Preventing multiple resistance above all: new insights for managing fungal adaptation

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Abstract (200 words)

Sustainable crop protection is crucial for food security, but is threatened by the adaptation of diverse, evolving pathogen population. Resistance can be managed by maximizing selection pressure diversity, by dose variation and the spatial and temporal combination of active ingredients. We explored the interplay between operational drivers for maximizing management strategy sustainability relative to the resistance status of fungal populations. We applied an experimental evolution approach to three artificial populations of an economically important wheat pathogen, *Zymoseptoria tritici,* differing in initial resistance status. We revealed that diversified selection pressure limited the selection of resistance in naïve populations and those with low frequencies of single resistances. Increasing the number of modes of action delayed resistance development most effectively — ahead of increasing the number of fungicides, fungicide choice based on resistance risk and temporal variation in fungicide exposure — but favored generalism in the evolved populations. However, the prior presence of multiple resistance and its subsequent selection in populations overrode the effects of diversity in management strategies, incidentally invalidating any universal ranking. Initial resistance composition must therefore be considered specifically in sustainable resistance management, to address real-world field situation.

Keywords: antifungal resistance; multiple resistance; population composition; generalism; resistance management strategy; experimental evolution; environmental variation; *Zymoseptoria tritici;* fungal adaptation

Introduction

Pesticides or drugs exert selection pressures on microbes and pest populations, leading to the emergence of uncontrolled resistant individuals, resulting in losses of efficacy and a need for dose increases, compromising crop, animal and human health and aggravating the environmental impact of the chemicals used [1, 2, 3, 4]. Resistance management strategies aim to delay the evolution of resistance by subjecting populations to diverse and heterogeneous selection pressures or by reducing their exposure to selection [5, 6]. Selection can thus be mitigated by increasing its *diversity* (*i.e.* number of active ingredients (AIs) or modes of action (MoAs) deployed, choosing AIs according to their intrinsic risk of resistance development), or by increasing its *heterogeneity* (*i.e.* playing with pattern of application over time, either concomitantly as in mixtures, or successively *as* in alternation or cycling, or over space as in mosaic) or by varying fungicide dose.

In agriculture, the ascomycete *Zymoseptoria tritici,* a hemibiotrophic plant pathogenic fungus responsible for *Septoria tritici* blotch (STB), the leading yield-reducing disease of wheat worldwide, provides an illustration of the debate about the relative performances of resistance management strategies [7, 8]. This pathogen is currently controlled principally by fungicide applications. This has led to its adaptation, to various degrees, to almost all the single-site MoAs authorized (benzimidazoles, which inhibit β-tubulin polymerization; QoIs, which inhibit complex III of mitochondrial respiration; DMIs, which inhibit sterol biosynthesis and SDHIs, which inhibit complex II of mitochondrial respiration), at least in Western Europe [9, 10]. Interestingly, this fungus displays various resistant phenotypes [11, 12, 13, 14, 15]. Specialist resistance results from the selection of mutations in genes encoding the target proteins of these fungicides (target-site resistance or TSR). The combination of multiple mutations in the same isolate, resulting in multiple resistance, is favored by the high rates of sexual reproduction in this pathogen [16]. Generalist resistance may result from non-target-site

62 resistance (NTSR), as in the multidrug resistance (MDR) caused by the enhanced efflux of unrelated
63 fungicides[17, 18]. The diversity of resistant phenotypes observed in *Z. tritici* in the field renders this
64 pathogen a relevant biological model for investigations of the interplay between the drivers of the
65 performance of anti-resistance strategies in contrasting field situations.
66 Several empirical and modelling studies in *Zymoseptoria tritici* have concluded that strategies
67 including alternation, mixtures, and modulations of the dose, number and timing of applications can
68 effectively limit the evolution of resistance, provided that they are optimized [5, 13, 19, 20, 21, 22]. However,
69 it remains a matter of debate which of these strategies is the most sustainable. Attempts to rank
70 strategies have suggested that mixture-based strategies may be the best, but the results obtained
71 were not unequivocal for *Z. tritici* or for many agricultural or clinical situations [5, 6]. There may be
72 several reasons for this. First, the results for strategy performance may depend on the criteria used
73 for assessment [23]. Indeed, the evolution or frequency of resistance are among the most widely used
74 criteria, but many other criteria, such as disease control or fungicide effective life, may be just as
75 relevant and may alter the ranking of performance between strategies. Furthermore, strategies must
76 be truly comparable to obtain meaningful results. Alternation and mixture strategies are rarely
77 compared at similar dose of AIs, and, when they are, the ranking is not decisive [20, 24]. Finally, the
78 composition of the pathogen population in terms of resistance types and frequencies (*i.e.* resistance
79 status) may also have an impact on strategy performance, as resistant individuals are fitter than
80 susceptible individuals in sprayed environments and higher doses of fungicide are required to control
81 them. The drivers of heterogeneity and diversity in selection pressure may, therefore, have different
82 impacts on resistance management, according to how and when they are implemented with respect
83 to pathogen resistance dynamics, which depends on the current composition of the pathogen
84 population. For example, strategies that effectively delay the initial emergence of resistance may be
85 different from those most effective at slowing the selection of resistances already present in the
86 pathogen population (see an example on mixture strategies [25]). The initial frequency and nature of
87 resistance mutations (*e.g.* inducing resistance to one or multiple MoAs) may also influence the

88 effective life of AIs [20]. Thus, the overall performance of a strategy depends on the respective
89 efficacies of its components for controlling both susceptible and resistant individuals over time and
90 space, whatever the composition of the pathogen population in terms of resistance status.
91 In this context, we investigated the interplay between the operational drivers maximizing the
92 sustainability of management strategies relative to the resistance status of the pathogen population.
93 We explored several assumptions, based on published studies, including the work of Ballu *et al.* [26, 27].
94 We found that the various sources of heterogeneity and diversity in selection pressures differed in
95 their efficacy for delaying resistance evolution. We found that combining these sources would
96 probably increase the overall sustainability of strategies [6], and that the efficacy of anti-resistance
97 strategies depends strongly on pathogen population structure in terms of resistance [20]. These results
98 have been achieved through experimental evolution, an innovative approach that allows for the
99 design of complex experiments but remains underused in fungi. *Z. tritici* is particularly suitable for
100 investigations with this approach because of its haploid yeast-like form (*i.e.* blastospores) that is easy
101 to culture in liquid medium. By evolving strains carrying field resistances, we were able to combine
102 some of the characteristics of natural populations with accelerated experimental evolution in
103 miniaturized controlled conditions [28, 29]. We followed the dynamics of populations and resistances
104 under several selection regimes, which helped to elucidate the impact of population composition on
105 the efficacy of the drivers of resistance management strategies. Several sources of diversity and
106 heterogeneity generally considered to promote the durable control of STB were tested: the number
107 of AIs used (*i.e.* 1-3), the number of MoAs (*i.e.* 1-3), the intrinsic vulnerability of these agents to the
108 development of resistance (*i.e.* low to high resistance risk), and the temporal heterogeneity of
109 fungicide exposure (*i.e.* mixture *vs.* alternation). We compared the performance of several selection
110 strategies, defined as combinations of one or several of these drivers, against artificial populations of
111 different compositions in terms of resistance status. The introduction of multiple resistance into
112 artificial populations mimicked some aspects of the recombination that occurs naturally in field
113 populations of *Z. tritici,* and made it possible to explore the potential effect of such events on

strategy performance [29]. A multi-criteria assessment of strategies, based on growth dynamics of populations and evolution of resistance phenotype profiles, confirmed that diversification of selection pressure, by alternation or mixture-based approaches, can limit the emergence and selection of resistance, at the cost of selecting generalist or multiple resistance. But above all, resistance status of pathogen population prior to the implementation of strategies was found to be the primary determinant of strategy performance, particularly when multiple resistance was already present in the initial population. These findings open up new perspectives for the informed tailoring of strategies, and show that population composition must be given priority over other drivers of selection in such strategies.

Results

Ancestral isolates display contrasting patterns of resistance in artificial populations

Experimental evolution was performed with artificial populations constituted from ancestral isolates currently found in European populations of *Z. tritici*. The resistant isolates (R_{SDHI}, R_{DMI} and $R_{SDHI/DMI}$) were isolated from a second-generation progeny of a cross between the susceptible (S) wild-type reference isolate and a field isolate bearing the *sdhC*^{H152R} change and several *cyp51* substitutions (*i.e.* V136A, A379G, I381V, Y461S and S524T; hereafter simplified as *cyp51*^{I381V}). We checked that the resulting changes to the target proteins of SDHI and DMI fungicides, respectively, were associated with the expected phenotypes and calculated their resistance factors (RFs) relative to the S isolate (**Fig. 1A**; **Supplementary Fig. 1**). We confirmed that the R_{SDHI} and $R_{SDHI/DMI}$ isolates bearing *sdhC*^{H152R} had RFs exceeding 90 for all SDHIs [30, 31] and that the R_{DMI} and $R_{SDHI/DMI}$ isolates bearing *cyp51*^{I381V} displayed moderate resistance to all DMIs tested (9<RFs<40), although the RFs for mefentrifluconazole were lower than those for other azoles [11, 32]. Complementary genotyping revealed that the R_{SDHI}, R_{DMI} and $R_{SDHI/DMI}$ isolates had inherited resistance to benzimidazoles from their parental field isolate, precluding any further use of these fungicides in resistance management strategies [33], and that only the multiresistant $R_{SDHI/DMI}$ isolate had inherited resistance to QoIs due to the *cytb*^{G143A} change [8]. All isolates were equally susceptible to the multisite agent chlorothalonil and to tolnaftate, an inhibitor of squalene epoxidase used to reveal multidrug resistance in *Z. tritici* [17].

The fitness of the ancestral isolates was assessed by establishing growth curves under the conditions used for experimental evolution and in the absence of fungicide selection (**Fig. 1B**), and the parameters of the logistic model were used to compare isolates (**Fig. 1C**). The time required to reach the half-maximal concentration of spores was significantly greater for the resistant isolates than for the reference isolate (2.94-3.08 days *vs.* 2.5 days), but maximal spore concentration was significantly

148 higher for resistant isolates than for the reference isolate at the end of the experiment (+4.1 -
149 +5.8%). However, in populations from control lines not exposed to fungicide (see below), the
150 resistance alleles initially present at low frequency were not detected at the end of the experimental
151 evolution period, suggesting that either the weak and early penalty associated with resistance
152 observed in our conditions was expressed, and/or that they were lost by chance because of the
153 regular bottlenecks affecting populations during their serial cultivation. As no other genotype
154 replicates were tested, it remains unclear whether this fitness penalty was related to the resistance
155 mutations or to the genetic background of the isolates. Nevertheless, these findings clarify and
156 complete previous observations concerning the fitness cost associated with the *sdhC*^{H152R} variant
157 expressed in culture *in vitro* and in competition experiments[34, 35], and restoration of the fitness cost
158 of the I381V variant when associated with a modification to the Y461 codon of *cyp51*, as in R_{DMI} and
159 $R_{SDHI/DMI}$[36]. We assumed that resistance to benzimidazoles did not bias fitness measurement, as no
160 associated fitness penalty has ever been observed for this resistance in any species, including *Z. tritici*
161 [33, 36]. Resistance to QoIs has been reported to decrease *Z. tritici* virulence *in planta* [36], but we
162 assumed that this cost did not operate *in vitro* for the $R_{SDHI/DMI}$ isolate.
163

164 Population composition affects the performance of resistance management strategies

165 We investigated the effect of population composition on the performance of strategies, by
166 comparing the evolution of three artificial populations containing 0% (PopS) or 5% (PopR and PopRR)
167 mixed resistant individuals, to reproduce the late stages of emergence of resistance as usually
168 detected with current monitoring tools in field situations (**Fig. 1D**). PopR contained only single
169 resistances (*i.e.* R_{SDHI} and R_{DMI}) and PopRR contained both single resistances and a small proportion of
170 multiresistant individuals (*i.e.* $R_{SDHI/DMI}$), to mimic the results of the sexual reproduction likely to occur
171 in field populations of *Z. tritici* [16]. These populations were subjected to selection pressure from
172 alternating treatments with benzovindiflupyr (a SDHI; hereafter referred to as B) and
173 prothioconazole-desthio (a DMI; hereafter referred to as P) or a mixture of these treatments, and

174 were then compared with untreated lines (**Fig. 2**; **Supplementary Table 2**). We quantified the
175 dynamics of resistance in each line as its mean normalized Malthusian growth, M_i^n, a proxy for the
176 increase in resistance under selection (**Fig. 3A**). The growth of PopS was significantly smaller (82%
177 and 84% smaller, under P and B mixture or alternation, respectively) than that of the control.
178 Significant decreases in growth relative to the control were also observed for the PopR population
179 treated with the P+B mixture (85%), and to a lesser extent, the PopR population subjected to an
180 alternation of P and B (46%). By contrast, the growth of PopRR was much more similar to that of the
181 control population, despite very slight, but nevertheless significant decreases of 3% in alternation
182 and 5% in mixture, revealing that both these strategies performed very poorly once multiple
183 resistance was present in the population. These observations mirrored the strong and significant
184 selection (increase by a factor of 53 to 157) of the resistant *sdhC*^{H152R} and *cyp51*^{I381V} alleles in all
185 PopRR lines (**Fig. 3B** and **3C**), and, more specifically, of the multiresistant isolate $R_{SDHI/DMI}$, as revealed
186 directly by the peak frequencies of *cytb*^{G143A} at the end of the experiment (**Supplementary Fig. 4**).
187 The more moderate increase (increase by a factor of 0 to 12) in the frequencies of *sdhC*^{H152R} and
188 *cyp51*^{I381V} in PopR lines confirmed that the various strategies, including mixture in particular, were
189 useful for mitigating the selection of single resistances, at least during the eight cycles of the
190 experiment (*i.e.* ≈ 50-55 generations).

191 We also established phenotype resistance profiles for all the lines at the end of the experiment, to
192 observe the emergence of resistances other than those introduced *via* the ancestral phenotypes.
193 Principal component analysis confirmed that the evolved lines differed mainly in their ancestral
194 composition rather than in selection regimes (**Fig. 5**). The PopS and PopRR lines retained their initial
195 phenotypes, but one PopR line evolved resistance to tolnaftate, an inhibitor of squalene epoxidase
196 often used to reveal multidrug resistance[17].

197

198 Mode-of-action diversity is a driver of strategy performance that should be considered
199 before increasing the number of AIs

200 We investigated the extent to which population composition interfered with the components
201 of strategies and determined their performance, by subjecting PopR and PopRR to 12 selection
202 regimes mimicking various degrees of selection heterogeneity and diversity, and comparing them
203 with untreated controls. We used two SDHI fungicides (B and F) potentially selecting for *sdhC*^{H152R},
204 and two DMI fungicides (P and M) potentially selecting for *cyp51*^{I381V} to investigate the effect of the
205 number of AIs (1 *vs.* 2) and of the number of MoAs (1 *vs.* 2) on resistance evolution (**Fig. 2** and
206 **Supplementary Table 2**). In PopRR lines, the maximal concentration of the multiresistant isolate was
207 estimated to be the lower of the concentrations of the two alleles. In situations in which *cytb*^{G143A} was
208 quantified, these two measurements were found to be highly correlated (Pearson's r = 0.998;
209 P<0.0001).

210 In strategies including two AIs with the same MoA, resistance was rapidly established, as shown by
211 the maximal Malthusian growth and the increase in resistance allele frequencies, as early as the
212 second cycle, as with the continuous use of a single AI, whatever the ancestral population (**Fig. 6A**).
213 Such strategies even significantly accelerated the selection of resistant alleles in PopR lines relative to
214 the continuous use of a single AI (+17.8% for *sdhC*^{H152R} on average in strategies combining two SDHIs;
215 +2.3% for *cyp51*^{I381V} on average in strategies combining two DMIs). The selection of single resistances
216 was also favored over that of multiple resistance in PopRR lines (a mean allele frequency of ≈97%
217 and ≈86% for *cyp51*^{I381V} and *sdhC*^{H152R}, respectively, over the experiment, *vs.* a mean frequency of
218 42% for multiresistant individuals) (**Fig. 6A** and **Supplementary Fig. 4**). Moreover, the relative ratio of
219 single *vs.* multiple resistance for each MoA was 2:1 at the beginning of the experiment. This ratio
220 greatly increased under DMI selection but stayed roughly stable under SDHI selection, at the end of
221 the experiment. This finding suggests a relative resistance cost for the $R_{SDHI/DMI}$ isolate in competition

222 with R_{DMI} isolate under DMI selection pressure. Finally, the absence of MoA diversity in selection
223 regimes resulted in the co-occurrence of single- and multiple-resistant isolates.

224 By contrast, the introduction of a second AI with a different MoA (SDHI or DMI) significantly
225 decreased population growth (by 2 - 85%, with a mean of 39%), whilst reducing the selection of
226 resistant alleles (-49% - -100%, mean -82% for *sdhC*^{H152R} and -43% - -100%, mean -87% for *cyp51*^{I381V},
227 relative to selection with a single AI) in lines in which only single resistances to the two MoAs were
228 initially introduced (**Fig. 6B**). The greatest reductions were observed with the P and B fungicides,
229 rather than with the M and F ones. This benefit of combining MoAs was much more limited (from -
230 4% to +4% of M_i^n, mean -0.6%) when multiple resistance was introduced, due to the early and rapid
231 generalization of the multiresistant mutant (increase by +85% - +596%; mean +241%), at the expense
232 of isolates with single resistances, revealing the greater relative fitness of the multiresistant mutant
233 when multiple selection is applied (**Fig. 6B** and **Supplementary Fig. 3**). These findings confirm that
234 diversifying modes of action, whatever the heterogeneity in the timing of their application (as a
235 mixture or in alternation) is beneficial only in the absence of multiple resistance.

236 We investigated whether increasing the number of AIs and of MoAs induced the selection of new
237 resistance profiles, by performing droplet tests on the 99 population lines evolved under one of the
238 31 selection regimes and collected at the end of the experiment, for 10 fungicide treatments and a
239 control. These treatments included the fungicides used for selection but also other independent AIs
240 (**Fig. 5** and **Supplementary Table 3B**). Population resistance profiles were generally consistent with
241 allele quantification, with the population displaying the resistance phenotype profile of the
242 predominantly selected ancestral isolate. In particular, in PopRR lines that had evolved under
243 diversified selection pressure from SDHIs combined with DMIs, low phenotypic diversity was
244 observed, confirming the generalization of the multiresistant mutant. By contrast, greater diversity
245 was observed after selection by a single MoA, reflecting the variation in resistance frequency
246 between replicates. In PopR lines, the lack of growth in the presence of DMI and SDHI mixtures of

247 lines evolved under a single selection pressure confirmed the preferential selection of single-
248 resistance alleles. Interestingly, not only did lines evolved under diversified selection regimes grow in
249 the presence of the two MoAs independently, confirming the co-selection of single-resistance alleles,
250 but some also displayed additional resistances, as suggested by their greater diversity in the PCA and
251 original patterns of resistance on heatmaps (**Fig. 5**). In particular, some lines selected under pressure
252 from a combination of M (DMI) and F (SDHI) fungicides displayed resistance to the non-DMI and non-
253 SDHI fungicides azoxystrobin and tolnaftate. Tolnaftate has been shown to reveal multidrug
254 resistance in *Z. tritici* [17]. We therefore checked for insertions in the promoter of the MFS1 transporter
255 gene, which is overexpressed in field isolates [37], in a selection of 20 strains isolated from these
256 populations with various scores for tolnaftate resistance. We found no change in the length of the
257 promoter sequence (data not shown). We then measured the expression of *mfs1* in the same isolates
258 after growth in the absence of fungicide. We confirmed that nine of these isolates displayed
259 moderately enhanced (about 3.3 - 10 times higher) fungicide efflux. By contrast, the level of
260 expression of *mfs1* in the MDR field isolate was about 30 times higher, suggesting only moderately
261 enhanced efflux due to MFS1 in some of our isolates, with still unknown determinism. Furthermore,
262 the poor correlation between tolnaftate resistance scores and *mfs1* expression strongly suggests that
263 unidentified generalist resistance mechanisms may be at work in our collection. Finally, this finding
264 suggests that diversified selection pressure mediates a trade-off between the growth rate of the
265 evolving population and the degree of generalism of the evolved isolates.

266

267 The performance of strategies depends primarily on the diversity of MoAs displayed,
268 but also on the particular characteristics of AIs

269 We further investigated the reasons for the better performance, in terms of effects on
270 population growth, of strategies combining several MoAs and AIs than of continuous use of the same
271 fungicide, by analyzing the effects of the respective intrinsic resistance risks of the MoAs and AIs on

272 resistance evolution. This analysis was made possible by the use of two different MoAs (SDHIs *vs.*
273 DMIs) and two AIs within each chemical class (B and F *vs.* P and M, respectively). It has been claimed
274 that the intrinsic risk of resistance is low-to-moderate for DMIs, but moderate-to-high for SDHIs [38].
275 AIs were compared at the same effective dose (EC_{97}) to ensure a fair comparison based on their
276 intrinsic properties.

277 DMI-based strategies yielded a 4.4% higher M_i^n mean normalized Malthusian growth than SDHI-
278 based strategies, regardless of the initial population structure, the number of AIs in the strategy or
279 the temporal heterogeneity of selection (**Fig. 6A**). This moderate difference suggests that the
280 respective resistance risks of DMIs and SDHIs were not markedly different in the experimental
281 conditions used here, at least for resistance dynamics, not anticipating field efficacy. In PopR, both
282 MoAs were equally effective for controlling simple mutants resistant to the other MoA (the
283 normalized concentration of $sdhC^{H152R}$ and of $cyp51^{I381V}$ after exposure to either DMIs or SDHIs,
284 respectively, both below the detection limit) whereas in PopRR, the estimated normalized
285 concentration of multiple resistance in the presence of DMIs was only half that with SDHIs only (16%
286 *vs.* 33%), suggesting that SDHIs were less efficient than DMIs for controlling $R_{SDHI/DMI}$. These findings
287 mirror the contrasting selection responses, which are largely dependent on the resistance allele
288 selected and its relative fitness. This finding is not, therefore, readily generalizable when diverse
289 mutations may arise under selection pressure from a single MoA in the field, as is the case for SDHIs
290 and DMIs.

291 The intrinsic resistance risk may also be AI-specific, and was therefore investigated for SDHIs and
292 DMIs. In our conditions, the M_i^n of strategies combining P and B was lower, by a factor of 1.45 - 5.7
293 (mean 2.4), than that of strategies combining M and F in PopR lines. This finding is consistent with
294 the -55% to -100% decrease in the concentrations of $sdhC^{H152R}$ or $cyp51^{I381V}$ in strategies including P
295 and B (**Fig. 6B**). The poorer control observed in M- and F-based strategies may also explain the
296 emergence of additional generalist resistance mechanisms, as described above, in these lines (**Fig.**

297 **5D**). The benefit of strategies combining P and B over those combining M and F was also significant in
298 PopRR lines, but to a much lesser extent (-2.6% of M_i^n; -1% in content of the multiresistant isolate).
299 These findings suggest that it may also be difficult to generalize resistance risk to a whole MoA.

300

301 New MoAs at appropriate doses are relevant partners for enhancing the performance
302 of strategies

303 Fungicide diversity was found to affect the selection of resistance for AIs for which resistance
304 was present at low frequencies in the initial populations. We also explored whether introducing a
305 "new" AI, *i.e.* an AI to which the whole populations were fully susceptible, could improve the
306 performance of strategies, and under which conditions. We therefore combined chlorothalonil (C), a
307 multisite inhibitor with a very low risk of resistance, with the B and P fungicides previously used (**Fig.**
308 **6B**).

309 Introducing C into a combination of B and P in PopR lines induced a slight increase of M_i^n (+46% for
310 the BP/C alternation and +13% for the BPC mixture when compared with the already high-
311 performing BP mixture), its overall value being close to the detection limit over the course of the
312 experiment. This finding can be explained by equal full control of both single-resistant isolates by C
313 that reinforces that of the BP mixture. By contrast, in PopRR, combining C with B and P had a
314 beneficial effect on resistance (-48% and -30% M_i^n respectively, for the BP/C alternation and BPC
315 mixture, compared with the poorly-performing BP mixture) and on the frequency of resistant alleles
316 (-63% for *sdhC*^{H152R} and -63% for *cyp51*^{I381V} average). However, resistance dynamics differed
317 depending on whether C was alternated or mixed with a BP mixture. In the mixture strategy, a half-
318 dose of C was not sufficient to control the increasing proportion of the multiresistant isolate in the
319 population and to compensate for the decrease in efficacy of P and B ($M_i^n = 0.66$ and mean double
320 resistant frequency = 38%). In the alternation strategy, C applied at full dose was fully effective
321 against the $R_{SDHI/DMI}$ ancestral isolate, restored efficacy, and selected against multiple resistance every

322 two cycles, enabling a better overall control of the population ($M_i^n = 0.49$ and mean double
323 resistant frequency = 25%). In populations in which resistance has emerged, the performance of
324 strategies involving a new AI may therefore be dependent on its dose and its redundancy in the
325 selection pattern.

326 In strategies combining B, C and P, the increase in MoA diversity due to the introduction of a third
327 MoA could potentially increase the degree of generalism in the evolved populations, as previously
328 described [26, 27]. This trade-off was not observed (*i.e.* no additional growth on tolnaftate; **Fig. 5**) in
329 PopRR lines where the multiresistant isolate was present and fitter, and invaded the population
330 rapidly, at the expense of the susceptible isolate. However, PopR populations subjected to
331 alternation or mixture involving C acquired generalist resistance, as revealed by their growth on
332 tolnaftate, unlike all but one population subjected to a combination of B and P. This emergence of
333 generalist resistance might have been permitted by the maintenance of higher diversity in
334 populations, more prone to mutation, especially in susceptible backgrounds. Therefore, when a new
335 MoA is introduced and combined with previously used MoAs, attention must be paid to the prior
336 presence or absence of certain resistances, which could strongly influence the risk of generalist
337 resistance emerging, as well as specialist resistance to the new MoA, depending on its own
338 resistance risk.

339

340 Mixtures do not systematically outperform alternation: population structure also
341 matters

342 As the search for the best anti-resistance strategies often pits mixtures against alternation,
343 we studied the effect of temporal heterogeneity (alternating *vs.* mixing AIs) on resistance dynamics
344 and phenotype evolution.

345 The temporal heterogeneity achieved by alternating instead of mixing AIs with the same MoA had no
346 (for DMIs) or little (-3% to -5%; for SDHIs) effect on M_i^n, whatever the composition of the ancestral

347 population (**Fig. 6A**). Within-MoA alternations and mixtures of SDHI and DMI fungicides led to the
348 rapid generalization of *sdhC*^{H152R} and *cyp51*^{I381V}, respectively, in a similar manner. The rate of
349 *sdhC*^{H152R} selection was slightly lower (-9% in PopR and -2% in PopRR) for the alternation of B and F
350 than for their mixture.

351 When SDHIs and DMIs were combined, their mixtures performed significantly but moderately better
352 than their alternation (in average, -34% for M_i^n; -70% for *sdhC*^{H152R} concentration; -99% for c*yp51*^{I381V}
353 concentration), only when single resistances were introduced (PopR lines). However, mixtures
354 combining M and F were less effective than those combining B and P, because of the lower level of
355 control exerted by their half-doses or differences in their inherent resistance risks, as explained
356 above (**Fig. 6B**).

357 However, when multiple resistance was present in the population (PopRR lines), it was rapidly and
358 intensively selected by both alternation and mixture, resulting in poor performances for both types
359 of strategy, for both M_i^n and the selection of single-resistant alleles, although slight differences were
360 observed between fungicide pairs. As described above, the addition of a third, new MoA (C) was
361 more effective in alternations than in mixtures (-26% for M_i^n; -32% for maximal multiresistant isolate
362 content) because it made it possible to use a full dose of C every second cycle.

363 Alternation and mixtures resulted in a diversity of resistance phenotype population profiles,
364 reflecting the variable frequencies of the resistances to SDHIs or DMIs, or their co-occurrence (**Fig. 5**).
365 No resistances other than those initially introduced from the ancestral strains were observed, with
366 the exception of the generalist resistance profiles of PopR lines, which were also able to grow on
367 tolnaftate and azoxystrobin, as previously described. These generalist profiles were found exclusively
368 in mixture and alternation selection regimes including two or three MoAs; neither alternation nor
369 mixing could be considered more likely to favor generalist resistance. Temporal heterogeneity may
370 not impact generalist resistance as much as the diversity of MoAs.

Overall, the advantage of mixtures over alternation selection regimes in terms of resistance dynamics and prevention of generalist resistance was driven mostly by the occurrence of multiple resistance in the ancestral population and the AIs used in the strategy and their doses.

Discussion

This experimental study was designed to explore the performance of some of the drivers of resistance management strategies (*i.e.* the number and type of MoAs, the number and type of AIs, their vulnerability to resistance and the temporal pattern of fungicide exposure) relative to the resistance status of the population of the plant pathogenic fungus *Zymoseptoria tritici*. We ran a comprehensive experimental evolution study that was designed to disentangle the respective influences of these drivers on the evolution of resistance in three populations of different initial compositions (a sensitive population, and two others, each including 5% fungicide-resistant individuals carrying either only single target-site resistance alleles or single and multiple target-site resistance alleles). We used multicriteria assessment (population growth rate, resistance allele content and pattern of cross-resistance in evolved populations) to evaluate the performance of 31 selection regimes. We found that strategies combining several MoAs exerted sufficiently diversified selection pressure to mitigate the emergence and selection of resistance if the initial populations had no resistance alleles or only single resistances at low frequency. By contrast, the introduction of multiple resistance abolished the beneficial impact of the best strategy drivers, indicating that population composition is the principal factor to be considered when tailoring anti-resistance strategies.

394 **Alternation can be as sustainable as mixture and may reconcile resistance management and**
395 **socioenvironmental expectations**

396 Using an original experimental evolution approach, we show here that maximizing the
397 diversity of selection while smartly combining drivers can effectively delay the evolution of
398 resistance, as previously reported in empirical studies comparing a limited number of field or
399 modeled situations, based on assumptions simplifying the biological reality [5, 6]. In particular, our
400 findings confirm that selection was weakest for the combination of several MoAs (*i.e.* when
401 maximizing inter-MoA diversity), and stronger when AIs with the same MoA were combined (*i.e.*
402 when maximizing intra-MoA diversity).
403 Indeed, the exclusive use of DMI or SDHI fungicides, with one or two AIs, rapidly led to the
404 establishment of resistance, associated with a loss of full control. This situation mirrors findings
405 previously obtained with the same approach concerning the emergence of resistance in a naïve
406 population [27]. Several studies have documented the poor efficacy of sequences of fungicides with the
407 same MoA for controlling target-site resistance in *Z. tritici,* in the field or in models [19, 20, 39, 40, 41].
408 Indeed, AIs with the same MoA generally display positive cross-resistance, as confirmed by the
409 phenotyping of our ancestral isolates. However, resistance intensity may differ between AIs for the
410 same resistance allele, or between alleles for the same AI, reflecting the variation in AI affinity for the
411 mutated target protein e.g. [12], ranging from strong to weak or a lack of resistance to some specific
412 AIs. AIs exceptionally not or less affected by cross-resistance may appear of interest from a practical
413 standpoint. However, this benefit is strongly limited by the high diversity of resistance mutations and
414 phenotypes in field populations [9, 11, 12, 17]. Our results suggest that although combinations of
415 fungicides sharing the same MoA involve potentially very diverse AIs, with the entire range of
416 authorized SDHIs and DMIs generally used in agricultural areas, they do not provide sufficiently
417 diverse selection pressure and globally favor the selection of resistance.

418 The use of multiple MoAs, rather than AIs, is another option to maximize the diversity of selection,
419 but this can be achieved either in mixture, with all generations of the pathogen submitted to the
420 same combination of selection pressures, or in alternation, in which a few generations are subjected
421 to a single selection pressure different from that imposed on their ancestors or progeny. The efficacy
422 of mixtures is based on redundant killing and the low probability of *de novo* multiple resistance
423 developing, but also possibly on higher dose rates (see below)[26], whereas alternation may delay the
424 build-up of resistance by spreading selection pressure out over longer periods, and by decreasing the
425 rate of evolution of resistance to the fungicides alternated [27]. Many studies have compared the
426 efficacy of these strategies for limiting the emergence of resistance and selection in fungi and other
427 taxa see reviews in [5, 6, 42]. Most, but not all, of these studies ranked mixtures above alternation in
428 terms of performance in theoretical and empirical studies and simplicity of implementation, notably
429 due to the availability of commercial ready-to-use mixtures. In most studies, mixtures were often
430 defined as the simultaneous application of two MoAs, both used at their effective dose [5]. In the field,
431 this would lead to the quantities of fungicide used almost doubling. In this study, we propose an
432 original and fair comparison of alternation and mixtures at constant environmental impact, *i.e.* with
433 equal cumulative amounts of fungicide. Mixtures were, thus, defined as half the selection dose of
434 each AI calculated for susceptible isolates. In this context, the mixture and alternation of SDHIs and
435 DMIs were equally effective for delaying the selection of resistance and controlling the growth of
436 initially naïve populations. Mixtures moderately outperformed alternation when single resistances
437 were present in the initial population, because the partial and possibly synergistic efficacies of both
438 partners remained sufficient to control emerging single resistances and keep them at low frequency
439 in the population. By contrast, in alternation, each resistance alternatively escaped control when
440 subjected only to a full dose of the corresponding AI every other cycle, because this strategy allowed
441 to increase frequency of both resistances, alternatively. Performance was also determined by the AIs
442 combined (*e.g.* at the chosen selection doses, M and F performed less well than P and B, whatever
443 the strategy used). Mixtures and alternation were equally poor when multiple resistance to both

partners was present in the initial population and selected, as both AIs were ineffective against the double-resistance mutant, which rapidly established itself within the population. Interestingly, in this context, the introduction of a new MoA to which the whole population was susceptible was more valuable in alternation than in mixtures, as alternation allowed maximal efficacy against multiple resistance due to the application of a full dose every other cycle. In situations in which multiple resistance is observed, the benefits of alternation may depend more on the dose, frequency of use and vulnerability to resistance of the new agent introduced, than on the AIs alternated. Here, the new fungicide introduced was a multisite inhibitor with a very low resistance risk. The introduction of a new, previously unused fungicide may be considered with caution for a single-site fungicide with a greater inherent risk of resistance, the efficacy of which may decrease over time. Overall, our findings demonstrate that at similar and constrained total dose, mixtures may not always outperform alternation and are in line with a recent modelling study [43] that concludes that the relative efficacies of these strategies clearly depend on the composition of the target population. This finding might be highly relevant in a context where the reduction of pesticide use, more achievable by using alternation than by using many current mixtures, constitutes a strong socioenvironmental expectation in an increasing number of countries.

The performance of strategies creating highly diversified selection pressure is limited by multiple and generalist resistance

Our analysis of the relevance of drivers of resistance management strategies for delaying the development of resistance unequivocally identifies population composition with regard to resistance as the primary determinant of the performance of strategies, ahead of the diversity of the MoAs applied and temporal heterogeneity. Multiple resistance, in particular, is an aggravating factor, as it is very poorly controlled by the fungicides that contributed to its selection, whether they were used alone or in combination. In strategies combining multiple MoAs, multiple resistance is preferentially

469 selected over single resistances, because it confers greater relative fitness under multidimensionnal
470 selection [44]. This study confirms *in vivo* the results of modeling studies considering multiple
471 resistance [20, 45]. Finally, this experimental evolution approach also provides evidence that increasing
472 the dimensionality of selection may favor generalist phenotypes over specialist ones, a question
473 rarely explored so far, except for a recent experimental work [46] whose results tend to agree with
474 ours, and that would deserve complementary theoretical investigation [47].
475 Generalist fungicide resistance is also observed when non target-site resistance is selected, that is,
476 when resistance mechanisms other than those encoded by mutations in the target-encoding genes
477 are selected and confer wide patterns of cross-resistance to unrelated fungicides [48, 49]. In an
478 experimental design in which resistance is already present, we expected to see the direct selection of
479 the resistance alleles, but at contrasting intensities, depending on the selection regime applied.
480 Surprisingly, the analysis of population phenotypes at the end of the evolution experiment revealed
481 the occurrence of *de novo* mutations in some lines. In populations into which only single resistances
482 were present, additional resistance to azoxystrobin, a QoI not used for selection, and tolnaftate, an
483 inhibitor of sterol-squalene epoxidase generally used to reveal enhanced fungicide efflux [17], formerly
484 absent, was detected, exclusively in some lines subjected to alternations or mixtures of SDHIs and
485 DMIs, associated or not to the multisite inhibitor C. This generalist resistance co-existed, within
486 populations, with the initially introduced DMI and SDHI resistances. Generalist resistance can even
487 be associated with target-site resistance allele, as highly probable in lines undergoing M and F
488 selection where $sdhC^{H152R}$ was fully established at the end of the experiment. The purification of
489 isolates resistant to tolnaftate made it possible to confirm the slight-to-moderate overexpression of
490 *mfs1*, encoding a MFS transporter responsible for multidrug resistance in *Z. tritici* in the field [37], in
491 some, but not all of the strains tested. The absence of the usual regulatory insertions in the promoter
492 of *mfs1*, and the poor correlation between susceptibility to tolnaftate and *mfs1* expression suggest
493 that unknown mutations and resistance mechanisms probably underlie the generalist resistance of
494 these isolates. This evolutionary trade-off in resistance management, with attempts to reduce

495 selection for specialist resistance traits promoting the evolution of generalist resistance, was
496 observed in a previous study dealing with alternation and effective-dose mixture strategies in naïve
497 populations [26, 27]. It may apply more generally to any strategy combining multiple MoAs in alternation
498 or mixtures and aiming to mitigate the evolution of resistance in other organisms, such as weeds [50, 51,
499 52] or bacteria [42]. This trade-off is still little considered in the field, with attention instead focused on
500 more quantitative criteria for measuring strategy performance, such as resistance frequency, or the
501 effective life-time of a fungicide. However, high frequencies of individuals with generalist resistance
502 in the pathogen population may irreversibly decrease the number of effective AIs even more than
503 TSR. In weeds and insects, NTSR and TSR generally occur together and are associated with high RFs
504 and unexpected resistance patterns [53, 54]. In *Z. tritici,* MDR *per se* is associated with moderate
505 resistance factors, but its combination with target-site alterations has been found to increase
506 resistance factors to DMIs or SDHIs significantly [55].

507 Given the importance of population composition for strategy performance and the trade-off
508 described above, these findings suggest that the debate should be refocused on the prevention of
509 multiple and, to a lesser extent, generalist resistance, rather than on the relative merits of mixtures
510 and alternation, as summarized in [44].

511

512 **The crux of resistance management should focus on limiting multiple resistance**

513 Multiple resistance occurs in populations for several reasons. First, it reflects the sequential
514 use of MoAs, following their successive discoveries and withdrawals following the development of
515 intensive cropping systems, which began just after World War II. Resistance led to new AIs often
516 being combined with their predecessors to improve residual efficacy, and resistance to new MoAs
517 was successively selected in genetic backgrounds that were already resistant to the older MoAs,
518 which were generally withdrawn once resistance became widely established. Multiple resistance has
519 been described as resulting from a stepwise accumulation of resistances in several plant pathogens

520 (*e.g. Venturia inaequalis* [56], *Monilinia fructicola* [57], *Botrytis cinerea* [58], and *Cercospora beticola* [59]),
521 and it may even favor the development of resistance to new MoAs. *Z. tritici* is no exception, as
522 resistance to benzimidazoles and QoIs remains widespread in populations from Western Europe,
523 despite the withdrawal of these fungicides from the market. The ongoing emergence of resistance to
524 SDHIs is almost systematically associated with DMI resistance, simply because it is selected within
525 DMI-resistant populations [30, 60].

526 Second, multiple resistance may result from the recombination of resistance alleles during sexual
527 reproduction. The primary inoculum of *Z. tritici,* including ascospores, is never a limiting factor for
528 epidemics and sexual reproduction. Indeed, population-level deep sequencing revealed intense
529 interplay between asexual and sexual reproduction [16]. In this airborne pathogen, the ascospores
530 resulting from sexual reproduction are dispersed over long distances and have been reported to
531 spread QoI resistance between fields locally [61] but also at the continental scale in Europe [62].

532 Despite these strong assumptions on the occurrence of multiple resistance in agricultural systems,
533 the performance of strategies has generally, with few exceptions, been modeled from initially
534 susceptible or single resistant populations. Yet, the reality in the field is that AIs are probably only
535 rarely confronted with the ideal situation in which all the AIs and MoAs used are fully effective. In the
536 real world, multiple resistance is the norm, and the initial frequency of single and multiple
537 resistances almost certainly differ at fine scales ; strategies need to be adapted accordingly [63]. In this
538 work, we assessed the performance of strategies in conditions mimicking real-life conditions, while
539 artificially introducing single and multiple resistances, to reproduce the combination of resistance
540 alleles likely to occur in the field by sexual reproduction, but hardly achievable in the laboratory.
541 However, the lack of this phenomenon in our experimental system precludes any assessment of the
542 time required for the emergence of multiple resistance by recombination, all the more since
543 preexisting resistances are often established by stepwise accumulation in populations.

544 One of the first conclusions to be drawn from our findings is that the design of resistance
545 management strategies, and the long-term durability of MoAs would greatly benefit from resistance
546 monitoring data (*i.e.* the composition of the local population) being made publicly available at the
547 finest possible scale. The high-throughput quantification of multiple resistance genotypes may
548 become possible in the near future with the development of next-generation sequencing techniques
549 for use in diagnostics for plant pathogen populations [14, 64]. This, in turn, should make it possible to
550 take informed decisions in spraying programs. Second, more attention should be paid to limiting the
551 development of multiple resistance, and of the combination of TSR and NTSR, as both could greatly
552 decrease the efficacy of any strategy. Recombination in airborne pathogens such as *Z. tritici* is an
553 aggravating factor, but it is one that is not really preventable. However, delaying the emergence of
554 single resistances and limiting the probability of recombination between them by ensuring that their
555 frequencies remain low might be a more realistic option. It has been estimated that *Z. tritic*i
556 produces at least 2.1-9.9 trillion pycnidiospores per hectare [65]. The number of *de novo* mutation
557 events associated with resistance occurring in a field may, therefore, be sufficiently high to prevent
558 all of these mutations being lost by chance, even if the mutation rate is low. The time to the
559 emergence of full resistance has been found to decrease monotonically with increasing dose, over
560 realistic ranges of pathogen life-history and fungicide dose-response parameters [22]. The maintenance
561 of low frequencies of resistance alleles during the selection phase would involve limitations on the
562 use of all MoAs, especially when the frequency of resistance reaches a critical threshold over a large
563 area. Disease control should more than ever be based on complementarity with other control
564 measures, such as the replacement of synthetic fungicides by biological agents, resistant cultivars,
565 prophylactic measures and agricultural practices, to increase the diversity of selection pressures and
566 limit the emergence of resistance by controlling population size. The overall sustainability of
567 integrated pest management may therefore rely on the vulnerability of each method to fungal
568 adaptation and then on our capacity to predict and monitor these phenomena. This would also lead
569 the community to consider criteria other than the effective life of fungicides when assessing the

durability of crop protection. Assessing qualitative changes in evolved population while evaluating the diversity of protection measures used would also help us to work towards current socioenvironmental expectations while better reflecting the multiple evolutionary processes at work. However, although adapting the way we conceive resistance management strategies to focus more on multiple resistance would be a huge step forward in itself, the implementation of these strategies in practice will probably prove an even greater challenge. Indeed, optimal disease control would imply using the best fungicides and at high rates in standardized programs. By contrast, resistance management with limited environmental impact would imply using the whole range of available AIs, whatever their intrinsic efficacy, within parsimonious combinations tailored to the current local composition of the population, associated with other control measures. These approaches are conceived for short (*i.e.* an agricultural season) and long (*i.e.* decades) time scales, respectively, and maximize profit or promote sustainable agriculture, respectively, potentially creating an intractable dilemma for growers. Education about resistance issues and communication between stakeholders and farmers are almost certainly the key to changing the paradigm and initiating a transition towards agroecology.

Materials and methods

Ancestral isolates

The susceptible ancestral isolate was the IPO-323 strain, the source of the reference genome for *Z. tritici* [66], hereafter referred to as 'S'. The bias in fitness due to genetic background was minimized by selecting resistant isolates from the progeny of a cross between 'S' and a resistant field strain [55]. The SDHI-resistant isolate, R_{SDHI}, was characterized by the substitution of an arginine (R) for the histidine (H) residue at codon 152 of the sdhC protein (*sdhC*^{H152R} resistance allele), one of the

593 subunits of succinate dehydrogenase, the target of SDHIs [12]. The DMI-resistant isolate, R_{DMI}, displayed
594 five amino-acid changes (V136A, A379G, I381V, Y461S and S524T) in the sterol 14α-demethylase
595 protein encoded by *cyp51* (the resistance allele is referred to hereafter as *cyp51*^{I381V}). A multiple
596 mutant isolate, $R_{SDHI/DMI}$, carried both resistance alleles.

597

598 Fungicide sensitivity of the ancestral isolates

599 We established the resistance profile of each strain by estimating EC_{50} values and resistance
600 factors (RFs) for a wide range of fungicides. The fungicides studied were all of technical grade and
601 included three SDHIs (benzovindiflupyr, boscalid and fluxapyroxad), four DMIs (prothioconazole-
602 desthio, metconazole, tebuconazole and mefentrifluconazole), a QoI (azoxystrobin), a benzimidazole
603 (carbendazim), a multisite inhibitor (chlorothalonil) and tolnaftate (an inhibitor of squalene
604 epoxidase, an enzyme involved in sterol biosynthesis, used in medicine and as a marker of multidrug
605 resistance). Fungicides were dissolved in DMSO and stock solutions were prepared so as to ensure
606 that the final medium contained no more than 0.5% DMSO.

607 EC_{50} values were calculated from dose-response curves plotted for data obtained in 96-well
608 microtiter plates (Sarstedt, Germany). Pure liquid cultures of each isolate were grown in 50 mL
609 borosilicate Erlenmeyer flasks containing 25 mL YPD medium (20 $g.L^{-1}$ dextrose, 20 $g.L^{-1}$ peptone, 10
610 $g.L^{-1}$ yeast extract; USBiological Salem, MA, USA), with carded cotton wool inserted into the neck. The
611 cultures were maintained at 18°C and a RH of 70% in the dark for 7 days, with shaking at 150 rpm.
612 These cultures were used to inoculate 200 µL of YPD medium per well in microtiter plates, at an
613 initial density of 2.5 x 10^5 $sp.mL^{-1}$. We tested 10 fungicide concentrations for each fungicide, in
614 addition to a control amended only with 0.5% DMSO. The design included three replicates per isolate
615 and per fungicide concentration. The plate was sealed with a gas-permeable membrane (Breathe
616 Easy®; Diversified Biotech) and placed on a shaker in the dark, in the conditions described above.
617 Optical density at 405 nm (OD_{405}) (SpectraMax M2 spectrophotometer, Molecular Devices) was

618 measured after five days, *i.e.* at the end of the exponential growth phase, with three technical
619 replicates of each well. Each experiment was repeated at least three times.
620 EC_{50} values were determined by logistic regression (logistic nonlinear mixed effects model, with the
621 SSlogis function of the Self-Starting Nls Logistic Model, available in the STATS package and R version
622 4.1.3), with dose as a fixed effect and biological replicate as a random effect.
623 RFs were calculated as the ratio of the EC_{50} of the resistant isolate to that of the susceptible isolate.
624 Standard errors were calculated as follows:

625 $$\sigma\mathrm{RF} = |RF| \times \sqrt{\left(\frac{\sigma EC_{50}R}{|EC_{50}R|}\right)^2 + \left(\frac{\sigma EC_{50}S}{|EC_{50}S|}\right)^2} \qquad (1)$$

626 where R is the resistant isolate and S is the susceptible isolate.
627

628 ## Fitness and resistance profiles of ancestral strains

629 Fitness was inferred for each isolate from its growth in the culture conditions used for
630 experimental evolution (detailed below) but without fungicides. Population size was measured daily
631 on the basis of OD_{405} data, for seven days, as previously described[26]. The experiment was performed
632 five times. The growth curve for each strain was modeled with a mixed non-linear logistic model, as
633 follows:

634 Spore concentration = *asym*/(1+exp((*xmid*-t)/*scal*)) (2)

635 where *t* is the day, *asym* the asymptote, *i.e.* maximum growth, *xmid* is the time required to
636 decrease the value of the asymptote by half and *scal* is the inverse of the slope.
637 The strains were considered as a fixed effect, with replicate as a random effect. We compared
638 parameters between strains with the Tukey method for pairwise comparisons. Estimates were
639 obtained with the NLME, CAR and EMMEANS packages of R version 4.1.3.
640

641 ## Artificial ancestral populations

642 Three ancestral populations were prepared for the first round of experimental evolution by
643 inoculating medium with pure cultures of ancestral isolates (grown as described in the previous
644 section) in various proportions so as to obtain a final density of 10^7 spores in total in 25 mL YPD
645 medium amended with 0.25% antibiotics (100 $mg.L^{-1}$ streptomycin and penicillin – to prevent
646 contamination in this long-term experiment) in 50 mL Erlenmeyer flasks (**Fig. 1C**). PopS was a naïve
647 population composed exclusively of the susceptible isolate S. PopR was composed of 95% S and of
648 5% R_{DMI} and R_{SDHI} (1:1). PopRR contained 95% S and 5% R_{DMI}, R_{SDHI} and $R_{SDHI/DMI}$ (2:2:1). This initial
649 population composition of PopR and PopRR was determined in preliminary experiments: a
650 proportion of 1% was found to be the smallest proportion providing the lowest mean and standard
651 deviation of experimental error in resistance allele quantification by qPCR or pyrosequencing (see
652 below) on a range of dilutions. As multiple resistance was expected to be scarce in natural
653 populations, we arbitrarily set the initial frequency of $R_{SDHI/DMI}$ at 1% and half that of single resistance.

654

655 Selection regimes

656 The 12 selection regimes differed in terms of four components and are described in detail in
657 **Supplementary Table 3**. The number of AIs ranged from one (straight selection) to three (diversified
658 selection). The number of MoAs ranged from one (intra-MoA selection) to three (inter-MoA
659 selection). We selected AIs representative of their MoAs from among those currently used to control
660 STB in the field. MoAs, and, thus, their representative AIs, differed in terms of their vulnerability to
661 resistance, *i.e.* intrinsic risk of resistance. They were considered to be at medium-to-high risk (SDHIs,
662 represented by benzovindiflupyr (B) and fluxapyroxad (F)), low-to-medium risk (DMIs, represented by
663 prothioconazole-desthio (P), the active metabolite of the commercialized prothioconazole, and
664 mefentrifluconazole (M)) or at very low risk (multisite inhibitor, represented by chlorothalonil (C)) of
665 resistance. We also studied the effect of temporal heterogeneity through comparisons of the
666 simultaneous application of AIs (intracycle heterogeneity, *i.e.* mixture) with the sequential

667 application of AIs (intercycle heterogeneity, *i.e.* alternation). All the alternation regimes began with
668 the AI with the lowest risk of resistance (or highest intrinsic activity for strategies with only one
669 MoA), as this has been shown to be the most effective approach [23, 67]. The two control lines included
670 only 0.5% of DMSO or ethanol 80%.

671 These 12 selection regimes and 2 control situations were applied to all or some of the three possible
672 ancestral populations, resulting in 31 possible interactions, and 100 independent lines, as there were
673 three replicates for each interaction (or four when chlorothalonil was involved) and five solvent-only
674 controls were included. Given the large size of this experiment, selection regimes were grouped
675 together into two batches (A and B) performed successively. Strategies that it was particularly
676 important to compare directly were included in the same batch. Controls were repeated in both
677 batches if necessary.

678 Fungicides were used as technical products purchased from Sigma Aldrich (US). They were dissolved
679 in 80% ethanol, with the exception of chlorothalonil, which was dissolved in DMSO due to its poor
680 solubility in ethanol. Controls in which only solvent (80% ethanol or DMSO) was added were included
681 in the experiment. Stock solutions of fungicides were prepared at the beginning of the experiment
682 and were stored at 4°C. The fungicides and solvents added to culture medium never exceeded 0.5%
683 of the final volume, to prevent toxic effects on the growth of *Z. tritici*.

684

685 Selection doses

686 For fungicides used alone (in sequence or in alternation), the selection dose was the EC_{97} of
687 each AI determined in a preliminary experiment for the S isolate grown in the conditions used for
688 experimental evolution (described below). EC_{97} values were established from dose-response curves
689 for each fungicide based on the logistic regression model used to describe the resistance patterns of
690 the ancestral isolates. The growth of the populations, inferred from OD_{450} measurement after 7 days
691 of culture, was normalized by dividing by that of the control line grown without fungicide. The

692 validation of this selection dose included culture of the S isolate in the conditions used for
693 experimental evolution at its theoretical EC_{97} and at close doses. The lowest dose providing at least
694 97% inhibition was finally defined as the selection dose.

695 The selection doses of two-way fungicide mixtures were set at half the selection dose of each AI used
696 alone, for the comparison of alternation and mixture regimes containing the same amounts of each
697 fungicide. The only three-way mixture studied contained half the EC_{97} of chlorothalonil, a quarter the
698 EC_{97} of benzovindiflupyr and a quarter the EC_{97} of prothioconazole-desthio.

699 The selection doses are detailed in **Supplementary Table 3A**.

700

701 Design of the experimental evolution experiment

702 Experimental evolution was performed over either six or eight cycles of seven days each (about six to
703 seven generations per cycle), depending on the experimental batch. This cycle duration corresponds
704 to about the time for which a control line remained in exponential growth phase before reaching a
705 plateau. On the first day of the experiment, ancestral population lines containing 10^7 spores were
706 prepared and incubated as described above, after amendment with the fungicides required for each
707 selection regime. OD_{405} was measured at the end of each cycle and transformed into population size,
708 as established in preliminary experiments (see more details in [27]). The OD_{405} of the treated lines was
709 normalized against the mean OD_{405} for the control solvent lines. At each transfer, 500 µL of the
710 evolving culture (*i.e.* 2% of the total volume of medium) or a maximum of 10^7 spores was transferred
711 to a new Erlenmeyer flask containing fresh medium. If the number of spores in the 500 µL of culture
712 medium was less than 10^7, as would be the case before the evolution of resistance, then the
713 appropriate number of spores from the appropriate source population (*i.e.* spores from the control
714 (PopS) or mixtures of spores reconstituting PopR or PopRR from the pure cultures of the different
715 isolates grown separately without fungicides) was added to make the number of spores present up

716 to 10^7. Thereby the starting population size was equalized between lines at the start of each new
717 cycle, and the process mimicked the immigration occurring in field situations. For each of the four
718 replicates, the same source population was used for immigration throughout the experiment. In
719 preliminary experiments, immigration was found to be useful for preventing population extinction
720 after weekly bottlenecks, and to minimize genetic drift.

721 At the end of each cycle, 2 mL of each line was mixed with glycerol (25%), frozen and stored at -80°C
722 for further analyses. If the normalized Malthusian growth (defined below) of a line reached at least
723 95% during three consecutives cycles, the line was ended. In such cases, we ensured fair comparison
724 between alternation and mixture approaches by stopping lines only after an even number of cycles
725 (*i.e.* not before the 4th cycle). In these situations, data (population size or allele content) for the
726 missing cycles during this establishment phase were extrapolated for analyses involving comparisons
727 between selection regimes.

728

729 Assessment of the rate of resistance evolution

730 The Malthusian growth[68] of each line was calculated as

731 $$M_{it} = \ln\left(\frac{Spore\ concentration\ of\ line\ i\ at\ the\ end\ of\ cycle\ t}{Spore\ concentration\ of\ line\ i\ at\ the\ beginning\ of\ cycle\ t}\right) \quad (3)$$

732 and normalized against that of the solvent control line $C\ (M_{Ct})$ such that $M_{it}^n = \frac{M_{it}}{M_{Ct}}$ (4).

733 For each selection regime, the mean Malthusian growth M_i^n is the mean of M_{it}^n calculated over the
734 total number of cycles the line was grown, and constitutes a quantitative summary of the increase in
735 resistance of the three replicates over the entire experiment.
736 We used beta regression (package BETAREG for R) on the M_i^n of each line to investigate the effects of
737 population composition and of the components of selection diversity and temporal heterogeneity,
738 included as fixed factors, with experimental batch as a random factor. These models are detailed in

739 SI6. We evaluated the impact of these factors with an analysis-of-variance-like (joint_tests function
740 of EMMEANS package) analysis, with the Tukey adjustment method for pairwise comparisons (package
741 EMMEANS).

742

743 Molecular quantification of resistance alleles in evolved lines

744 DNA was extracted from samples stored in microtiter plates with the DNeasy® Plant Kit
745 (Qiagen), according to the manufacturer's procedure.

746 The *sdhC*^{H152R} allele, included in the R_{SDHI} and $R_{SDHI/DMI}$ ancestral isolates, was quantified by qPCR.
747 Allele-specific primers were designed with the goal of amplifying only this allele, with a primer
748 discriminating between this and all other known alleles. The allele-specific forward primer CGT TGA
749 ATG GAG TGA GGC A and the reverse primer TGT ACC ATC TCT CTT CAT CCT C amplified the product
750 of the wild-type allele H152, whereas the allele-specific forward primer GTT GAA TGG AGT GAG GCG,
751 used with the same reverse primer, amplified the product of the mutant allele R152. The non-specific
752 forward primer TCG TTG AAT GGA GTG AGG C was used to estimate the concentrations of the
753 reference gene (wildtype, mutant). The reactions were performed with FastStart Universal SYBR
754 Green Master (ROX) (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) on a
755 Bio-Rad CFX-384 machine. The PCR was run under the following conditions: initial denaturation at
756 95°C for 10 min, 40 cycles at 95°C for 15 s, 58°C for 30 s and 75°C for 30 s (measurement of
757 fluorescence). A melting curve, from 65°C to 95°C was recorded after the PCR. The detection
758 threshold was estimated at 1%.

759 The *cyp51*^{I381V} allele, included in the R_{DMI} and $R_{SDHI/DMI}$ ancestral isolates, was quantified by
760 pyrosequencing. A 216 bp fragment containing the 381 codon was amplified with the primer pair CCC
761 GAC ATC CAA GAC GAA C and Biotin-TGG AAT GAC GTA TGC CGT ACC. The PCR conditions were as
762 follows: initial denaturation at 95°C for 2 min, 50 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30

763 s and a final elongation at 72°C for 5 min (GoTaq® Hot Start Polymerase, Promega, Madison, WI). We
764 prepared 20 µL of PCR product for pyrosequencing reactions with the PyroMark Q96 Vacuum
765 Workstation (Qiagen) and Streptavidin Sepharose High-Performance Beads (GE Healthcare Bio-
766 Sciences AB, Uppsala, Sweden), as described in the workstation instructions and in the PyroMark Q96
767 ID user manual. The single-stranded DNA templates were transferred to 40 µL annealing buffer
768 (Qiagen) containing the sequencing primer. The primer used in the reaction was ACC CTT CGT ATT
769 CAC G [0.4 µM] for sequencing the codon 381 mutation site. The sequencing run was set up with
770 PyroMark-Software v.1.0 (Qiagen). Samples were incubated at 80° C for 2 min and then allowed to
771 cool to room temperature. The sequencing reaction was then performed with PyroMark Gold Q96
772 reagents on a PyroMark Q96 ID machine (both from Qiagen). The dispensation order of the
773 nucleotides was AGC GTC AGA TCA. The detection threshold was estimated at 5% for the I381V
774 substitution in preliminary experiments.

775 The *cytb*^{G143A} allele, included only in the $R_{SDHI/DMI}$ ancestral isolate was quantified by qPCR. The
776 forward primers were allele-specific discriminating for the wild type allele G143 (ACC TTA TGG TCA
777 AAT GTC TTT ATG ATG) and for the mutant allele A143 (ACC TTA TGG TCA AAT GTC TTT ATG ATC).
778 The conserved reverse primer used was AGC AAA GAA TCT GTT CAA TGT TGC. The reactions were
779 performed with FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics GmbH, Roche
780 Applied Science, Mannheim, Germany) on a Bio-Rad CFX-384 machine. The PCR was run under the
781 following conditions: initial denaturation at 95°C for 10 min, 40 cycles at 95°C for 15 s, 60°C for 30 s
782 and 71°C for 30 s (measurement of fluorescence). The detection threshold was estimated at 1%.

783 From qPCR and pyrosequencing data, we calculated RC_{it}^{n}, the normalized concentration of the
784 resistant mutants R_{SDHI} and R_{DMI} in the population, as the proportion of the resistance alleles *sdhC*^{H152R}
785 and *cyp51*^{I381V}, respectively, multiplied by the size of the population (in spores.mL^{-1}):

786 $$RC_{it} = Resistance\ allele\ frequency * C_{it} \quad (5)$$

787 where C_{it} is the concentration of the line *i* at time *t*.

788 We then divided this value by the spore concentration of the control lines, C_{Ct}, to obtain the
789 normalized concentration of resistance (hereafter referred to as the normalized allelic
790 concentration):

791 $$RC_{it}^{n} = \frac{RC_{it}}{C_{Ct}} \quad (6)$$

792 The qPCR and pyrosequencing methods used could not distinguish combinations of *sdhC*^{H152R} and
793 *cyp51*^{I381V} within the same individual (*i.e.* as in $R_{SDHI/DMI}$) from co-occurrence of the same alleles in
794 different individuals (*i.e.* as in the R_{SDHI} and R_{DMI} isolate). We therefore calculated the maximum
795 possible concentration of this double resistant mutant as the proportion of the two resistant alleles
796 together. This calculation may overestimate the concentration of the $R_{SDHI/DMI}$ isolate in PopRR. For
797 validation of this estimate, we compared these data with those for *cytb*^{G143A} quantification on
798 samples collected from the last cycles of PopRR lines.
799 For each of the three resistance alleles, we used beta regression (package BETAREG for R) on the mean
800 normalized allele concentrations of each line to investigate the effects of population structure, with
801 the components of selection diversity and temporal heterogeneity treated as fixed factors, and
802 experimental batch as a random factor. We evaluated the impact of factors with an analysis-of-
803 variance-like (joint_tests function of EMMEANS package) approach with the Tukey adjustment method
804 for pairwise comparisons (package EMMEANS). Details of these models are provided in SI6.

805

806 Phenotype resistance profiles of evolved lines

807 The cross-resistance patterns of all populations collected at the end of the experimental
808 evolution were established in droplet tests. We deposited suspensions containing 10^7, 10^6, 10^5, and
809 10^4 spores.mL^{-1} as drops organized into columns, on 12 cm square Petri dishes. These plates
810 contained YPD supplemented with discriminatory doses of fungicides, as detailed in **Supplementary**
811 **Table 3B**. The 10 treatments tested encompassed a control with 0.5% solvent and fungicides B, P, F,
812 M and C at their selection doses and their mixtures. Finally, for the detection of generalist resistance,

813 we also included fungicides not used for experimental evolution (azoxystrobin and tolnaftate).
814 Tolnaftate, in particular, identifies individuals displaying enhanced fungicide efflux leading to non-
815 specific multidrug resistance (MDR), as described in field isolates of *Z. tritici* [17]. Ancestral isolates, and
816 a MDR isolate collected in the field were included as control strains. After seven days of incubation,
817 drops were scored 0 or 1, according to the presence or absence of fungal growth. A total score was
818 established for each strain, ranging from 0 (susceptible) to 4 (resistant if >0), according to the
819 number of dilution droplets for which growth was observed. For each strain, the resistance
820 phenotype profile was determined by concatenating the scores established for the 10 sets of
821 fungicide conditions.

822 The distributions of resistance phenotype profiles between and within selection regimes were
823 plotted as heatmaps. The Euclidean pairwise distance was used for the hierarchical clustering of
824 these profiles, with dendrograms for the rows and columns, in R version 4.1.3. A principal
825 component analysis (PCA) was also performed on the same phenotypes.

826

827 Characterization of enhanced efflux

828 The 17 strains isolated from lines growing on tolnaftate in droplet tests were selected for
829 further investigation. We checked for changes in the length of the promoter of *mfs1* by PCR, as
830 previously described [37]. Several types of insertion into the promoter of this gene encoding a MFS
831 transporter have been shown to cause enhanced efflux leading to multidrug resistance in field
832 isolates of *Z. tritici*. We quantified *mfs1* expression in the same isolates by RT-qPCR [18]. The protocols
833 used are detailed in SI5.

Data availibity

Data and scripts were deposited on the Research Data Gouv repository under DOI https://doi.org/10.15454/DCVDZN.

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Supporting Information

Supplementary information 1: Phenotype of ancestral isolates

Supplementary information 2: Selection regimes used for experimental evolution

Supplementary information 3: Doses of fungicides used for experimental evolution

Supplementary information 4: cytb^{G143A} content of evolved lines for PopRR

Supplementary information 5: Expression of *mfs1* in evolved lines of *Zymoseptoria tritici*

Supplementary information 6: Models used for data analysis

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Author contributions

AB, ASW, FC and CU conceived and designed the study. AB and CU performed the experimental evolution experiment with the help of CD. CU, AB and CD isolated evolved strains and established their phenotypes and genotypes. AN analysed the expression of *mfs1* in evolved isolates. JW and ST quantified alleles content in evolved populations. AB and FC performed the statistical analysis, with contributions from ASW and AD. The paper was written by AB and ASW, with additional contributions from all authors.

Competing interests

The authors declare no competing interests.

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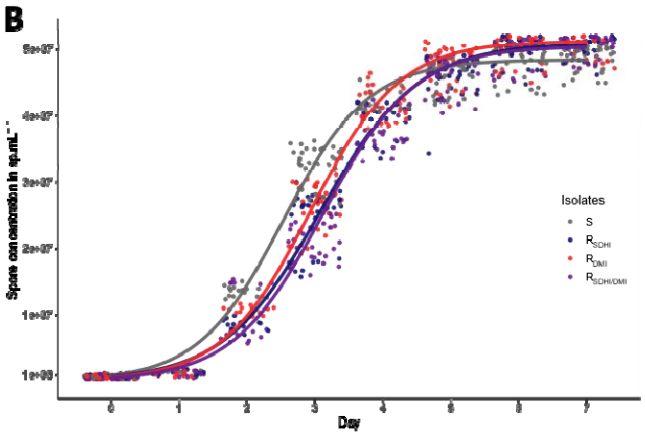
Figures

A

Chemical class	Fungicide	EC_{50} (mg.L^{-1})	Resistance factor (RF)		
		S	R_{SDHI}	R_{DMI}	$R_{SDHI/DMI}$
SDHIs	Benzovindiflupyr	0.028 (0.003)	92.61 (18.4)	3.73 (0.53)	247.8 (191.6)
	Boscalid	0.242 (0.013)	> 410	1.53 (0.13)	> 410
	Fluxapyroxad	0.053 (0.005)	> 950	2.9 (0.53)	> 950
SBIs class I (DMIs)	Mefentrifluconazole	0.00097 (0)	0.98 (0.07)	9 (1.44)	13.22 (2.82)
	Metconazole	0.0038 (0.001)	1.06 (0.19)	22.76 (5.04)	34.78 (4.74)
	Prothioconazole-desthio	0.0016 (0)	0.86 (0.13)	25.61 (3.51)	27.7 (5.93)
	Tebuconazole	0.0076 (0.002)	1.03 (0.48)	40.15 (14.06)	5.86 (2.55)
Benzimidazoles	Carbendazim	0.115 (0.037)	> 430	> 430	> 430
QoIs	Azoxystrobin	0.047 (0.004)	0.61 (0.11)	0.7 (0.11)	454.1 (787)
Multisites	Chlorothalonil	1.185 (0.104)	0.42 (0.04)	0.67 (0.08)	0.78 (0.09)
SBIs class IV	Tolnaftate	0.23 (0.022)	1.47 (0.33)	1.34 (0.2)	0.5 (0.07)

B



C

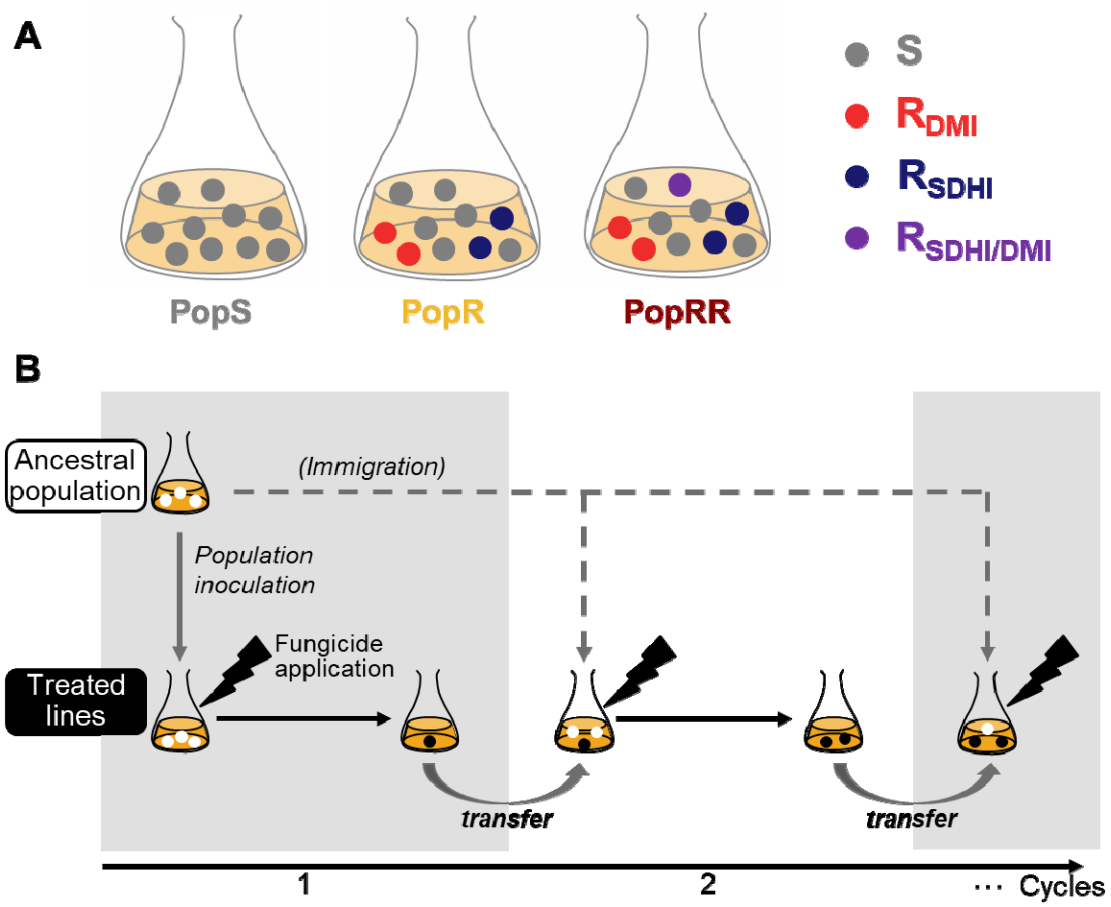
Isolates	xmid	slope	asym
S	2.54 (0.05) a	1.61 (0.02) a	4.84E+07 (4.37E+05) a
R_{SDHI}	3.08 (0.05) bc	1.41 (0.05) b	5.08E+07 (4.43E+05) b
R_{DMI}	2.94 (0.05) b	1.54 (0.02) ab	5.12E+07 (4.40E+05) b
$R_{SDHI/DMI}$	3.13 (0.05) c	1.49 (0.02) ab	5.04E+07 (4.42E+05) b

D

Isolates	Genotypes	Phenotypes		Population composition		
		SDHI	DMI	PopS	PopR	PopRR
S	*sdhc*^{WT}; *cyp51*^{WT}	S	S	100%	95%	95%
R_{SDHI}	*sdhc*^{H152R}; *cyp51*^{WT}	R	S	-	2.50%	2%
R_{DMI}	*sdhc*^{WT}; *cyp51*^{I381V}	S	R	-	2.50%	2%
$R_{SDHI/DMI}$	*sdhc*^{H152R}; *cyp51*^{I381V}	R	R	-	-	1%

Figure 1: ***Features of the ancestral isolates and populations of Z. tritici. used in experimental evolution. A. Profiles of susceptibility to multiple fungicides of ancestral isolates of Z. tritici.*** The S isolate is the fully susceptible isolate IPO-323 (wild-type genotype). R_{SDHI}, R_{DMI} and $R_{SDHI/DMI}$ were isolated from a second-generation progeny with IPO-323 and a field isolate as ancestors. This ancestral field isolate carried the H152R substitution in SdhC (*i.e.* a subunit of succinate dehydrogenase, the target of SDHI fungicides), causing high-level resistance to SDHIs [30] and the V136A, A379G, I381V, Y461S and S524T substitutions in cyp51 (*i.e.* sterol

1114 14α-demethylase, the target of DMI fungicides), causing moderate resistance to DMIs [11]. These genotypes were
1115 simplified to *sdhC*^{H152R}and *cyp51*^{I381V}, respectively, as these were the specific substitutions quantified
1116 molecularly in subsequent experiments. The ancestral isolates used in the ancestral populations displayed
1117 either none (S), one (R_{SDHI}, R_{DMI}) or two ($R_{SDHI/DMI}$) of these changes. The susceptibility to multiple fungicides of
1118 these ancestral isolates was expressed as *the* EC_{50} and RFs (*i.e.* $EC_{50\ R\ isolate}$ / $EC_{50\ S\ isolate}$) calculated after the
1119 establishment of dose-response curves with cultures in microtiter plates and a logistic non-linear mixed-effects
1120 model. Dose-response curves were plotted one to five times, depending on the AI, with four technical repeats.
1121 The standard error is specified in brackets. Within- and between-MoA differences in intrinsic activity were
1122 visible for the S isolate, but resistant isolates had the resistance profiles expected from their genotypes. Cross-
1123 resistance was confirmed within DMIs and SDHIs. In addition, the R_{SDHI}, R_{DMI} and $R_{SDHI/DMI}$ isolates inherited
1124 resistance to benzimidazoles (*e.g.* due to *tub2*^{E198A} [33]) from their field ancestor. Similarly, the $R_{SDHI/DMI}$ isolate
1125 inherited the *cytb*^{G143A} substitution, leading to resistance to QoIs [62]. ***B. Fitness of ancestral isolates in the***
1126 ***conditions used for experimental evolution.*** Growth was measured in 50 mL Erlenmeyer flasks as the increase
1127 of spore concentration (spores.mL^{-1}) over time (7 days), without fungicide, at 18°C, with shaking at 150 rpm, in
1128 the dark, for cultures of single isolates. The experiment was performed five times and the data were modeled
1129 with a mixed non-linear logistic regression model. ***C. Estimated parameters of the growth curves for ancestral***
1130 ***isolates.*** The standard error is shown in brackets. For each curve, *asym* is the asymptote (*i.e.* maximum
1131 growth); *xmid* is the time required to decrease the asymptote by 50%. The slope is the inverse of the *scal*
1132 parameter provided in the model; the standard error and statistics are shown for *scal.* Pairwise comparisons
1133 were adjusted with the Tukey method for multiple comparisons to determine the significance of differences
1134 (P<0.05) between isolates, with significant differences indicated by different group letters. ***D. Artificial***
1135 ***populations of* Z. tritici *used for experimental evolution.*** The evolution of a fully susceptible PopS population
1136 was compared to that of the PopR and PopRR populations, each containing 5% resistant isolates. PopR
1137 contained only isolates bearing single resistances, whereas PopRR contained isolates with both single and
1138 multiple resistances.
1139



C Modes of action

SDHI **DMI**
(concerned by resistance)

B — Benzovindiflupyr
P — Prothioconazole-desthio
F — Fluxapyroxad
M — Mefentrifluconazole

Multisite
(naive)

C — Chlorothalonil

Selection regimes (4-8 cycles)

Unisite(s) **Single-site+multisite**

Sequence …
Alternation … …
Mixture … …

1140

1141 **Figure 2: Experimental evolution design.** ***A. Ancestral populations.*** Isolate S (susceptible) in gray; R_{SDHI}
1142 (resistant to SDHIs) in blue; R_{DMI} (resistant to DMIs) in red; $R_{SDHI/DMI}$ (resistant to SDHIs and DMIs) in purple. No
1143 more than 5% of the isolates introduced into the PopR and PopRR populations were resistant. ***B. General***
1144 ***design of the experimental evolution.*** The three ancestral populations were used to found 100 lines, with each
1145 Erlenmeyer flask inoculated with 10^7 spores. Fungicides were added to the treated lines to mimic 26 patterns
1146 of selection. After seven days, 2% of the population was used to inoculate fresh medium to start the next cycle,
1147 supplemented, if necessary, by immigration from the untreated control line to achieve a total of 10^7 spores.
1148 This procedure was repeated for up to six-eight cycles in three replicate lines per fungicide treatment. ***C.***
1149 ***Fungicides and selection regimes.*** Five fungicides (indicated by specific labels and colors) were studied: two
1150 SDHIs, benzovindiflupyr and fluxapyroxad (B and F; shades of blue; moderate resistance risk); two DMIs,
1151 prothioconazole-desthio and mefentrifluconazole (P and M; shades of red; low resistance risk); and the
1152 multisite inhibitor chlorothalonil (C; green; no risk of resistance). Fungicides were used in sequence, alternation
1153 or in mixture (see **Supplementary Table 2** for detailed description of selection regimes).

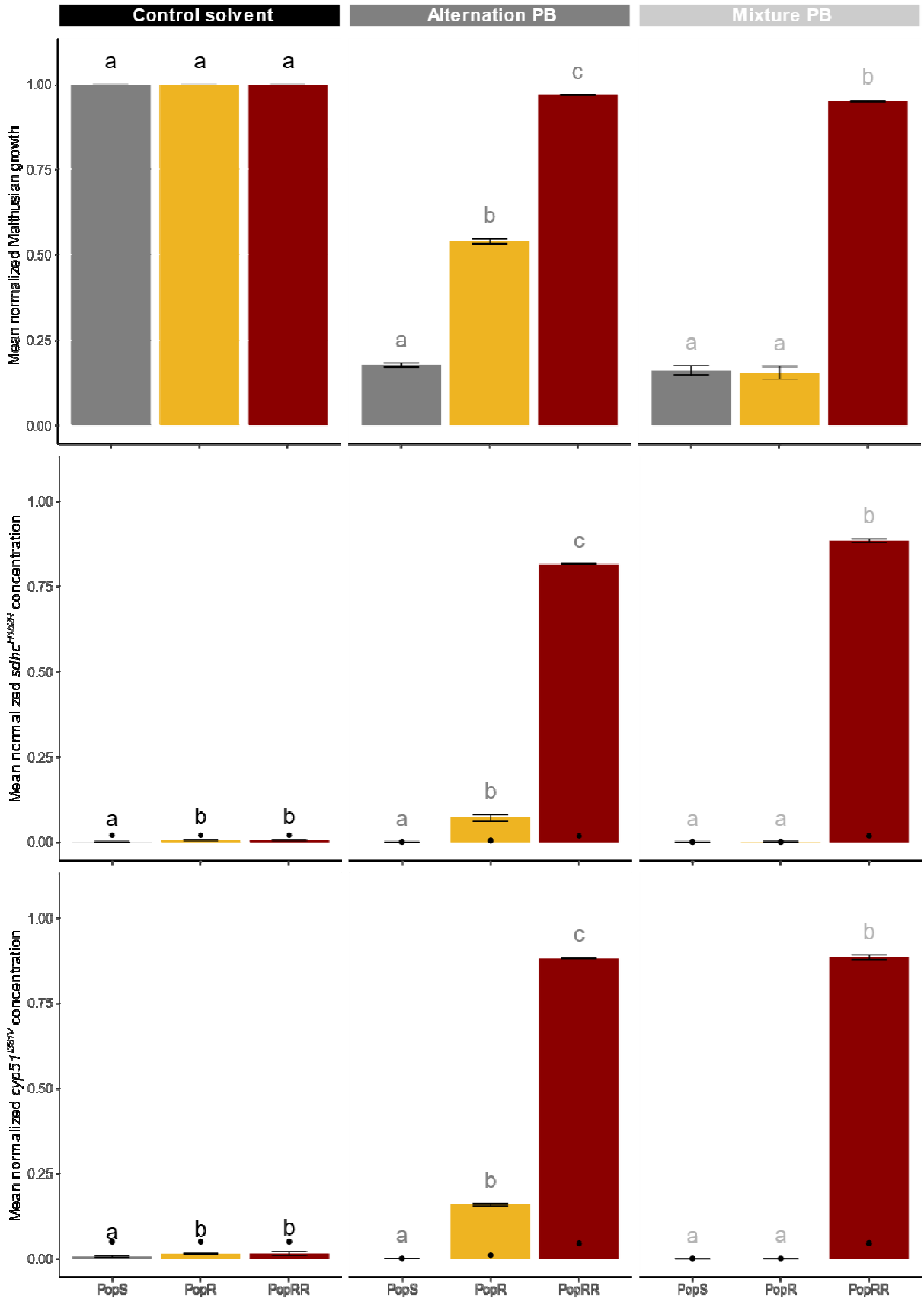

Control solvent
Alternation PB
Mixture PB
Mean normalized Malthusian growth
Mean normalized sdhc^H152R concentration
Mean normalized cyp51^I381V concentration
1.00
0.75
0.50
0.25
0.00
a
b
c
PopS
PopR
PopRR

Figure 3: Performance of management strategies according to the resistance composition of the ancestral population of *Z. tritici*. Selection regimes include the mixture or alternation of benzovindiflupyr (B) and prothio-desthio (P), contrasted with a control amended only with 0.5% solvent. Ancestral populations (PopS, PopR and PopRR, respectively in gray, yellow and dark red) undergoing selection as described in **Supplementary Table 2**. Each selection regime was applied to three independent lines, for each ancestral population. ***A: Mean normalized Malthusian growth.*** For each strategy, pairwise comparisons of lines evolved from different ancestral populations were adjusted with the Tukey method for multiple comparisons (MODM0; SI2). Significant differences (P<0.05) are indicated by different group letters. The similar growth observed in all control lines (0.9988<P<0.9998) suggests that all populations were similarly fit. The PopS and PopR lines were controlled to a similar extent in the mixture strategy (P=0.7284) but not in the alternation strategy (P=0.0001). PopRR lines were never controlled by fungicides. Overall, population composition strongly determined the performance of strategies, and the extent of this effect was also dependent on the presence or absence of multiple resistance. ***B: Mean normalized concentration of sdhc^{H152R}.*** This allele confers high-level resistance to fungicide B and was quantified by qPCR. Black dots indicate the detection limit of this allele. For each strategy, pairwise comparisons of lines evolved from distinct ancestral population were adjusted with the Tukey method for multiple comparisons (MODR0; SI2). Significant differences (P<0.05) are indicated by different group letters. *sdhC^{H152R}* was intensively selected by B, used in mixture or alternation, in ancestral populations containing multiple resistance (P<0.001). By contrast, the selection of this allele was moderate even if significant (P<0.001) in PopR lines subjected to an alternation of B and P and this allele was not selected in the presence of a mixture of B and P (P=0.0731). ***C. Mean normalized concentration of cyp51^{I381V}.*** This allele confers moderate resistance to P and was quantified by pyrosequencing. Red dots represent the detection limit of this allele. Statistics as for ***B*** (MODV0; SI2). *cyp51^{I381V}* was intensively selected by P, whether used in mixture or alternation, in ancestral populations containing multiple resistance (P<0.001). By contrast, the selection of this allele was moderate even if significant (<0.001) in PopR lines subjected to an alternation of B and P and this allele was not selected in the presence of a mixture of B+P (P=0.2317).

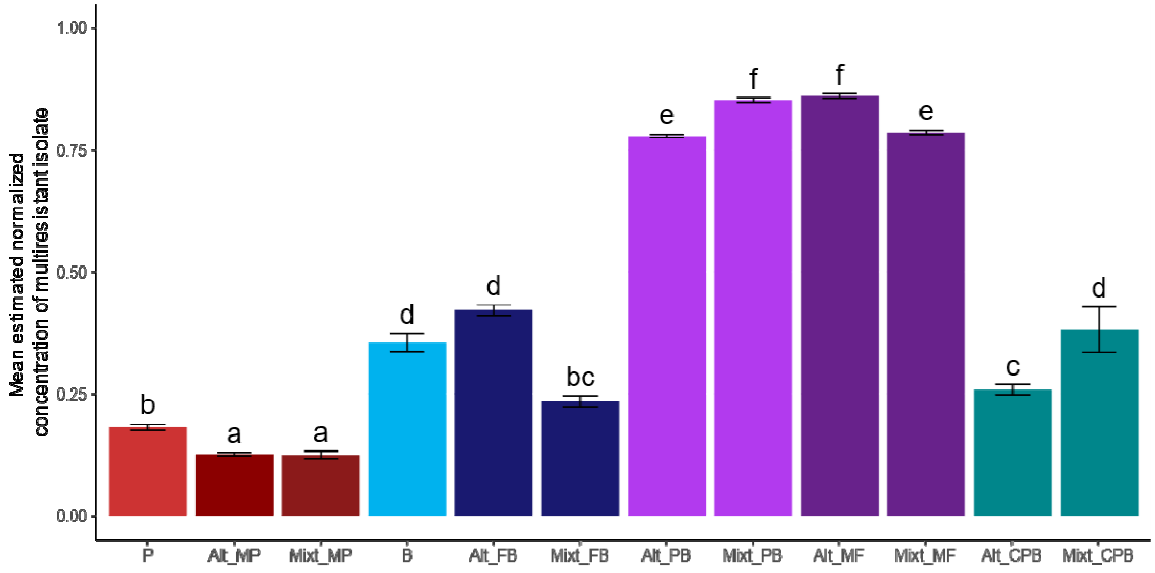


Figure 4: Mean maximal concentration of the multiresistant isolate in lines evolved from the PopRR ancestral population. Molecular methods used to quantify allele content could not distinguish combinations of *sdhC*^{H152R} and *cyp51*^{I381V} within the same individual (*i.e.* as in $R_{SDHI/DMI}$) from co-occurrence of the same alleles in different individuals (*i.e.* as in the R_{SDHI} and R_{DMI} isolates). The maximum possible concentration of this double mutant is shown as the lower proportion of the two resistant alleles quantified independently and is averaged over the different cycles of the experiment. Pairwise comparisons of lines evolved under different selection regimes were adjusted with the Tukey method for multiple comparisons (MODDM0; SI2). Multiple resistance was preferentially selected in lines subjected to diversified SDHI and DMI selection, as opposed to straight selection in the presence of a single fungicide or treatment with a mixture or alternation of fungicides sharing the same mode of action ($P<0.001$; MODDM1). These findings reveal a resistance cost associated with the $R_{SDHI/DMI}$ ancestral isolate in competition with isolates carrying a single resistance gene in the presence of homogeneous selection pressure.

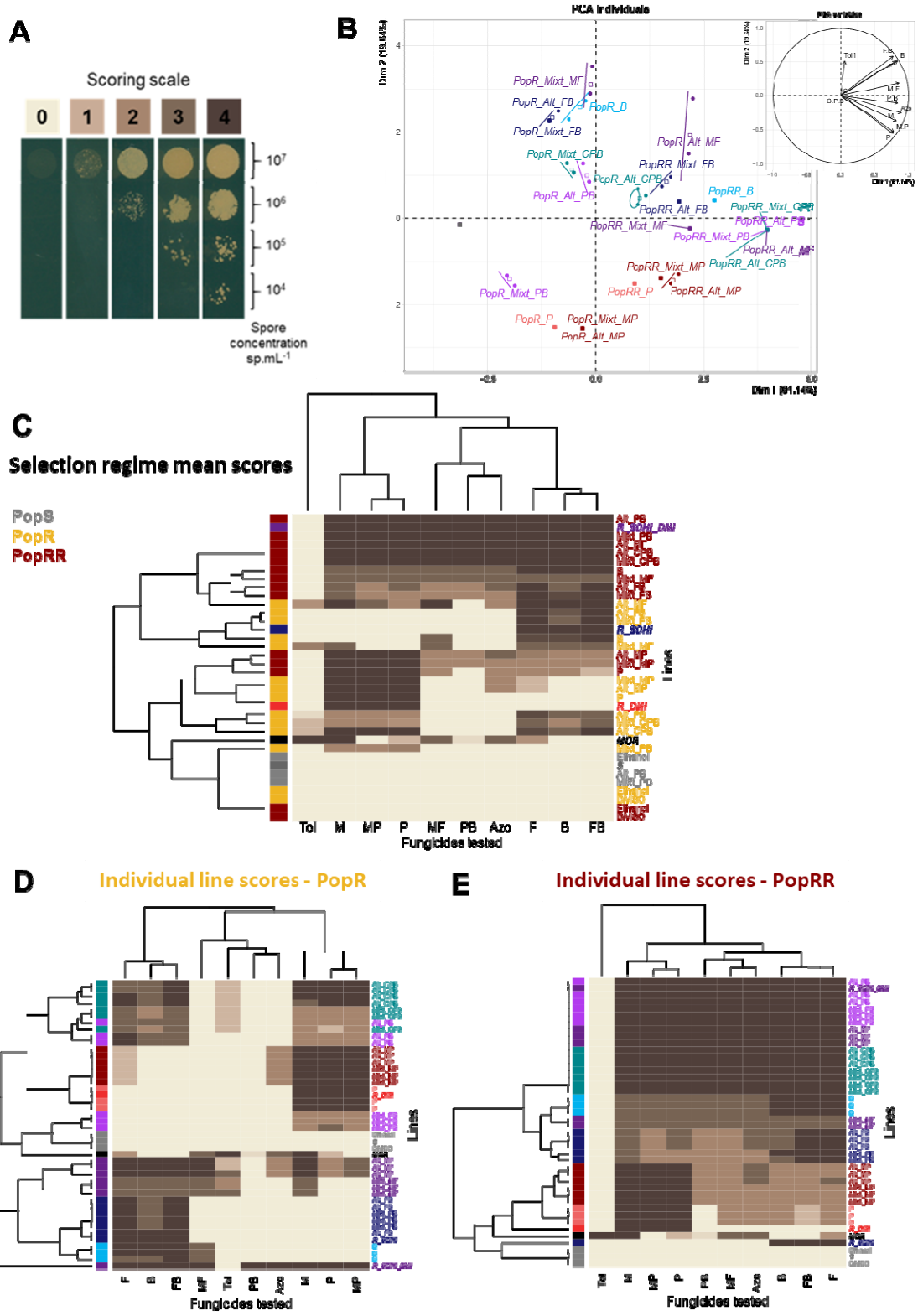


Figure 5: Phenotype resistance profiles of *Z. tritici* observed at the end of experimental evolution. The names of the ancestral isolates and selection regimes are as in **Fig. 1** and **Supplementary Table 2**. MDR: MDR isolate

collected in the field [17] serving as a reference for generalist resistance and exhibiting high resistant to tolnaftate. Scoring after the 4th or 6th cycle, according to selection regime. ***A. Scoring scale used to assess the growth of lines on solid medium supplemented with discriminatory doses of fungicides (droplet tests).*** For each population, 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 benzovindiflupyr (B), fluxapyroxad (F), prothioconazole-desthio (P) and mefentrifluconazole (M) at their selection doses (EC_{97}) and in mixture (1/2 EC_{97} of each), as well as azoxystrobin (Azo) and tolnaftate (Tol) which were not used for experimental evolution, at the minimal dose controlling the ancestral susceptible ancestral isolate. ***B: Individual graph from the principal component analysis performed on the phenotype resistance profiles of the evolved lines***. Colors according to selection regimes (ellipses for each ancestral population line x selection regime). The inset graphic shows the PCA variables. The first axis of the PCA discriminated the evolved lines according to their ancestral populations, based on their resistance phenotype profiles. ***C: Heatmaps of average resistance phenotype profile scores for all selection regimes and ancestral populations.*** Mean of the scores established for the three replicates subjected to the same selection regime. Resistance phenotype profile is mostly driven by ancestral isolates at the end of the experiment and variations of the score for a given test modality may reflect contrasting population compositions. ***D: Heatmaps of phenotype resistance profile scores for individual PopR lines***. Ancestral or field strains are shown in bold. Profiles of lines presenting no or few variability between repeats are shown as their mean in order to improve heatmap visibility and are marked with an asterisk. Individual scores for the three replicates of each selection regime are shown otherwise. New resistance to azoxystrobin and tolnaftate was selected in lines subjected to mixtures or an alternation of M and F. ***E: Heatmaps of phenotype resistance profile scores for individual PopRR lines***. Ancestral or field strains are shown in bold. Profiles of lines presenting no or few variability between repeats are shown as their mean in order to improve heatmap visibility and are marked with an asterisk. Individual scores for the three replicates of each selection regime are shown otherwise. No additional resistance to tolnaftate, which would have suggested MDR, was selected.

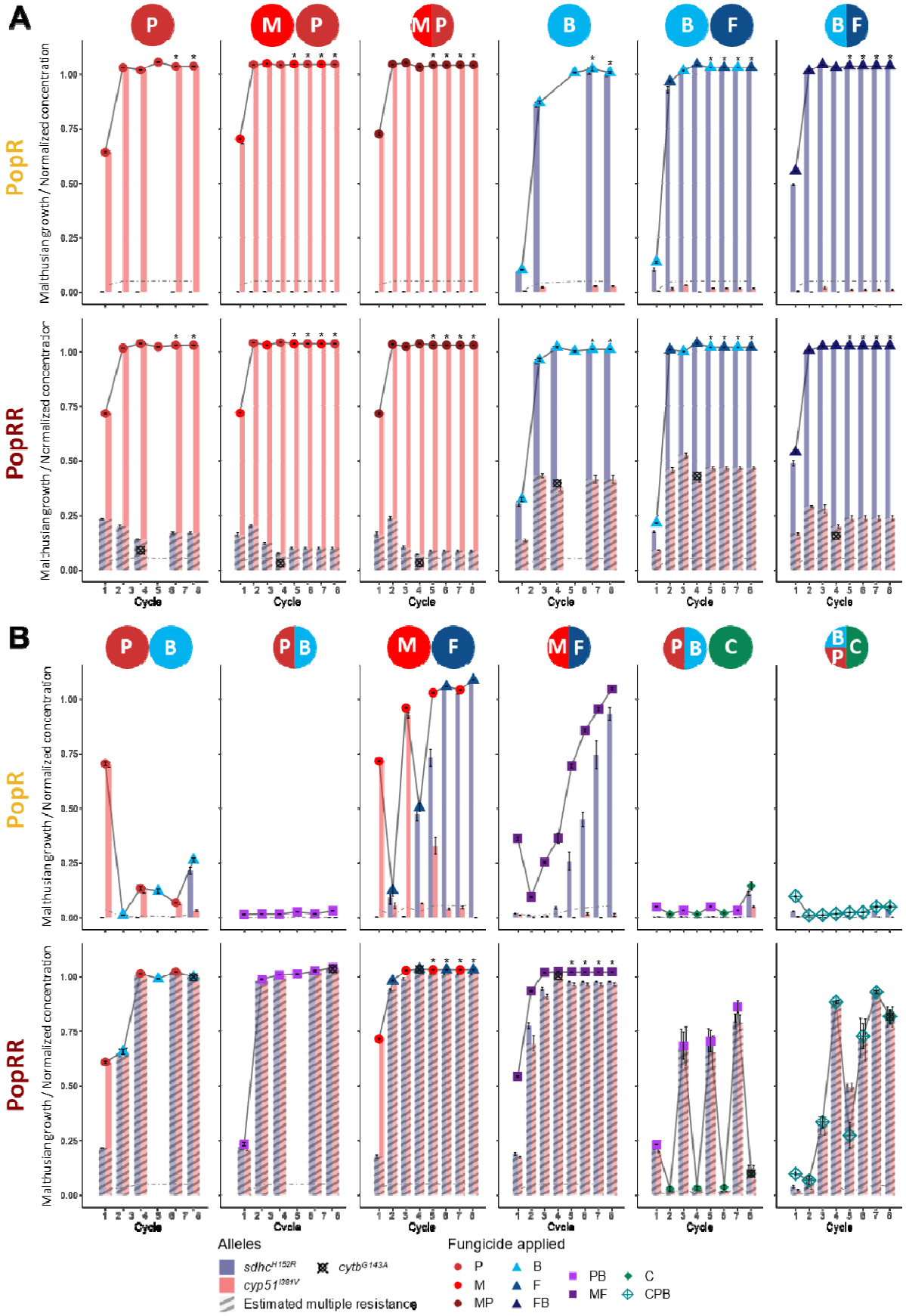

A
B
PopR
PopRR
Malthusian growth / Normalized concentration
Cycle
Alleles
sdhc^H152R
cyp51^I381V
cytb^G143A
Estimated multiple resistance
Fungicide applied
P
M
MP
B
F
FB
PB
MF
C
CPB

Figure 6: Performance of management strategies according to the selection regime applied to the ancestral population of *Z. tritici*. Light gray lines show the change in Malthusian growth in evolved lines over the eight cycles of experimental evolution. Selection regimes are indicated at the top of the panels and refer to the sequence (one fungicide dot), alternation (two successive fungicide dots) or mixture (one shared dot) of fungicides applied. The color and shape of the symbols in the graphs indicate the selecting fungicide used at a given cycle. Bars show the change in the concentration of resistance alleles (*sdhC*^{H152R} in blue and *cyp5*^{I381V} in red). The dashed portion of these bars corresponds to the maximum possible proportion of the multiresistant ancestral strain $R_{SDHI/DMI}$. The final concentration of *cytb*^{G143A} measured in PopRR lines is indicated by a black crossed circle. Dashed gray lines represent the detection limit of the alleles. ***A. Performance of strategies with poorly diverse selection pressure.*** Increasing the number of AIs within a strategy did not significantly improve Malthusian growth (MODM1, *P*=0.5464), normalized resistance allele concentration (MODR1, *P*=0.0706; MODV1, *P*=0.1384) or the normalized concentration of the multiple mutant (MODDM1, *P*=0.1155). In particular, combining AIs sharing the same MoA (SDHI or DMI) did not reduce the growth of the population, actually even increasing it (MODM1.1, *P*=0.9358 for DMIs and *P*<0.0001 for SDHIs), whilst also accelerating the selection of single resistance alleles (MODR1.1, *P*<0.0001; MODV1.1, *P*=0.0236) in PopR lines, and limiting the selection of multiple resistance in PopRR lines, particularly under DMI exposure (MODDM1.1, *P*=0.3022). DMI-based strategies resulted in 1.04 times higher mean normalized Malthusian growth than SDHI-based strategies, whatever the initial population structure (PopR and PopRR, MODM1.1, *P*<0.0001), the number of AIs (1 or 2, MODM1.1, P<0.0001) or the temporal pattern of fungicide application (alternation and mixture, MODM2.1, *P*<0.0001). In PopRR lines, SDHIs controlled the isolate with multiple resistance less efficiently than DMIs (MODDM1.1, *P*<0.0001). The temporal pattern of fungicide application had little or no effect on Malthusian growth when AIs with the same MoA were combined (MODM2.1, *P*<0.0001 for SDHIs only, *P*=0.7783 for DMIs only) and on the selection of single resistance alleles (MODR2.1, in the presence of SDHIs P=0.7833, in the presence of DMIs only *P*=0.9597; MODV2.1, in the presence of DMIs only *P*=0.8505). ***B. Performance of strategies with highly diverse selection pressure.*** Combining AIs with different MoAs (SDHIs and DMIs) significantly reduced growth (modM1, *P*<0.0001) and the selection of resistance alleles (MODR1, *P*<0.0001; MODV1, *P*<0.0001) when only single resistances were introduced into the initial population. This benefit was not observed in PopRR lines (MODM1, *P*=0.2287), due to the early and rapid selection of the multiple resistant ancestral strain as opposed to its counterselection in the presence of a single MoA (MODDM1, *P*<0.0001;

MODG143A1, *P*<0.0001). Strategies combining P and B were more efficient than those combining M and F for decreasing Malthusian growth (MODM2.2, *Ps*<0.0001) or the selection of single resistance alleles (MODR2.2 or MODV2.2, *Ps*<0.001) in PopR lines, and, to a much lesser extent, in PopRR lines (MODDM2.2, *P*= 0.0296). The addition of a previously unused AI (C) maintained (PopR lines; MODM3, *P*<0.0001) or restored (PopRR lines; MODM3, *P*<0.0001) the performance of strategies, but the utility of this driver depended strongly on its dose and redundancy in the strategy in which both B and P lost efficacy due to the establishment of multiple resistance (PopRR lines). Mixtures outperformed alternation when SDHIs and DMIs were combined (Malthusian growth MODM2.2, P<.001) but only if exclusively single resistances were present in the initial population. Mixture and alternation strategies performed similarly otherwise, but with alternation limiting the Malthusian growth of the multiresistant mutant more efficiently when a third previously unused MoA was added to SDHIs and DMIs (MOD3, *P*<0.001).

3 Supplementary Information

Supplementary information 1: Phenotype of ancestral isolates

EC_{50} *(mg.L^{-1})*	S	R_{SDHI}	R_{DMI}	$R_{SDHI/DMI}$
Benzovindiflupyr	0.028	2.556	0.103	6.84
	(±0.003) (*n*=2)	(±0.407) (*n*=3)	(±0.008) (*n*=2)	(±5.224) (*n*=4)
Boscalid	0.242	>100	0.37	>100
	(±0.013) (*n*=4)	(*n*=1)	(±0.025) (*n*=4)	(*n*=1)
Fluxapyroxad	0.053	>50	0.152	>50
	(±0.005) (*n*=3)	(*n*=1)	(±0.024) (*n*=3)	(*n*=1)
Mefentrifluconazole	0.001	0.001	0.009	0.013
	(±0) (*n*=3)	(±0) (*n*=3)	(±0.001) (*n*=2)	(±0.003) (*n*=3)
Metconazole	0.004	0.004	0.087	0.132
	(±0.001) (*n*=2)	(±0) (*n*=2)	(±0.015) (*n*=2)	(±0.003) (*n*=1)
Prothioconazole-desthio	0.002	0.001	0.042	0.045
	(±0) (*n*=3)	(±0) (*n*=3)	(±0.004) (*n*=3)	(±0.009) (*n*=3)
Tebuconazole	0.008	0.008	0.305	0.044
	(±0.002) (*n*=2)	(±0.003) (*n*=2)	(±0.068) (*n*=2)	(±0.015) (*n*=2)
Carbendazim	0.115	>50	>50	>50
	(±0.037) (*n*=3)	(*n*=3)	(*n*=3)	(*n*=3)
Azoxystrobin	0.047	0.029	0.033	21.401
	(±0.004) (*n*=4)	(±0.004) (*n*=3)	(±0.004) (*n*=5)	(±37.046) (*n*=4)
Chlorothalonil	1.185	0.494	0.789	0.927
	(±0.104) (*n*=3)	(±0.021) (*n*=2)	(±0.061) (*n*=3)	(±0.076) (*n*=2)
Tolnaftate	0.23	0.338	0.307	0.116
	(±0.022) (*n*=4)	(±0.068) (*n*=3)	(±0.036) (*n*=4)	(±0.011) (*n*=3)

Supplementary Table 1: Susceptibility of ancestral isolates to various fungicides. The susceptibility of the ancestral isolates is expressed as the *EC_{50}s (mg.L^{-1}) calculated from dose-response curves established with cultures in microtiter plates and a* logistic non-linear mixed-effects model. Dose-response curves were plotted one to five times, depending on the AI (number of replicates specified in brackets), with four technical replicates. The standard error is specified in brackets.

Supplementary information 2: Selection regimes used for experimental evolution

Population tested	Number of AIs		Number of MoAs	Vulnerability to resistance (resistance risk)					Temporal pattern of fungicide application		Name of the selection regime	Experimental batch
	Number of fungicides	*Includes a multisite inhibitor*		*Medium/High (SDHI)*		*Medium/Low (DMI)*		*Very low (multisite)*	*Intra-cycle (mixture)*	*Inter-cycle (alternation)*		
				Benzovindiflupyr (B)	*Fluxapyroxad (F)*	*Prothioconazole-desthio (P)*	*Mefentrifluconazole (M)*	*Chlorothalonil (C)*				
PopS	0		0								PopS_Ethanol	A
PopS	2		2	X		X			X		PopS_Mixt_PB	A
PopS	2		2	X		X				X	PopS_Alt_PB	A
PopR	0		0								PopR_Ethanol	A/B
PopR	0		0								PopR_DMSO	B
PopR	1		1	X							PopR_B	A
PopR	1		1			X					PopR_P	A
PopR	2		1	X	X				X		PopR_Mixt_BF	B
PopR	2		1	X	X					X	PopR_Alt_BF	B
PopR	2		1			X	X		X		PopR_Mixt_MP	B
PopR	2		1			X	X			X	PopR_Alt_MP	B
PopR	2		2	X		X			X		PopR_Mixt_PB	A
PopR	2		2	X		X				X	PopR_Alt_PB	A
PopR	2		2		X		X		X		PopR_Mixt_MF	B
PopR	2		2		X		X			X	PopR_Alt_MF	B
PopR	3	X	3	X		X		X	X		PopR_Mixt_CPB	B
PopR	3	X	3	X		X		X	X	X	PopR_Alt_CPB	B

PopRR	0		0								PopRR_Ethanol	A/B
PopRR	0		0								PopRR_DMSO	B
PopRR	1		1	X							PopRR_B	A
PopRR	1		1			X					PopRR_P	A
PopRR	2		1	X	X				X		PopRR_Mixt_BF	B
PopRR	2		1	X	X					X	PopRR_Alt_BF	B
PopRR	2		1			X	X		X		PopRR_Mixt_MP	B
PopRR	2		1			X	X			X	PopRR_Alt_MP	B
PopRR	2		2	X		X			X		PopRR_Mixt_PB	A
PopRR	2		2	X		X				X	PopRR_Alt_PB	A
PopRR	2		2		X		X		X		PopRR_Mixt_MF	B
PopRR	2		2		X		X			X	PopRR_Alt_MF	B
PopRR	3	X	2	X		X		X	X		PopRR_Mixt_CPB	B
PopRR	3	X	2	X		X		X	X	X	PopRR_Alt_CPB	B

Supplementary Table 2: Selection regimes and their respective components and codes. Thirty-one selection regimes were applied to three artificial populations of *Z. tritici* — PopS, PopR and PopRR — as defined in **Table 1A**. These regimes were organized into two batches (A and B). The selection regimes differed in their components, which defined the diversity and heterogeneity of selection. Control regimes (not including fungicides) were amended with 0.5% ethanol or DMSO (*i.e.* the amount of fungicide solvent used in treated lines). Straight selection regimes involved the continuous use of a single fungicide. The regimes displaying diverse selection included two or three fungicides differing in their inherent resistance risk and mode of action, and their temporal heterogeneity over the course of the experiment.

20 **Supplementary information 3: Doses of fungicides used for experimental**
21 **evolution**
22
23

	SDHI fungicides		DMI fungicides		Multisite
	benzovin-diflupyr	**fluxapyroxad**	**prothioconazole-desthio**	**mefentriflu-conazole**	**chloro-thalonil**
Used alone	0.7	0.7	0.005	0.002	5.5
Used in two-way mixtures	0.35	0.35	0.0025	0.001	-
Used in three-way mixtures	0.175	-	0.00125	-	2.75

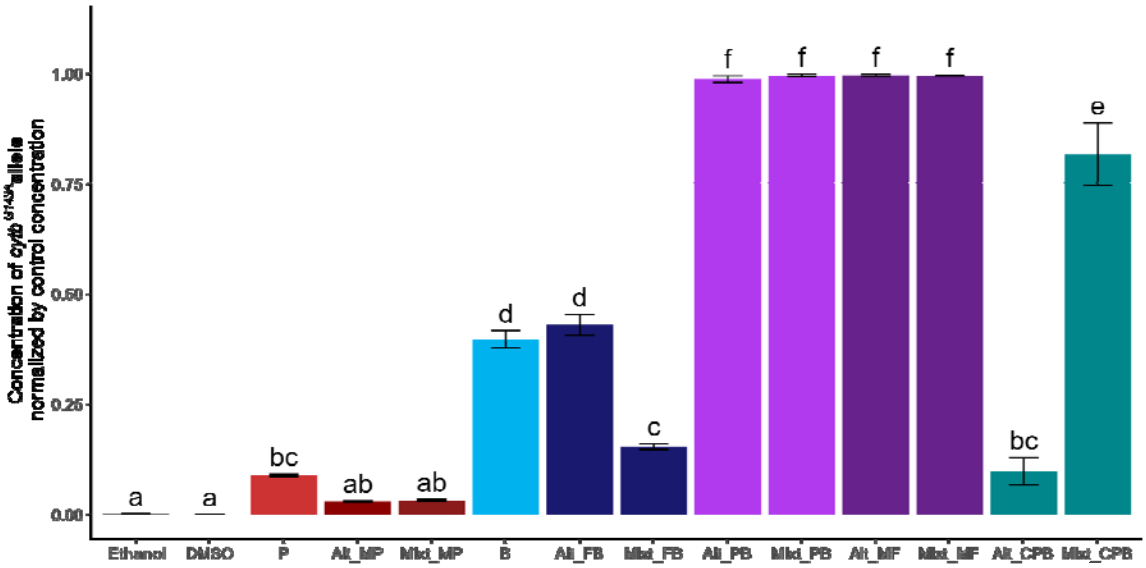
24 **Supplementary Table 3A: Selection doses of fungicides used for experimental evolution** (mg.L^{-1}).
25
26
27

Acronym in Fig. 5	Fungicides	Fungicide class	Dose
B	benzovindiflupyr	SDHI	0.7
F	fluxapyroxad	SDHI	0.7
FB	fluxapyroxad + benzovindiflupyr	SDHI+SDHI	0.7+0.7
P	prothioconazole-desthio	DMI	0.005
M	mefentrifluconazole	DMI	0.002
MP	mefentrifluconazole + prothioconazole-desthio	DMI+DMI	0.002+0.005
PB	prothioconazole-desthio + benzovindiflupyr	DMI+ SDHI	0.005+0.7
MF	mefentrifluconazole + fluxapyroxad	DMI+ SDHI	0.002+0.7
Azo	azoxystrobin	QoI	0.5
Tol	tolnaftate	SBI class IV	1.5
C	chlorothalonil	Multisite	5.5

28 **Supplementary Table 3B: Discriminatory doses used in droplet tests** (mg.L^{-1}).

29 ***Supplementary information 4:*** **cytb^{G143A} content of evolved lines for PopRR**

30



Supplementary Figure 4: Normalized concentration of *cytb^{G143A}* in lines evolved from the PopRR ancestral population. *cytb^{G143A}* confers high-level resistance to QoI fungicides (not used in the strategies compared here). It was quantified by qPCR when optical density reached 90% of that of the control for a minimum of three consecutive cycles (4th, 6th or 8th cycle, depending on the line). This allele was carried exclusively by the $R_{SDHI/DMI}$ ancestral isolate and was used for indirect quantification of the proportion of isolates displaying multiple resistance in PopRR lines. Indeed, Pearson's correlation coefficient for the relationship between the concentration of *cytb^{G143A}* and the estimated concentration of multiple resistance (**Fig. 4**) in the same population was 0.998 (P<0.0001). Pairwise comparisons of lines evolved under different selection regimes were adjusted with the Tukey method for multiple comparisons (MODG143A0; SI2). Multiple resistance was preferentially selected in lines subjected to diversified SDHI and DMI selection as opposed to straight selection under a single fungicide or the mixture or alternation of fungicides with the same mode of action (P<0.001; MODG143A1). There was, therefore, a resistance cost associated with the $R_{SDHI/DMI}$ ancestral isolate in competition with isolates carrying single resistance alleles in the presence of homogeneous selection pressure.

31 **Supplementary information 5: Expression of *mfs1* in evolved lines of**
32 ***Zymoseptoria tritici***

33

34 The expression of the drug transporter MFS1 was quantified, as enhanced efflux is a recognized
35 mechanism of generalist resistance in field isolates of *Z. tritici*[1].

36

37 **1. Origin of the isolates tested**

38 We purified 20 isolates from lines displaying MDR, on the basis of scores in droplet tests. The origins
39 of these isolates and of the four control reference isolates are indicated in the table below.

Isolate	Origin
IPO323	Ancestral susceptible strain (negative control)
MDR	Field type I MDR[2] isolate (positive control)
H152R	Ancestral SDHI-resistant isolate (negative control)
G31	Ancestral DMI-resistant isolate (negative control)
PopR_Alt_PB_I	Evolved under Alt_PB selection regime
PopR_Alt_PB_II	Evolved under Alt_PB selection regime
PopR_Alt_PB_III	Evolved under Alt_PB selection regime
PopR_Mixt_PB_I	Evolved under Mixt_PB selection regime
PopR_Mixt_PB_II	Evolved under Mixt_PB selection regime
PopR_Mixt_PB_III	Evolved under Mixt_PB selection regime
PopR_Alt_MF_I	Evolved under Alt_MF selection regime
PopR_Alt_MF_II	Evolved under Alt_MF selection regime
PopR_Alt_MF_III	Evolved under Alt_MF selection regime
PopR_Mixt_MF_I	Evolved under Mixt_MF selection regime
PopR_Mixt_MF_II	Evolved under Mixt_MF selection regime
PopR_Mixt_MF_III	Evolved under Mixt_MF selection regime
PopR_Alt_CPB_I	Evolved under Alt_CPB selection regime
PopR_Alt_CPB_II	Evolved under Alt_CPB selection regime
PopR_Alt_CPB_III	Evolved under Alt_CPB selection regime
PopR_Alt_CPB_IV	Evolved under Alt_CPB selection regime
PopR_Mixt_CPB_I	Evolved under Mixt_CPB selection regime
PopR_Mixt_CPB_II	Evolved under Mixt_CPB selection regime
PopR_Mixt_CPB_III	Evolved under Mixt_CPB selection regime
PopR_Mixt_CPB_IV	Evolved under Mixt_CPB selection regime

40 **2. Protocols**

41 Isolates were grown in the dark at 18°C, with a RH of 70%, with shaking at 150 rpm for four days, in
42 50 mL borosilicate Erlenmeyer flasks containing 25 mL YPD medium and no fungicide. Two
43 independent biological replicates, grown successively, were produced for each isolate. Fungal cells
44 were freeze-dried and RNA was extracted with a commercial kit (Monarch Total RNA Miniprep; New

45 England Biolabs) in accordance with the manufacturer's recommendations. Reverse transcription
46 was performed with the ProtoScript II First Strand cDNA Synthesis kit (New England
47 BioLabs) according to the manufacturer's protocol. qPCR was performed as follows, with three
48 technical replicates per sample, and the primers detailed below.

49

Primer name	Primer sequence (5'-3')	Reference	Use
MFS1_F MFS1_R	CAATGGGGCGGCAGCAAATAC TTGGATGGTGGCGAAGATGAGG	Omrane *et al,* 2017	Expression of *mfs1,* encoding a MFS drug transporter
UBC_Fw UBC_RV	GTCTGCGGACCACAATACC CGACCTTTCCTTGCCTCTG	Omrane *et al,* 2017	Ubiquitin housekeeping gene
EF1α_Fw EF1α_Rv	AAGATTGGTGGTATCGGAACAG GACTTGACTTCGGTGGTGAC	Omrane *et al,* 2017	Elongation factor EF1α housekeeping gene

50

Mix	1 reaction (µL)
cDNA	2
Primer FW (10 µM)	0.4
Primer RV (10 µM)	0.4
Master Mix (2X)	10
Deionized H_2O	7.2
Total volume	20

51 DNA polymerase: MESA GREEN qPCR MasterMix Plus for SYBR Assay (Eurogentec)

52

53 Regular qPCR program for *mfs1*

Step	Temperature	Duration	
Initial denaturation	95°C	5 min	
Denaturation	95°C	15 s	45 cycles
Annealing - Extension	60°C	1 min	

54

55 **3. Determination of relative gene expression, $2^{-\Delta\Delta Ct}$**

56 We first calculated ΔCt values as follows:

$$\Delta \mathrm{Ct} = Ct_{\mathrm{i,\ \ target\ gene}} - Ct_{\mathrm{i,\ \ housekeeping\ gene}}$$

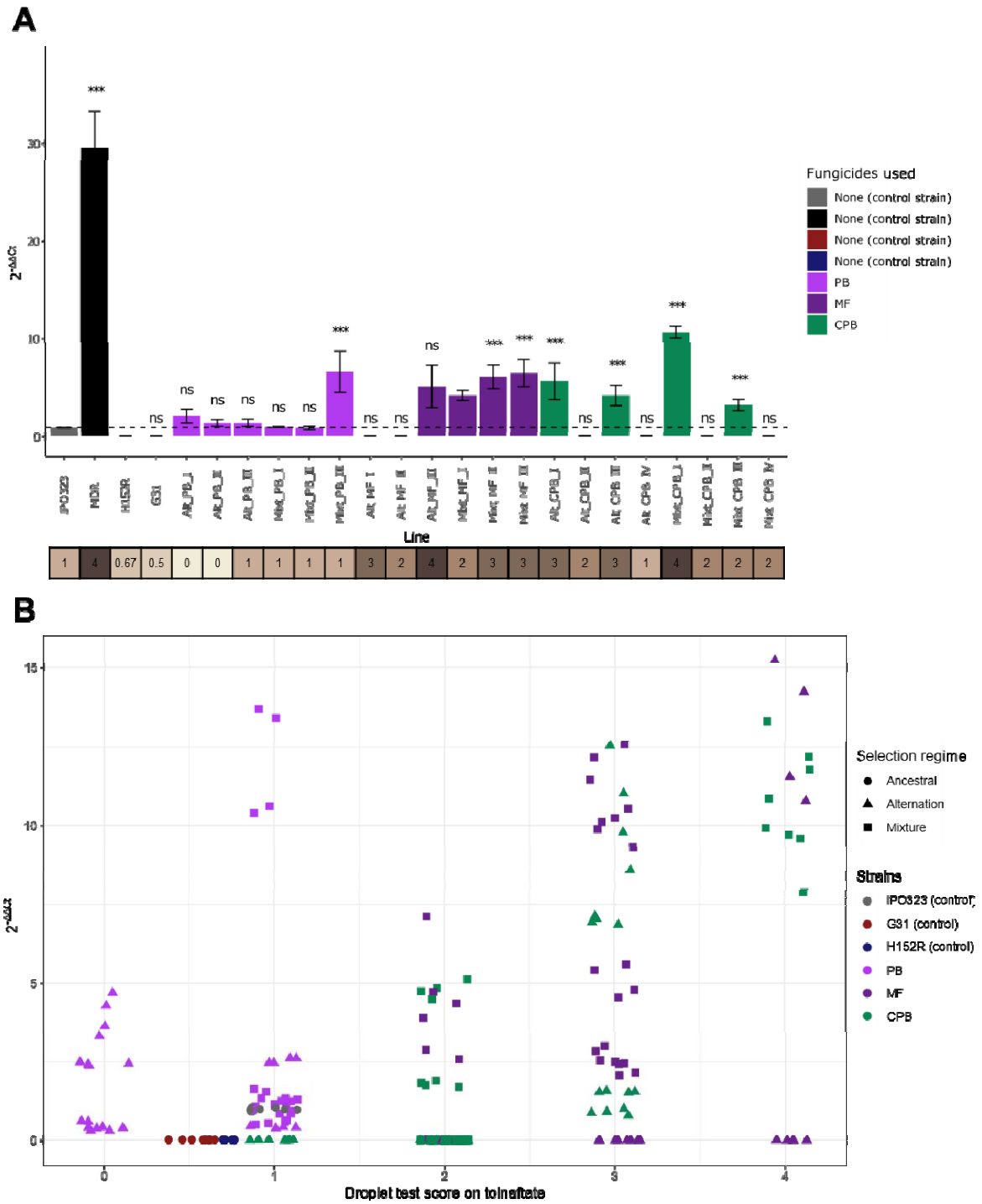
where Ct is the value of the cycle threshold and *i* is the isolate tested.

ΔΔCt values were calculated after normalization against the control ancestral strain IPO323:

$$\Delta\Delta\mathrm{Ct} = \Delta\mathrm{Ct}_{\mathrm{i}} - \Delta\mathrm{Ct}_{\mathrm{IPO323}}$$

4. Results

We normalized *mfs1* expression (expressed as $2^{-\Delta\Delta Ct}$) against the expression of the genes encoding EF1α and ubiquitin, and the mean value for *mfs1* expression for each isolate is presented in **Supplementary Figure 5A**. Strong *msf1* expression (up to 30-fold overexpression) was observed for the reference field isolate displaying high levels of resistance to tolnaftate. Moderate expression (up to 3.3-10.6-fold overexpression) was observed for nine of the 20 isolates displaying low to high resistance to tolnaftate. These isolates were indifferently collected from lines having undergone alternation or mixture selection regimes, including two or three MoAs. The correlation between *mfs1* expression and growth on tolnaftate established from a droplet test for the same isolate was poor (**Supplementary Figure 5B**). The overexpression of *mfs1* is not necessarily associated with a tolnaftate resistant phenotype (*e.g.* score of 1 to 4). Genotyping of the promoter regions of these isolates revealed no insertions resembling those observed in MDR field isolates. These findings suggest that *mfs1* overexpression may be involved in the resistance of these evolved isolates, but that this mechanism is not exclusive. Other unknown additional mechanisms must also be involved.



Supplementary Figure 5: Expression of *mfs1* in evolved lines of *Zymoseptoria tritici.*

A.* mfs1 *expression. Scores in droplet tests are shown in shades of brown, at the top of each graph. ***B. Relation between* mfs1 *expression and resistance to tolnaftate.*** Resistance to tolnaftate, known to reveal multidrug resistance in field isolates of *Z. tritici,* was screened through droplet tests. The control MDR isolate is not shown here, to allow the better visualization of variations between evolved isolates.

81

82 **5. References**

83 [1]Omrane, S., et al. (2015). "Fungicide efflux and the MgMFS1 transporter contribute to the multidrug
84 resistance phenotype in *Zymoseptoria tritici* field isolates." Environmental Microbiology **17**(8): 2805-
85 2823.

86 [2] Omrane, S., et al. (2017). "Plasticity of the *MFS1* promoter leads to multidrug resistance in the
87 wheat pathogen *Zymoseptoria tritici*." mSphere **2**(5).

Supplementary information 6: Models used for data analysis

All analyses were performed 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).

Supplementary Information 6A: Mathematical models used to analyze mean Malthusian growth data and combined results tables

Model	Degrees of freedom	Sampling design	Fixed effect(s)	Random effect	Used to study the effect of:
MODM0	10	Only strategies common to the 3 ancestral populations (batch A) (*i.e.* control, PB alternation and PB mixture) n=54	Regime_cplt*pop	None	- Population composition
MODM1	14	Strategies with only 1 or 2 AIs PopS excluded n=90	(NB_AI*DMI)*Pop +Pop:NB_MoA +NB_MoA/Fung_batch	Exp_batch	- Number of MoAs - Number of AIs - Population composition
MODM1.1	10	Strategies with only 1 MoA PopS excluded n=54	NB_AI*Pop*DMI	Exp_batch	- Number of AIs - MoA choice
MODM2	15	Strategies with only 2 AIs PopS excluded n=60	(DMI+NB_MoA)*Regime *Pop+Fung_batch	Exp_batch	- Number of MoAs
MODM2.1	9	Strategies with only 1 MoA PopS excluded n=24	DMI*Regime*Pop	None	- Temporal heterogeneity - MoA choice

MODM2.2	10	Strategies with only 2 MoAs PopS excluded *n*=36	Fung_batch*Regime*Pop	Exp_batch	- Temporal heterogeneity - AI choice
MODM3	10	Regimes of alternation and mixture with PB or CPB PopS excluded *n*=40	Multisite*Regime*Pop	Exp_batch	- Temporal heterogeneity - Naïve MoA

Supplementary Table 6A: Description of the models used to analyze Malthusian growth.

Regime_cplt: factor with 3 levels (control, alternation of B and P, mixture of B+P; **Supplementary Table 2**).

Pop: factor with 3 levels (three populations: PopS, PopR and PopRR; **Fig. 1D**).

Regime: factor with 2 levels (degree of temporal heterogeneity i.e. alternation *vs.* mixture).

Fung_batch: factor with 2 levels (batches of AIs compared).

Exp_batch: factor with 2 levels (batches of selection regimes used in the same experiment; A or B; **Supplementary Table S2**).

NB_AI: factor with 2 levels (the number of different AIs introduced in the strategy; **Supplementary Table S2**).

NB_MoA: factor with 2 levels (number of different MoAs introduced in the selection regime; **Supplementary Table S2**).

DMI: factor with 2 levels (presence or absence of a DMI fungicide in the selection regime; **Supplementary Table S2**). In our models, the absence of DMI fungicides necessarily means that SDHI fungicides are present.

Multisite: factor with 2 levels (presence or absence of the multisite agent chlorothalonil used in populations naïve to this MoA).

Combined data for model MODM0

Model term	df1	*F* ratio	*P* value
Regime_cplt	2	10453.366	0
Pop	2	6583.227	0
Regime_cplt:Pop	4	3546.801	0

Combined data for model MODM1

NOTE: A nesting structure was detected in the fitted model: Fung_batch %in% NB_MoA

Model term	df1	*F* ratio	*P* value
NBmol	1	0.364	0.546
DMI	1	39.639	0.000
Pop	1	65.020	0.000
NB_MoA	1	48.043	0.000
NBmol:DMI	1	0.466	0.495
NBmol:Pop	1	0.363	0.547
DMI:Pop	1	10.669	0.001
Pop:NB_MoA	1	65.149	0.000
NB_MoA:Fung_batch	2	60.721	0.000
NBmol:DMI:Pop	1	0.235	0.628

Combined data for model modM1.1

Model term	df1	*F* ratio	*P* value
NBmol	1	52.867	0
Pop	1	42.391	0
DMI	1	350.310	0
NBmol:Pop	1	43.428	0
NBmol:DMI	1	56.943	0
Pop:DMI	1	42.157	0
NBmol:Pop:DMI	1	33.903	0

Combined data for model modM2

Model term	df1	*F* ratio	*P* value
DMI	1	372.365	0.000
NB_MoA	1	912.781	0.000
Regime	1	9.625	0.002
Pop	1	701.906	0.000
Fung_batch	1	133.858	0.000
DMI:Regime	1	64.839	0.000
DMI:Pop	1	36.147	0.000
NB_MoA:Regime	1	18.139	0.000
NB_MoA:Pop	1	629.142	0.000
Regime:Pop	1	0.002	0.963
DMI:Regime:Pop	1	6.122	0.013
NB_MoA:Regime:Pop	1	3.135	0.077

Combined data for model modM2.1

Model term	df1	*F* ratio	*P* value
DMI	1	2288.000	0.000
Regime	1	953.292	0.000
Pop	1	11.347	0.001
DMI:Regime	1	965.427	0.000

DMI:Pop	1	34.087	0.000
Regime:Pop	1	57.706	0.000
DMI:Regime:Pop	1	37.849	0.000

Combined data for model modM2.2

Model term	df1	*F* ratio	*P* value
Fung_batch	1	2529.069	0
Regime	1	644.618	0
Pop	1	5539.574	0
Fung_batch:Regime	1	203.211	0
Fung_batch:Pop	1	2021.288	0
Regime:Pop	1	505.323	0
Fung_batch:Regime:Pop	1	190.371	0

Combined data for model modM3

Model term	df1	*F* ratio	*P* value
Multisite	1	323.441	0.000
Regime	1	24.103	0.000
Pop	1	1175.345	0.000
Multisite:Regime	1	84.420	0.000
Multisite:Pop	1	66.429	0.000
Regime:Pop	1	108.352	0.000

Multisite:Regime:Pop	1	6.191	0.013

121 **Supplementary Information 6B: Mathematical models used for the quantification of the *sdhC*^{H152R} and *cyp51*^{I381V} resistant alleles and combined results**
122 **tables**

NB: models were run similarly on *sdhC*^{H152R} and *cyp51*^{I381V} data. Their names include an "R" or a "V", respectively, with no other change for the analysis of effects.

Model	Degrees of freedom	Sampling design	Fixed effect(s)	Random effect	Used to study the effect of
MODR0/ MODV0	10	Regimes common to the 3 populations (*i.e.*) (batch A) (ethanol, PB alternation and PB mixture) *n*=27	Regime_cplt*Pop	None	- Population structure
MODR1/ MODV1	13	Regimes with only 1 or 2 AIs PopS excluded *n*=60	(NB_AI*DMI+NB_MoA)*Pop+Fung_batch	Exp_batch	- Number of MoAs - Number of AIs - Population structure
MODR1.1/ MODV1.1	10	Regimes with only 1 MoA PopS excluded *n*=36	NB_AI*Pop*DMI	Exp_batch	- Number of AIs - MoA choice
MODR2/ MODV2	15	Regimes with only 2 AIs PopS excluded *n*=48	(DMI+NB_MoA)*Regime*Pop+Fung_batch	Exp_batch	- Number of MoAs
MODR2.1/ MODV2.1	9	Regimes with only 1 MoA PopS excluded *n*=24	DMI*Regime*Pop	None	- Temporal heterogeneity - MoA choice
MODR2.2/ MODV2.2	10	Regimes with only 2 MoAs PopS excluded *n*=24	Fung_batch*Regime*Pop	Exp_batch	- Temporal heterogeneity - AI choice
MODR3/ MODV3	10	Regimes of alternation or mixture with PB or CPB PopS excluded	Multisite*Regime*Pop	Exp_batch	- Temporal heterogeneity - Naïve MoA

		n=28			

Supplementary Table 6B: Description of the models used to analyze DMI and SDHI resistance allele contents.

Regime_cplt: factor with 3 levels (control, alternation of B and P, mixture of B+P; **Supplementary Table S2**).

Pop: factor with 3 levels (three populations: PopS, PopR and PopRR; **Fig. 1D**).

Regime: factor with 2 levels (degree of temporal heterogeneity i.e. alternation *vs.* mixture).

Fung_batch: factor with 2 levels (batches of AIs compared).

Exp_batch: factor with 2 levels (batches of selection regimes used in the same experiment; A or B; **Supplementary Table S2**).

NB_AI: factor with 2 levels (the number of different AIs introduced in the strategy; **Supplementary Table S2**).

NB_MoA: factor with 2 levels (number of different MoAs introduced in the selection regime; **Supplementary Table S2**).

DMI: factor with 2 levels (presence or absence of a DMI fungicide in the selection regime; **Supplementary Table S2**). In our models, the absence of DMI fungicides necessarily means that SDHI fungicides are present.

Multisite: factor with 2 levels (presence or absence of the multisite agent chlorothalonil used in populations naïve to this MoA).

Allele H152R

Combined data for model MODR0

Model term	df1	*F* ratio	*P* value
Regime_cplt	2	24295.38	0
Pop	2	29180.83	0
Regime_cplt:Pop	4	14147.43	0

Combined table for model modR1

Model term	df1	*F* ratio	*P* value
NBmol	1	3.268	0.071
DMI	1	355.188	0.000

NB_MoA	1	122.741	0.000
Pop	1	63.898	0.000
Fung_batch	1	12.848	0.000
NBmol:DMI	1	7.035	0.008
NBmol:Pop	1	0.058	0.810
DMI:Pop	1	41.185	0.000
NB_MoA:Pop	1	11.913	0.001
NBmol:DMI:Pop	1	0.823	0.364

Combined data for model modR1.1

Model term	df1	*F* ratio	*P* value
Pop	1	124.961	0.000
DMI	1	4373.215	0.000
NBmol	1	24.357	0.000
Pop:DMI	1	9.471	0.002
Pop:NBmol	1	1.984	0.159
DMI:NBmol	1	31.306	0.000
Pop:DMI:NBmol	1	0.973	0.324

Combined data for model modR2

Model term	df1	*F* ratio	*P* value
DMI	1	4085.760	0.000
NB_MoA	1	711.202	0.000
Regime	1	8.279	0.004
Pop	1	339.275	0.000
Fung_batch	1	7.470	0.006
DMI:Regime	1	8.869	0.003
DMI:Pop	1	126.180	0.000
NB_MoA:Regime	1	9.692	0.002
NB_MoA:Pop	1	83.052	0.000
Regime:Pop	1	0.446	0.504
DMI:Regime:Pop	1	21.362	0.000
NB_MoA:Regime:Pop	1	21.611	0.000

Combined data for model modR2.1

Model term	df1	*F* ratio	*P* value
DMI	1	2544.805	0.000
Regime	1	0.027	0.870
Pop	1	36.098	0.000
DMI:Regime	1	0.055	0.815
DMI:Pop	1	3.393	0.065
Regime:Pop	1	5.851	0.016

DMI:Regime:Pop	1	5.619	0.018

Combined data for model modR2.2

Model term	df1	*F* ratio	*P* value
Fung_batch	1	767.236	0
Regime	1	379.681	0
Pop	1	32100.864	0
Fung_batch:Regime	1	380.580	0
Fung_batch:Pop	1	971.780	0
Regime:Pop	1	496.973	0
Fung_batch:Regime:Pop	1	18.829	0

Combined data for model modR3

Model term	df1	*F* ratio	*P* value
Multisite	1	1291.559	0.00
Regime	1	17.037	0.00
Pop	1	5492.403	0.00
Multisite:Regime	1	16.940	0.00
Multisite:Pop	1	1049.720	0.00
Regime:Pop	1	67.709	0.00
Multisite:Regime:Pop	1	0.739	0.39

Allele I381V

Combined data for model modV0

Model term	df1	*F* ratio	*P* value
Regime_cplt	2	13036.522	0
Pop	2	24308.895	0
Regime_cplt:Pop	4	9660.256	0

Combined data for model modV1

Model term	df1	*F* ratio	*P* value
NBmol	1	2.196	0.138
DMI	1	365.672	0.000
NB_MoA	1	66.460	0.000
Pop	1	115.528	0.000
Fung_batch	1	3.163	0.075
NBmol:DMI	1	1.458	0.227
NBmol:Pop	1	1.811	0.178
DMI:Pop	1	5.560	0.018
NB_MoA:Pop	1	42.020	0.000
NBmol:DMI:Pop	1	1.069	0.301

Combined data for model modV1.1

Model term	df1	*F* ratio	*P* value
Pop	1	488.424	0.000
DMI	1	11781.110	0.000
NBmol	1	0.932	0.334
Pop:DMI	1	514.497	0.000
Pop:NBmol	1	1.588	0.208
DMI:NBmol	1	7.035	0.008
Pop:DMI:NBmol	1	0.480	0.488

Combined data for model modV2

Model term	df1	*F* ratio	*P* value
DMI	1	8397.286	0.000
NB_MoA	1	1482.835	0.000
Regime	1	215.577	0.000
Pop	1	776.966	0.000
Fung_batch	1	2.353	0.125
DMI:Regime	1	3.876	0.049
DMI:Pop	1	57.196	0.000
NB_MoA:Regime	1	97.361	0.000
NB_MoA:Pop	1	357.692	0.000

Regime:Pop	1	20.944	0.000
DMI:Regime:Pop	1	36.546	0.000
NB_MoA:Regime:Pop	1	0.648	0.421

Combined data for model modV2.1

Model term	df1	*F* ratio	*P* value
DMI	1	21437.891	0
Regime	1	65.087	0
Pop	1	736.946	0
DMI:Regime	1	67.255	0
DMI:Pop	1	815.068	0
Regime:Pop	1	53.681	0
DMI:Regime:Pop	1	40.999	0

Combined data for model modV2.2

Model term	df1	*F* ratio	*P* value
Fung_batch	1	419.532	0.000
Regime	1	5353.541	0.000
Pop	1	103239.842	0.000
Fung_batch:Regime	1	1606.077	0.000
Fung_batch:Pop	1	496.945	0.000
Regime:Pop	1	1220.062	0.000

Fung_batch:Regime:Pop	1	0.168	0.682

Combined data for model modV3

Model term	df1	*F* ratio	*P* value
Multisite	1	1756.624	0.000
Regime	1	1.528	0.216
Pop	1	5475.879	0.000
Multisite:Regime	1	80.749	0.000
Multisite:Pop	1	1034.761	0.000
Regime:Pop	1	86.294	0.000
Multisite:Regime:Pop	1	1.889	0.169

166 **Supplementary Information 6C: Mathematical models used for quantification of the multiple resistant mutant and combined results tables**

167

Model	Degrees of freedom	Sampling design	Fixed effect(s)	Random effect	Used to study the effect of
modDM0	14	All regimes of PopRR *n*=38	Regime_cplt	Exp_batch	- Regimes
modDM1	8	Regimes with only 1 or 2 AIs PopS and PopR excluded *n*=30	NB_AI*DMI+NB_MoA +Fung_batch	Exp_batch	- Number of MoAs - Number of AIs
modDM1.1	6	Regimes with only 1 MoA PopS and PopR excluded *n*=18	NB_AI*DMI	Exp_batch	- Number of AIs - MoA choice
modDM2	9	Regimes with only 2 AIs PopS and PopR excluded *n*=24	(DMI+NB_MoA)*Regime+Fung_batch	Exp_batch	- Number of MoAs
modDM2.1	5	Regimes with only 1 MoA PopS and PopR excluded *n*=12	DMI*Regime	None	- Temporal heterogeneity - MoA choice
modDM2.2	6	Regimes with only 2 MoAs PopS and PopR excluded *n*=12	Fung_batch*Regime	Exp_batch	- Temporal heterogeneity -AI choice
modDM3	6	Regimes of alternation and mixture with PB or CPB PopS and PopR excluded *n*=14	Multisite*Regime	Exp_batch	- Temporal heterogeneity - Naïve MoA

168 **Supplementary Table 6C: Description of the models used to analyze multiple resistance content of PopRR lines**

169 Regime_cplt: factor with 3 levels (control, alternation of B and P, mixture of B+P; **Supplementary Table S2**).

170 Regime: factor with 2 levels (degree of temporal heterogeneity i.e. alternation *vs.* mixture).

171 Fung_batch: factor with 2 levels (batches of AIs compared).
172 Exp_batch: factor with 2 levels (batches of selection regimes used in the same experiment; A or B; **Supplementary Table S2**).
173 NB_AI: factor with 2 levels (the number of different AIs introduced in the strategy; **Supplementary Table S2**).
174 NB_MoA: factor with 2 levels (number of different MoAs introduced in the selection regime; **Supplementary Table S2**).
175 DMI: factor with 2 levels (presence or absence of a DMI fungicide in the selection regime; **Supplementary Table S2**). In our models, the absence of DMI
176 fungicides necessarily means that SDHI fungicides are present.
177 Multisite: factor with 2 levels (presence or absence of the multisite agent chlorothalonil used in populations naïve to this MoA).

178

179 **Combined data for model modDM1**

Model term	df1	*F* ratio	*P* value
NBmol	1	2.478	0.115
DMI	1	95.570	0.000
NB_MoA	1	1234.036	0.000
Fung_batch	1	0.034	0.854
NBmol:DMI	1	1.018	0.313

180

181 **Combined data for model modDM1.1**

Model term	df1	*F* ratio	*P* value
DMI	1	98.994	0.000
NBmol	1	4.679	0.031
DMI:NBmol	1	0.255	0.613

Combined data for model modDM2

Model term	df1	*F* ratio	*P* value
DMI	1	1037.636	0.000
NB_MoA	1	8795.630	0.000
Regime	1	319.285	0.000
Fung_batch	1	0.074	0.785
DMI:Regime	1	102.421	0.000
NB_MoA:Regime	1	1.733	0.188

Combined data for model modDM2.1

Model term	df1	*F* ratio	*P* value
DMI	1	819.395	0
Regime	1	177.430	0
DMI:Regime	1	170.253	0

Combined data for model modDM2.2

Model term	df1	*F* ratio	*P* value
Fung_batch	1	4.732	0.030
Regime	1	0.001	0.974
Fung_batch:Regime	1	408.374	0.000

Combined data for model modDM3

Model term	df1	*F* ratio	*P* value
Multisite	1	637.592	0.00
Regime	1	24.290	0.00
Multisite:Regime	1	1.326	0.25

189 **Supplementary Information 6D: Mathematical models used to quantify the *cytb*^{G143A} resistance allele and combined results tables**

190

Model	Degrees of freedom	Sampling design	Fixed effect(s)	Random effect	Used to study the effect of
modG143A0	15	All regimes of PopRR *n*=47	Regime_cplt	Exp_batch	- Regimes
modG143A1	8	Regimes with only 1 or 2 AIs of PopRR *n*=30	NB_AI*DMI+NB_MoA +Fung_batch	Exp_batch	- Number of MoAs - Number of AIs
modG143A1.1	6	Regimes with only 1 MoA of PopRR *n*=18	NB_AI*DMI	Exp_batch	- Number of AIs - MoA choice
modG143A2	9	Regimes with only 2 AIs of PopRR *n*=24	(DMI+NB_MoA)*Regime+Fung_batch	Exp_batch	- Number of MoAs
modG143A2.1	5	Regimes with only 1 MoA of PopRR *n*=12	DMI*Regime	None	- Temporal heterogeneity - MoA choice
modG143A2.2	6	Regimes with only 2 MoAs of PopRR *n*=12	Fung_batch*Regime	Exp_batch	- Temporal heterogeneity -AI choice
modG143A3	6	Regimes of alternation and mixture with PB or CPB of PopRR *n*=14	Multisite*Regime	Exp_batch	- Temporal heterogeneity - Naïve MoA

191

192 **Supplementary Table 6D: Description of the models used to analyze the *cytb*^{G143A} content of PopRR lines**

193 Regime_cplt: factor with 3 levels (control, alternation of B and P, mixture of B+P; **Supplementary Table S2**).

Regime: factor with 2 levels (degree of temporal heterogeneity i.e. alternation *vs.* mixture).
Fung_batch: factor with 2 levels (batches of AIs compared).
Exp_batch: factor with 2 levels (batches of selection regimes used in the same experiment; A or B; **Supplementary Table S2**).
NB_AI: factor with 2 levels (the number of different AIs introduced in the strategy; **Supplementary Table S2**).
NB_MoA: factor with 2 levels (number of different MoAs introduced in the selection regime; **Supplementary Table S2**).
DMI: factor with 2 levels (presence or absence of a DMI fungicide in the selection regime; **Supplementary Table S2**). In our models, the absence of DMI fungicides necessarily means that SDHI fungicides are present.
Multisite: factor with 2 levels (presence or absence of the multisite agent chlorothalonil used in populations naïve to this MoA).

Combined data for model modG143A1

Model term	df1	*F* ratio	*P* value
NBmol	1	0.362	0.5476
DMI	1	115.469	<.0001
NB_MoA	1	1322.617	<.0001
Fung_batch	1	2.576	0.1085
NBmol:DMI	1	0.671	0.4127

Combined data for model modG143A1.1

Model term	df1	*F* ratio	*P* value
DMI	1	136.650	<.0001
NBmol	1	10.247	0.0014
DMI:NBmol	1	2.837	0.0921

Combined data for model modG143A2

Model term	df1	*F* ratio	*P* value
DMI	1	43.614	<.0001
NB_MoA	1	1935.462	<.0001
Regime	1	40.236	<.0001
Fung_batch	1	2.953	0.0857
DMI:Regime	1	39.966	<.0001
NB_MoA:Regime	1	38.419	<.0001

Combined data for model modG143A2.1

Model term	df1	*F* ratio	*P* value
DMI	1	1306.984	<.0001
Regime	1	359.259	<.0001
DMI:Regime	1	370.394	<.0001

Combined data for model modG143A2.2

Model term	df1	*F* ratio	*P* value
Fung_batch	1	1.516	0.2182
Regime	1	1.274	0.2589
Fung_batch:Regime	1	3.185	0.0743

Combined data for model modG143A3

Model term	df1	*F* ratio	*P* value
Multisite	1	356.135	<.0001
Regime	1	146.349	<.0001
Multisite:Regime	1	139.696	<.0001