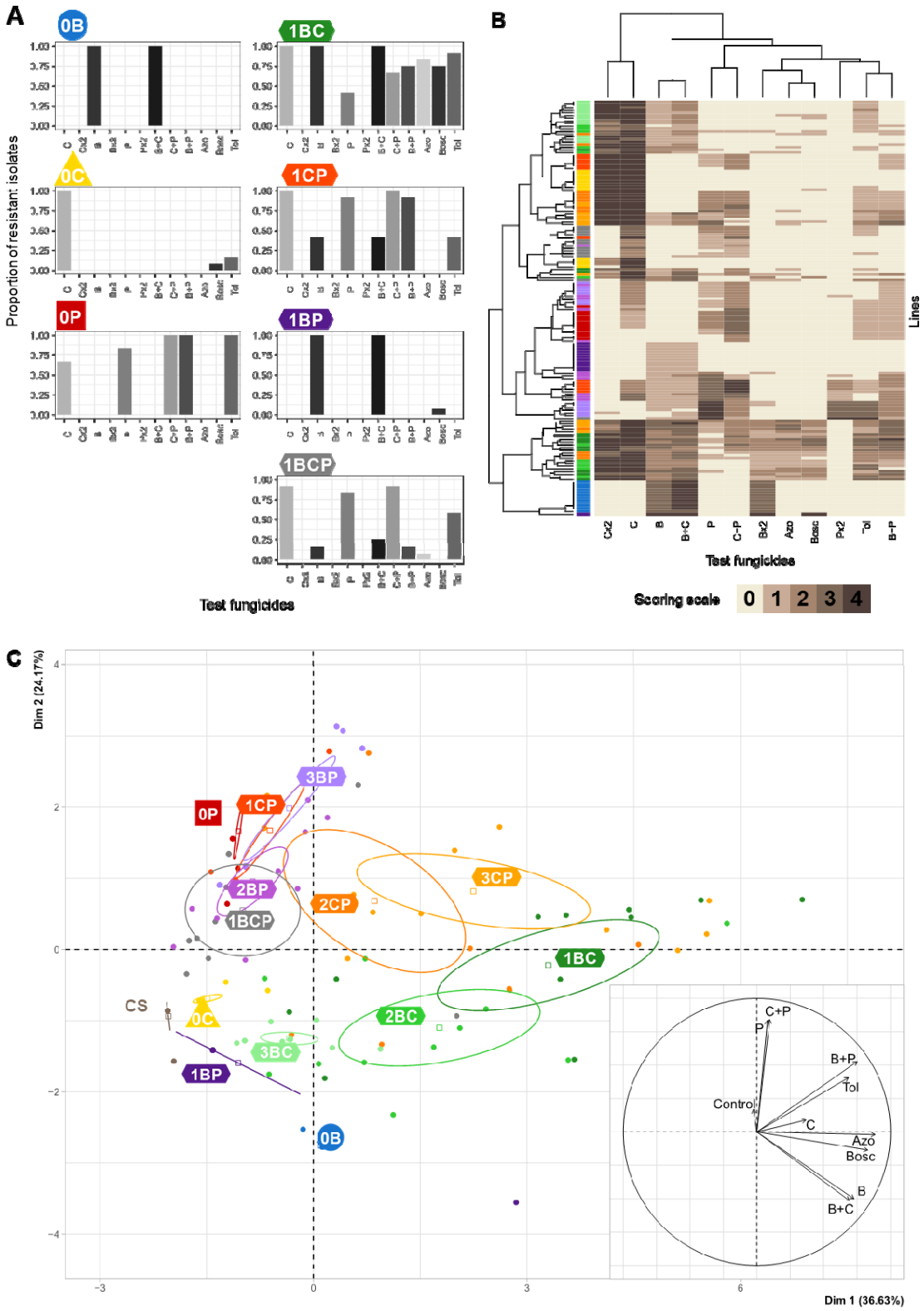
737 **Figure 3**



738

739

Figure 4


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

742 # Figure captions

743 **Figure 1. Experimental evolution of fungicide resistance under treatments with contrasting**
744 **temporal heterogeneities.** (**a**) The sensitive strain IPO-323 of Z. *tritici* was used to found 56 lines,
745 with each Erlenmeyer flask inoculated with 10^7 spores. Fungicides were added to the treated lines to
746 mimic 14 patterns of selection. After seven days, 2% of the population was used to inoculate fresh
747 medium to start the next cycle, supplemented, if necessary, by immigration from the untreated
748 control line to achieve a total of 10^7 spores. This procedure was repeated for 12 cycles in four
749 replicate lines per fungicide treatment. (**b**) Three fungicides and all the possible combinations of two
750 and three fungicides were studied: carbendazim (C; yellow; high risk), benzovindiflupyr (B; blue;
751 moderate risk), prothioconazole-desthio (P; red; low risk). For each pair of fungicides, three
752 alternation rhythms were tested (1, 2 or 3, depending on the number of cycles with the same
753 selection). (**c**) Some fungicide alternations effectively attenuated resistance dynamics. For each
754 treated line, normalized Malthusian growth, estimated at the end of the cycle, is shown as a function
755 of time. Colors indicate the treated lines and symbols (triangle, circle and square) indicate the
756 fungicide applied (C, B and P, respectively) during a given cycle. For each cycle and selection regime,
757 these large symbols represent the mean of observations, with individual observations shown as light
758 dots. Overall, resistance evolved and became generalized within six cycles in lines with continuous
759 exposure, whereas generalization in lines subjected to alternating exposure was either delayed or
760 had not yet been reached after 12 cycles (1BP, 2BP).

761

762 **Figure 2. Effects of alternation components on the evolution of resistance. (a)** ρ_f, the mean rate of
763 resistance evolution for time of exposure to fungicide *f,* is shown as a function of alternating AIs on
764 the left, and for each alternation pair, as a function of alternation rhythm on the right. The error bars
765 represent the standard error. *P*-values for pairwise comparisons were calculated with linear models
766 (Tukey's *post-hoc* correction). "ns" not significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$,

767 ***P < 0.001. Alternation decreased resistance evolution relative to continuous exposure, except
768 for the low-risk fungicide (P) in alternation with the high-risk fungicide (C). Fast alternations (low
769 rhythm) are recommended, even if the effect of rhythm alternation is often weak. (**b**) The effect of
770 alternation components on Malthusian growth M_{it}^{n} is shown as the exposure time required to reach
771 several thresholds. These results were estimated by Cox analysis and are expressed as cycles of
772 fungicide f exposure gained or lost to reach these critical values relative to the lines continuously
773 exposed to f. From top to bottom: C, B and P. Squares represent the estimated effects and errors
774 bars their 95% interval. Black and white squares correspond to significant and non-significant effects,
775 respectively. Alternation had a positive effect on Malthusian growth (*i.e.* the time taken to reach a
776 particular threshold was greater in lines subjected to alternating regimes), mostly during the
777 generalization phase (resistance >90%), regardless of the alternation partner for the high-risk
778 fungicide C, but depending on alternation partner for B and P. For the low-risk fungicide P, the effect
779 of alternation on Malthusian growth was negative (*i.e.* alternation with partners with a higher
780 resistance risk decreased the time taken to reach thresholds) during the early phases of alternation.

781

782 **Figure 3. Evolution of phenotype resistance profiles during experimental evolution.** (**a**) Scoring
783 scale used to assess the growth of isolates on solid medium supplemented with discriminatory doses
784 of fungicides (droplet tests). For each isolate, four spore concentrations were tested on the same
785 plate. 0: no growth (susceptible isolate). 1-4: growth varying with spore concentration (resistant
786 isolate). The fungicides tested included B, C and P at their selection doses, at twice this dose and in
787 mixtures, as well as fungicides not used in the experiment (boscalid, azoxystrobin, tolnaftate).
788 Tolnaftate was used to identify isolates displaying multidrug resistance due to enhanced efflux. (**b**),
789 (**c**) and (**d**) Heatmaps of phenotype resistance profiles scored for isolates collected during
790 experimental evolution, and for lines continuously exposed to C, B and P, respectively. For each line
791 and fungicide test, the resistance rating scores (0-4; represented by the brown scale) of 12 isolates
792 collected from the four replicates are shown for three to four critical values of spore concentration in

793 lines subjected to alternating regimes relative to control lines (≥15% final frequency; represented by
794 the gray scale). The structure of the lines under continuous B, C or P selection revealed significant
795 changes between these different stages of resistance evolution (AMOVA; $0.273<F_{st}<0.453$; $P<0.001$).
796
797 **Figure 4. Phenotypic resistance profiles selected at the end of experimental evolution** (**a**) The
798 average phenotypic resistance profile obtained with the different selection regimes (*i.e.* after
799 continuous or alternating exposure; alternation rhythm = 1 cycle) at the end of the experiment.
800 Diagrams show the proportion of isolates resistant (rating score >0) to the test fungicides among the
801 12 collected from the four replicate lines of each selection regime. The test and scores are described
802 in the legend to Fig **3**. (**b**) Heatmap of the phenotypic resistance profiles at the end of experimental
803 evolution. The resistance rating scores (0-4; represented by the brown scale) are shown for each of
804 the 12 isolates collected for each of the 13 selection regimes (4 replicate lines per regime;
805 represented by the rainbow scale, as described in Fig **1**) and for each fungicide tested. The structure
806 of the lines differed significantly between selection regimes (AMOVA; $F_{st}=0.455$, $P<0.001$).
807 Alternation favored generalist phenotypes: the number of AIs to which a line is resistant (excluding
808 tolnaftate) increased with the number of AIs used in the selection regime ($\chi^2=36.5$; df=2; $P=10^{-8}$) and
809 was also dependent, to a lesser extent, on alternation partner. Similarly, resistance to tolnaftate, an
810 indicator of multidrug resistance in several pathogens, increased with the number of AIs used in the
811 selection regime ($\chi^2=4.91$; df=2; $P=8 \times 10^{-3}$) and alternation rhythm ($\chi^2=3.85$; df=2; $P=0.022$). The
812 intensity of resistance, assessed with rating scores, was significantly affected by all components of
813 alternation. In particular, alternations including the medium- and low-risk fungicides decreased the
814 intensity of resistance (F=70.17; df=1,225; $P=6 \times 10^{-15}$ and F=57.06; df=1; $P=10^{-12}$, respectively). By
815 contrast, alternation rhythm and the number of AIs used in the selection regime slightly increased
816 the intensity of resistance (F=4.91; df=2; $P=0.008$ and F=3.85; df=2; $P=0.02$, respectively). (**c**) PCA on
817 the phenotypic resistance profiles for all lines at the end of the experiment showed structuring

818 according to independent AIs (not used for selection during the evolution experiment; Azo and Bosc)

819 and according to the low-risk fungicide (P). CS: control solvent lines.

820

Supplementary Information Methods S1: Statistical programs used for data analysis

All analyses were performed and all figures were generated with R 4.0.4 and R Studio 1.4.1106.

R Core Team. *R: A Language and Environment for Statistical Computing*. (R Foundation for Statistical Computing, 2021).

RStudio Team. *RStudio: Integrated Development Environment for R*. (RStudio, PBC, 2021).

The additional packages used for this work are listed below.

- **car**: Fox, J. & Weisberg, S. *An R Companion to Applied Regression*. (Sage, 2019).
- **cowplot:** Wilke, C. O. *cowplot: Streamlined Plot Theme and Plot Annotations for 'ggplot2'*. (2020).
- **coxed:** Kropko, J. & Harden, J. J. *coxed: Duration-Based Quantities of Interest for the Cox Proportional Hazards Model*. (2020).
- **emmeans**: Lenth, R. V. *emmeans: Estimated Marginal Means, aka Least-Squares Means*. (2021).
- **FactoMineR**: Lê, S., Josse, J. & Husson, F. FactoMineR: A Package for Multivariate Analysis. *Journal of Statistical Software* **25**, 1–18 (2008).
- **ggplot2:** Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*. (Springer-Verlag New York, 2016).
- **ggpubr:** Kassambara, A. *ggpubr: 'ggplot2' Based Publication Ready Plots*. (2020).
- **ggdendro:** Vries, A. de & Ripley, B. D. *ggdendro: Create Dendrograms and Tree Diagrams Using 'ggplot2'*. (2020).
- **Multcomp:** Hothorn, T., Bretz, F. & Westfall, P. Simultaneous Inference in General Parametric Models. *Biometrical Journal* **50**, 346–363 (2008).
- **nlme:** Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & R Core Team. *nlme: Linear and Nonlinear Mixed Effects Models*. (2021).

- **survival**: Therneau, T. M. *A Package for Survival Analysis in R*. (2020).

848 **Supplementary Information Methods S2: Sequencing fungicide target genes in *Z. tritici***

849

850 **1. Primers used to sequence fungicide target genes and to detect alterations of the *MFS1***

851 **promoter in *Z. tritici***

Primer name	**Primer sequence**	**Tm** (°C)	**Reference**
Tub2			
Tub5	CAGTCACTTGCAAACGGGTA (F)	63.7	This work
Tub14	GAAGCCTCCTGGTACTGCTG (R)	63.9	-
Tub15	GTCGACCAGGTTCTCGATGT (F)	64.1	-
Tub13	AACCGACCATGAAGAAGTGG (R)	63.8	-
cyp51			
CypFra2r	TCCTTCTCCTCCCTCCTCTC (R)	64	Brunner *et al* (2008)
EBI112	TTCAGCACGCTCGCCATCCTCC (F)	76.5	Brunner *et al* (2008)
Cyp9	TCTATACTGCCAGCCGATCA (R)	63.3	This work
Cyp12	CACTAACAGGCCAACTGCAA (F)	63.8	-
NB1	CACTCTTCATCTGCGACCGAGTC (R)	69.4	Leroux *et al* (2007)
sdhB			
Mg SDHB	TTCGGCACGTCGAGTTTCTGGCCATGCTGGCGG (F)	89.2	This work
Mg SDHB Rv	CCACAATGCCGCAAAATCGGCACAACTGCTCCC (R)	86.1	-
Mg SeqSDHB Fw	ACATCTACCGATGGAACCC (F)	61.2	-
Mg SeqSDHB Rv	TGTGGCATCGGTACAAGC (R)	63.5	-
sdhC			
Mg SDHC Fw	TCTCTTCGTCGACATCACCA (F)	64.7	This work
Mg SDHC Rv	ACGCACTCGCAACACTCA (R)	64.7	-
sdhD			

Mg SDHD Fw Mg SDHD Rv	ACAGCTTCCGAGGTTTCGCG (F) GCCATCTGTATATACACGCCC (R)	70.8 63.0	This work -
mfs1			
MFS1_consensus_2 MFS1_consensus_4	GCAAGGATTCGGACTTGACG (F) CTGCCGGTATCGTCGATGAC (R)	66.9 67.8	Omrane *et al* (2017) -

852

853 Brunner, P. C., et al. (2008). "Evolution of the CYP51 gene in *Mycosphaerella graminicola*: evidence for
854 intragenic recombination and selective replacement." Molecular Plant Pathology **9**(3): 305-316. Leroux, P., et
855 al. (2007). "Mutations in the CYP51 gene correlated with changes in sensitivity to sterol 14 alpha-
856 demethylation inhibitors in field isolates of *Mycosphaerella graminicola*." Pest Management Science **63**(7):
857 688-698.
858 Omrane, S., et al. (2017). "Plasticity of the *MFS1* promoter leads to multidrug resistance in the wheat pathogen
859 *Zymoseptoria tritici*." mSphere **2**(5).

860

861 **2. Regular PCR Mix**

Mix	1 tube (µL)
DNA	1
dNTP (25mM)	0.5
FW (10 µM)	1
RV (10 µM)	1
Titanium buffer (10X)	5
Titanium *Taq* polymerase (50X)	0.5
Deionized H_2O	41
Total volume	50

862 DNA polymerase: Titanium *Taq* (Takara Bio)

863

864

865 **3. Regular PCR program**

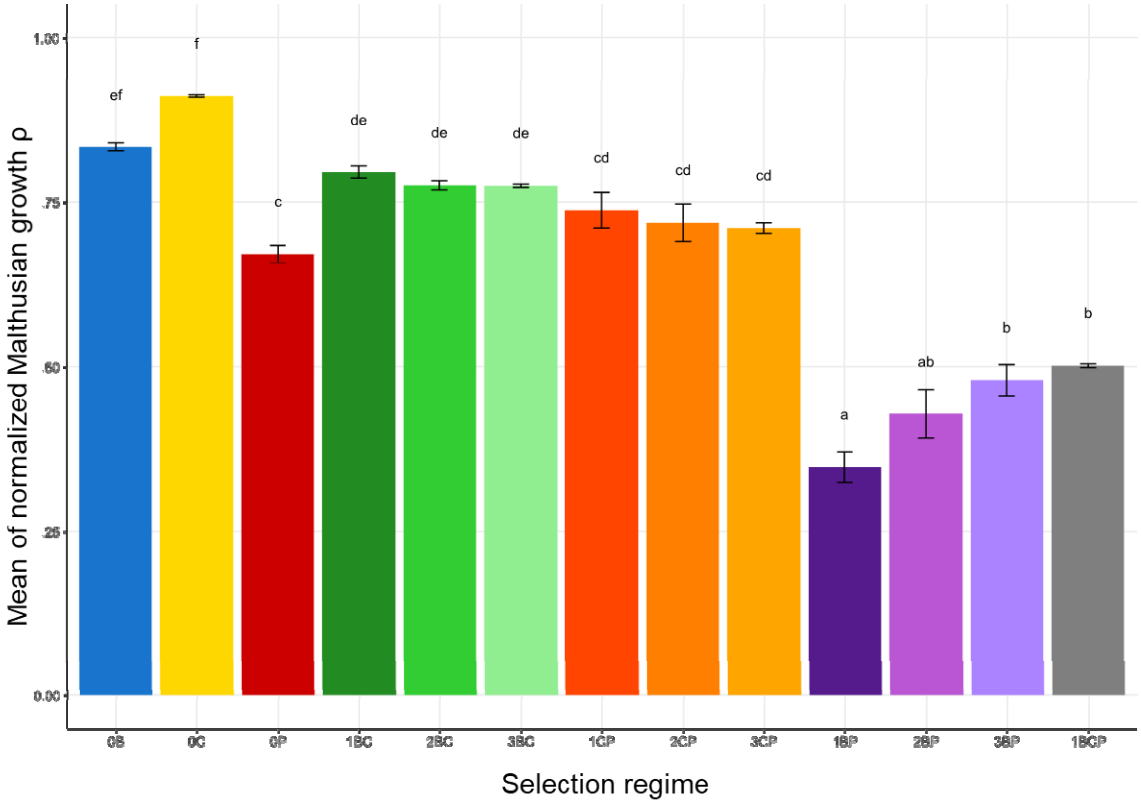
Initiation	95°C	3 min	
Denaturation	95°C	30 s	35 cycles
Annealing	Tm°C	30 s	
Elongation	72°C	1min	
Final	72°C	5 min	
	10°C	To the end of the cycle	

866 **Supplementary Information Figure S1: Variation of resistance evolution in selection regimes**

867

868 ρ, the mean resistance evolution rate, is shown for each selection regime. The error bars represent
869 the standard error *P*-values for pairwise comparisons were obtained with linear models (Tukey's
870 *post-hoc* correction). Alternations involving B, C or P had a beneficial or neutral effect on resistance
871 evolution rate relative to the continuous use of the same fungicide. The benefit of alternation
872 depends on the intrinsic resistance risks of the paired AIs.

873



874

875

876

877