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

Short title: Multiple resistance and management of fungal adaptation

Agathe Ballu[1], Claire Ugazio[1], Clémentine Duplaix[1], Alicia Noly[1], Juerg Wullschleger[2], Stefano F.F. Torriani[2], Anne Dérédec[1], Florence Carpentier[3,4*†], Anne-Sophie Walker[1*†]

[1]Université Paris-Saclay, INRAE, UR BIOGER, 91123 Palaiseau, France

[2]Syngenta, Crop Protection AG, 4332 Stein, Switzerland

[3]Université Paris-Saclay, INRAE, UR MaIAGE, 78350 Jouy-en-Josas, France

[4]AgroParisTech, 91123 Palaiseau Cedex, France

[*]Corresponding authors: anne-sophie.walker@inrae.fr; Florence.carpentier@agroparistech.fr

[†]These authors contributed equally

ORCID numbers:

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

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

Agathe Ballu: https://orcid.org/0000-0001-7104-6493

Abstract

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. 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 resistantresistant isolates and their 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

Originality-Significance Statement

Pesticides are part of microbe environment in agricultural systems and may select for resistance. This adaptation of pathogens is a burden for plant health. Using an original multicriteria assessment and experimental evolution, we revealed that multi-fungicide use, selecting for multiple resistance, trumped any other driver of selection, invalidating any universal ranking of management strategies, a dispute extensively illustrated in literature and still inconclusive, especially in agriculture. This outcome refocuses the debate on population diversity and evolution rather than on the intrinsic properties of strategies, as mostly acclaimed in literature. From a practical point of view, our results highlight the importance of considering local population composition when recommending spraying programs. This practice is currently not generalized in agriculture but may be timely to effectively delay resistance evolution *and* reduce pesticide load in agricultural systems, a growing social demand, since resistance monitoring at relatively fine spatial scales and at low frequency may become commonplace in a near future with the rise of new molecular biology technologies

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 (McKenna, 2013; Fisher et al., 2018; Gould et al., 2018; Murray et al., 2022). 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 (REX_Consortium, 2013; van den Bosch et al., 2014a). 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 its intensity (*i.e.* modulation of fungicide dose or number of sprays).

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 (Fones and Gurr, 2015; Torriani et al., 2015). 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 (Hellin et al., 2020; Garnault et al., 2021). Interestingly, this fungus displays various resistant phenotypes (Heick et al., 2017a; Huf et al., 2018; Jorgensen et al., 2018; Rehfus et al., 2018; Samils et al., 2021). Specialist

88 resistance results from the selection of mutations in genes encoding the target proteins of these
89 fungicides (target-site resistance or TSR). The combination of multiple mutations in the same isolate,
90 resulting in multiple resistance, is favored by the high rates of sexual reproduction in this pathogen
91 (Singh et al., 2021). Generalist resistance may result from non-target-site resistance (NTSR), as in the
92 multidrug resistance (MDR) caused by the enhanced efflux of unrelated fungicides (Leroux and
93 Walker, 2011; Omrane et al., 2015). The diversity of resistant phenotypes observed in *Z. tritici* and
94 their contrasting local frequencies in the field renders this pathogen a relevant biological model for
95 investigations of the interplay between the drivers of the performance of anti-resistance strategies .
96 Several empirical and modelling studies in *Zymoseptoria tritici* have concluded that strategies
97 including alternation, mixtures, and modulations of the dose, number and timing of applications can
98 effectively limit the evolution of resistance, provided that they are optimized (Hobbelen et al., 2013;
99 van den Bosch et al., 2014a; Dooley et al., 2016a; Heick et al., 2017a; Jørgensen et al., 2017;
100 Mikaberidze et al., 2017). However, it remains a matter of debate which of these strategies is the
101 most sustainable. Attempts to rank strategies have suggested that mixture-based strategies may be
102 the best, but the results obtained were not unequivocal for *Z. tritici* or for many agricultural or
103 clinical situations (REX_Consortium, 2013; van den Bosch et al., 2014a). There may be several reasons
104 for this. First, the results for strategy performance may depend on the criteria used for assessment
105 (Elderfield et al., 2018). Indeed, the evolution or frequency of resistance are among the most widely
106 used criteria, but many other criteria, such as disease control or fungicide effective life, may be just
107 as relevant and may alter the ranking of performance between strategies. Furthermore, strategies
108 must be truly comparable to obtain meaningful results. In most of the empirical data available,
109 alternation and mixture strategies are rarely compared at similar dose of AIs, but rather at their field
110 rates, in most instances designed not solely for fungicide resistance management but for a
111 combination of factors, including spectrum of control, (eco)toxicological aspects and cost-
112 effectiveness of the product. And when alternation and mixture strategies are fairly compared, the
113 ranking is not decisive (Hobbelen et al., 2013; van den Bosch et al., 2014b). Finally, the composition

114 of the pathogen population in terms of resistance types and frequencies (*i.e.* resistance status) may
115 also have an impact on strategy performance, as resistant individuals are fitter than susceptible
116 individuals in sprayed environments and higher doses of fungicide are required to control them.
117 Furthermore, when single-resistant genotypes are already present, the acquisition of multiple
118 resistance to two independent MoAs only requires the subsequent selection of a single new
119 mutation. The emergence of multiple resistance is therefore more likely than when a combination of
120 the same MoAs is used on a totally susceptible population. The drivers of heterogeneity and diversity
121 in selection pressure may, therefore, have different impacts on resistance management, according to
122 how and when they are implemented with respect to pathogen resistance dynamics, which depends
123 on the current composition of the pathogen population. For example, strategies that effectively
124 delay the initial emergence of resistance may be different from those most effective at slowing the
125 selection of resistances already present in the pathogen population (see an example on mixture
126 strategies (Hobbelen et al., 2014)). The initial frequency and nature of resistance mutations (*e.g.*
127 inducing resistance to one or multiple MoAs) may also influence the effective life of AIs (Hobbelen et
128 al., 2013). Thus, the overall performance of a strategy depends on the respective efficacies of its
129 components for controlling both susceptible and resistant individuals over time and space, whatever
130 the composition of the pathogen population in terms of resistance status.
131 In this context, we investigated the interplay between the operational drivers maximizing the
132 sustainability of management strategies relative to the resistance status of the pathogen population.
133 We explored several assumptions, based on published studies, including the work of Ballu *et al* (Ballu
134 et al., 2021; Ballu et al., 2023). We found that the various sources of heterogeneity and diversity in
135 selection pressures differed in their efficacy for delaying resistance evolution. We found that
136 combining these sources would probably increase the overall sustainability of strategies
137 (REX_Consortium, 2013; Ballu et al., 2023), and that the efficacy of anti-resistance strategies depends
138 strongly on pathogen population composition in terms of resistance (Hobbelen et al., 2013). These
139 results have been achieved through experimental evolution, an innovative approach that allows for

140 the design of complex experiments but remains underused in fungi. *Z. tritici* is particularly suitable
141 for investigations with this approach because of its haploid yeast-like form (*i.e.* blastospores) that is
142 easy to culture in liquid medium. By evolving strains carrying field resistances, we were able to
143 combine some of the characteristics of natural populations with accelerated experimental evolution
144 in miniaturized controlled conditions (Bailey and Bataillon, 2016; Fisher and Lang, 2016). We
145 followed the dynamics of populations and resistances under several selection regimes, which helped
146 to elucidate the impact of population composition on the efficacy of the drivers of resistance
147 management strategies. Several sources of diversity and heterogeneity generally considered to
148 promote the durable control of STB were tested: the number of AIs used (*i.e.* 1-3), the number of
149 MoAs (*i.e.* 1-3), the intrinsic vulnerability of these agents to the development of resistance (*i.e.* low to
150 high resistance risk), and the temporal heterogeneity of fungicide exposure (*i.e.* mixture *vs.*
151 alternation). We compared the performance of several selection strategies, defined as combinations
152 of one or several of these drivers, against artificial populations of different compositions in terms of
153 resistance status. We were especially interested in the introduction of multiple resistance, which is
154 associated to the combination, in a single individual, of independent resistance determinants each
155 involving resistance to a single mode of action, into artificial populations, to mimic some aspects of
156 the recombination that occurs naturally in field populations of *Z. tritici.* This made it possible to
157 explore the potential effect of such events on strategy performance in an artificial experimental
158 design (Fisher and Lang, 2016). A multi-criteria assessment of strategies, based on growth dynamics
159 of populations and evolution of resistance phenotype profiles, confirmed that diversification of
160 selection pressure, by alternation or mixture-based approaches, can limit the emergence and
161 selection of resistance, at the cost of selecting generalist or multiple resistance. But above all,
162 resistance status of pathogen population prior to the implementation of strategies was found to be
163 the primary determinant of strategy performance, particularly when multiple resistance was already
164 present in the initial population. These findings open up new perspectives for the informed tailoring

165 of strategies and show that population composition must be given priority over other drivers of
166 selection in such strategies.

Materials and methods

Ancestral isolates

The susceptible ancestral isolate was the IPO-323 strain, the source of the reference genome for *Z. tritici* (Goodwin et al., 2011), hereafter referred to as 'S'. The bias in fitness due to genetic background was minimized by selecting resistant isolates from the F1 progeny of a cross between 'S' and a resistant field strain (Walker and Gazeau, 2019). 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 subunits of succinate dehydrogenase, the target of SDHIs (Rehfus et al., 2018). The DMI-resistant isolate, R_{DMI}, displayed five amino-acid changes (V136A, A379G, I381V, Y461S and S524T) in the sterol 14α-demethylase protein encoded by *cyp51* (the resistance allele is referred to hereafter as *cyp51*^{I381V}). An isolate exhibiting multiple resistance, $R_{SDHI/DMI}$, carried both unrelated resistance alleles, as well as the G143A change in cytochrome b (the target of QoI fungicides), inherited from its wild parent. The R_{SDHI} and R_{DMI} isolates might be referred hereafter as "single-resistant isolates", whereas $R_{SDHI/DMI}$ might be referred as "the multiple-resistant isolate".

Fungicide sensitivity of the ancestral isolates

We established the resistance profile of each strain by estimating EC_{50} values and resistance factors (RFs) for a wide range of fungicides. The fungicides studied were all of technical grade and included three SDHIs (benzovindiflupyr, boscalid and fluxapyroxad), three DMIs (prothioconazole-desthio, metconazole and mefentrifluconazole), a QoI (azoxystrobin), a benzimidazole (carbendazim), a multisite inhibitor (chlorothalonil) and tolnaftate (an inhibitor of squalene epoxidase, an enzyme involved in sterol biosynthesis, used in medicine and as a marker of multidrug resistance). Fungicides

190 were dissolved in DMSO and stock solutions were prepared so as to ensure that the final medium
191 contained no more than 0.5% DMSO.

192 EC_{50} values were calculated from dose-response curves plotted for data obtained in 96-well
193 microtiter plates (Sarstedt, Germany). Pure liquid cultures of each isolate were grown in 50 mL
194 borosilicate Erlenmeyer flasks containing 25 mL YPD medium (20 $g.L^{-1}$ dextrose, 20 $g.L^{-1}$ peptone, 10
195 $g.L^{-1}$ yeast extract; USBiological Salem, MA, USA), with carded cotton wool inserted into the neck. The
196 cultures were maintained at 18°C and a RH of 70% in the dark for 7 days, with shaking at 150 rpm.
197 These cultures were used to inoculate 200 µL of YPD medium per well in microtiter plates, at an
198 initial density of 2.5 x 10^5 $spores.mL^{-1}$. We tested 10 fungicide concentrations for each fungicide, in
199 addition to a control amended only with 0.5% DMSO. The design included three replicates per isolate
200 and per fungicide concentration. The plate was sealed with a gas-permeable membrane (Breathe
201 Easy®; Diversified Biotech) and placed on a shaker in the dark, in the conditions described above.
202 Optical density at 405 nm (OD_{405}) (SpectraMax M2 spectrophotometer, Molecular Devices) was
203 measured after five days, *i.e.* at the end of the exponential growth phase, with three technical
204 replicates of each well. Each experiment was repeated at least three times.
205 EC_{50} values were determined by logistic regression (logistic nonlinear mixed effects model, with the
206 SSlogis function of the Self-Starting Nls Logistic Model, available in the STATS package and R version
207 4.1.3), with dose as a fixed effect and biological replicate as a random effect.
208 RFs were calculated as the ratio of the EC_{50} of the resistant isolate to that of the susceptible isolate.
209 Standard errors were calculated as follows:

210 $$\sigma\mathrm{RF} = |RF| \times \sqrt{\left(\frac{\sigma EC_{50}R}{|EC_{50}R|}\right)^2 + \left(\frac{\sigma EC_{50}S}{|EC_{50}S|}\right)^2} \qquad (1)$$

211 where R is the resistant isolate and S is the susceptible isolate.

212

213 Fitness and resistance profiles of ancestral strains

214 Fitness was inferred for each isolate from its growth in the culture conditions used for
215 experimental evolution (detailed below) but without fungicides. Population size was measured daily
216 on the basis of OD_{405} data, for seven days, as previously described (Ballu et al., 2021). The experiment
217 was performed five times. The growth curve for each strain was modeled with a mixed non-linear
218 logistic model, as follows:

219 Spore concentration = $asym/(1+\exp((xmid-t)/scal))$ (2)

220 where *t* is the day, *asym* the asymptote, *i.e.* maximum growth, *xmid* is the time required to
221 decrease the value of the asymptote by half and *scal* is the inverse of the slope.
222 The strains were considered as a fixed effect, with replicate as a random effect. We compared
223 parameters between strains with the Tukey method for pairwise comparisons. Estimates were
224 obtained with the NLME, CAR and EMMEANS packages of R version 4.1.3.
225

226 Artificial ancestral populations

227 Three ancestral populations were prepared for the first round of experimental evolution by
228 inoculating medium with pure cultures of ancestral isolates (grown as described in the previous
229 section) in various proportions so as to obtain a final density of 10^7 spores in total in 25 mL YPD
230 medium amended with 0.25% antibiotics (100 $mg.L^{-1}$ streptomycin and penicillin – to prevent
231 contamination in this long-term experiment) in 50 mL Erlenmeyer flasks (**Fig. 1C**). PopS was a naïve
232 population composed exclusively of the susceptible isolate S. PopR was composed of 95% S and of
233 5% R_{DMI} and R_{SDHI} (1:1) and therefore contained only single-resistance isolates although both
234 resistances coexisted in the same population. PopRR contained 95% S and 5% R_{DMI}, R_{SDHI} and $R_{SDHI/DMI}$
235 (2:2:1) and therefore contained a collection of single- and multiple-resistant isolates. This initial
236 population composition of PopR and PopRR was determined in preliminary experiments: a
237 proportion of 1% was found to be the smallest proportion providing the lowest mean and standard
238 deviation of experimental error in resistance allele quantification by qPCR or pyrosequencing (see

239 below) on a range of dilutions. As multiple resistance was expected to be scarce in natural
240 populations, we arbitrarily set the initial frequency of $R_{SDHI/DMI}$ at 1% and half that of single-resistant
241 isolates.

242

243 Components of selection regimes diversity

244 The 12 selection regimes differed in terms of four components and are described in detail in
245 **Supplementary Information 2**.

246 The number of AIs ranged from one (straight selection) to three (diversified selection).

247 The number of MoAs ranged from one (intra-MoA selection) to three (inter-MoA selection).

248 We selected AIs representative of their MoAs from among those currently used to control STB in the
249 field. MoAs, and, thus, their representative AIs, differed in terms of their vulnerability to resistance,
250 *i.e.* intrinsic risk of resistance (Brent and Hollomon, 2007). In our view, the risk of resistance is seen
251 globally and may include the time to resistance emergence, the speed of resistance selection and
252 resistance intensity and pattern, as determined by the specific resistance mechanism(s) selected.
253 Selected AIs were considered to be at medium-to-high risk (SDHIs, represented by benzovindiflupyr
254 (B) and fluxapyroxad (F)), low-to-medium risk (DMIs, represented by prothioconazole-desthio (P), the
255 active metabolite of the commercialized prothioconazole, and mefentrifluconazole (M)) or at very
256 low risk (multisite inhibitor, represented by chlorothalonil (C)) of resistance.

257 We also studied the effect of temporal heterogeneity through comparisons of the simultaneous
258 application of AIs (intracycle heterogeneity, *i.e.* mixture) with the sequential application of AIs
259 (intercycle heterogeneity, *i.e.* alternation). All the alternation regimes began with the AI with the
260 lowest risk of resistance (or highest intrinsic activity for strategies with only one MoA), as this has
261 been shown to be the most effective approach (van den Bosch and Gilligan, 2008; Elderfield et al.,
262 2018).

263

264 Regimes of selection applied to artificial populations

265 These 12 selection regimes and 2 control situations were applied to all or some of the three
266 possible ancestral populations, resulting in 31 possible interactions, and 100 independent lines, as
267 there were three replicates for each interaction (or four when chlorothalonil was involved) and five
268 solvent-only controls were included (**Supplementary Information 2)**. Given the large size of this
269 experiment, selection regimes were grouped together into two batches (A and B) performed
270 successively. Strategies that it was particularly important to compare directly were included in the
271 same batch. The two control lines included only 0.5% of DMSO or ethanol 80%. Controls were
272 repeated in both batches if necessary.

273

274 Fungicides preparation

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

281

282 Selection doses

283 For fungicides used alone (in sequence or in alternation), the selection dose was the EC_{97} of
284 each AI determined in a preliminary experiment for the S isolate grown in the conditions used for
285 experimental evolution (described below). EC_{97} values were established from dose-response curves
286 for each fungicide based on the logistic regression model used to describe the resistance patterns of

287 the ancestral isolates. The growth of the populations, inferred from OD_{405} measurement after 7 days
288 of culture, was normalized by dividing by that of the control line grown without fungicide. The
289 validation of this selection dose included culture of the S isolate in the conditions used for
290 experimental evolution at its theoretical EC_{97} and at close doses. The lowest dose providing at least
291 97% inhibition was finally defined as the selection dose.

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

296 The selection doses are detailed in **Supplementary Information 2**.

297

298 Design of the experimental evolution experiment

299 Experimental evolution was performed over either six or eight cycles of seven days each (about six to
300 seven generations per cycle), depending on the experimental batch. This cycle duration corresponds
301 to about the time for which a control line remained in exponential growth phase before reaching a
302 plateau. On the first day of the experiment, ancestral population lines containing 10^7 spores were
303 prepared and incubated as described above, after amendment with the fungicides required for each
304 selection regime. OD_{405} was measured at the end of each cycle and transformed into population size,
305 as established in preliminary experiments (See more details in Ballu et al., 2023). The OD_{405} of the
306 treated lines was normalized against the mean OD_{405} for the control solvent lines. At each transfer,
307 500 µL of the evolving culture (*i.e.* 2% of the total volume of medium) or a maximum of 10^7 spores
308 was transferred to a new Erlenmeyer flask containing fresh medium. If the number of spores in the
309 500 µL of culture medium was less than 10^7, as would be the case before the evolution of resistance,
310 then the appropriate number of spores from the appropriate source population (*i.e.* spores from the

311 control (PopS) or mixtures of spores reconstituting PopR or PopRR from the pure cultures of the
312 different isolates grown separately without fungicides) was added to make the number of spores
313 present up to 10^7. Thereby the starting population size was equalized between lines at the start of
314 each new cycle, and the process mimicked the immigration occurring in field situations. For each of
315 the three replicates, a dedicated source population was used for immigration throughout the
316 experiment. In preliminary experiments, immigration was found to be useful for preventing
317 population extinction after weekly bottlenecks, and to minimize genetic drift.

318 At the end of each cycle, 2 mL of each line was mixed with glycerol (25%), frozen and stored at -80°C
319 for further analyses. If the normalized Malthusian growth (defined below) of a line reached at least
320 95% during three consecutives cycles, the line was ended. In such cases, we ensured fair comparison
321 between alternation and mixture approaches by stopping lines only after an even number of cycles
322 (*i.e.* not before the 4th cycle). In these situations, data (population size or allele content) for the
323 missing cycles during this establishment phase were extrapolated for analyses involving comparisons
324 between selection regimes. Extrapolated data were calculated using the average value of the last
325 three consecutive cyles with generalized resistance normalized by divided by the value of the control
326 at the considered cycle.

327

328 Assessment of the rate of resistance evolution

329 The Malthusian growth (Fisher, 1999) of each line was calculated as

330 $$M_{it} = \ln\left(\frac{Spore\ concentration\ of\ line\ i\ at\ the\ end\ of\ cycle\ t}{Spore\ concentration\ of\ line\ i\ at\ the\ beginning\ of\ cycle\ t}\right) \quad (3)$$

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

332 For each selection regime, the mean Malthusian growth M_i^n is the mean of M_{it}^n calculated over the
333 total number of cycles the line was grown, and constitutes a quantitative summary of the increase in
334 resistance of the three replicates over the entire experiment.
335 We used beta regression (package BETAREG for R) on the M_i^n of each line to investigate the effects of
336 population composition and of the components of selection diversity and temporal heterogeneity,
337 included as fixed factors, with experimental batch as a random factor. These models are detailed in
338 **Supplementary Information 7**. We evaluated the impact of these factors with an analysis-of-
339 variance-like (joint_tests function of EMMEANS package) analysis, with the Tukey adjustment method
340 for pairwise comparisons (package EMMEANS).

341

342 Molecular quantification of resistance alleles in evolved lines

343 The *sdhC*^{H152R} allele, included in the R_{SDHI} and $R_{SDHI/DMI}$ ancestral isolates, was quantified by
344 qPCR. The *cyp51*^{I381V} allele, included in the R_{DMI} and $R_{SDHI/DMI}$ ancestral isolates, was quantified by
345 pyrosequencing. The *cytb*^{G143A} allele, included only in the $R_{SDHI/DMI}$ ancestral isolate was quantified by
346 qPCR. Details on these procedures are provided as **Supplementary Information 4**.

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

350 $$RC_{it} = Resistance\ allele\ frequency * C_{it} \quad (5)$$

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

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

355 $$RC_{it}^n = \frac{RC_{it}}{C_{Ct}} \quad (6)$$

356 The qPCR and pyrosequencing methods used could not distinguish combinations of *sdhC*^{H152R} and
357 *cyp51*^{I381V} within the same individual (*i.e.* as in $R_{SDHI/DMI}$) from co-occurrence of the same alleles in
358 different individuals (*i.e.* as in the R_{SDHI} and R_{DMI} isolates). We therefore set the maximum possible
359 concentration of this multiple-resistant isolate as the lower proportion of the two resistant alleles
360 compared together. This calculation may overestimate the concentration of the $R_{SDHI/DMI}$ isolate in
361 PopRR. For validation of this estimate, we compared these data with those for *cytb*^{G143A}
362 quantification on samples collected from the last cycles of PopRR lines.
363 For each of the three resistance alleles, we used beta regression (package BETAREG for R) on the mean
364 normalized allele concentrations of each line to investigate the effects of population composition,
365 with the components of selection diversity and temporal heterogeneity treated as fixed factors, and
366 experimental batch as a random factor. We evaluated the impact of factors with an analysis-of-
367 variance-like (joint_tests function of EMMEANS package) approach with the Tukey adjustment method
368 for pairwise comparisons (package EMMEANS). Details of these models are provided in **Supplementary**
369 **Information 7** and models are referred hereafter as MOD"*NAME*".

370

371 Phenotype resistance profiles of evolved lines

372 The cross-resistance patterns of all populations collected at the end of the experimental
373 evolution were established in droplet tests. We deposited population suspensions containing 10^7,
374 10^6, 10^5, and 10^4 spores.mL^{-1} as drops organized into columns, on 12 cm square Petri dishes. These
375 plates contained YPD supplemented with discriminatory doses of fungicides, as detailed in
376 **Supplementary Information 3**. The 10 treatments tested encompassed a control with 0.5% solvent
377 and fungicides B, P, F, M and C at their selection doses and their mixtures. Finally, for the detection
378 of generalist resistance, we also included fungicides not used for experimental evolution (tolnaftate
379 and azoxystrobin although this fungicide is of limited interest for PopRR lines where the *cytb*^{G143A} was
380 introduced with the $R_{SDHI/DMI}$ isolate). Tolnaftate, in particular, identifies individuals displaying

381 enhanced fungicide efflux leading to non-specific multidrug resistance (MDR), as described in field
382 isolates of *Z. tritici* (Leroux and Walker, 2011). Ancestral isolates, and a MDR isolate collected in the
383 field were included as control strains. After seven days of incubation, drops were scored 0 or 1,
384 according to the presence or absence of fungal growth. A total score was established for each strain,
385 ranging from 0 (susceptible) to 4 (resistant if >0), according to the number of dilution droplets for
386 which growth was observed. For each strain, the resistance phenotype profile was determined by
387 concatenating the scores established for the 10 sets of fungicide conditions.

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

392

393 Characterization of enhanced efflux

394 The 17 strains isolated from lines growing on tolnaftate in droplet tests were selected for
395 further investigation. We checked for changes in the length of the promoter of *mfs1* by PCR, as
396 previously described (Omrane et al., 2017). Several types of insertion into the promoter of this gene
397 encoding a MFS transporter have been shown to cause enhanced efflux leading to multidrug
398 resistance in field isolates of *Z. tritici*. We quantified *mfs1* expression in the same isolates by RT-qPCR
399 (Omrane et al., 2015). The protocols used are detailed in **Supplementary Information 6**.

400

401

402 # Results

403

404 ## Ancestral isolates display contrasting patterns of resistance in artificial populations

405 Experimental evolution was performed with artificial populations constituted from ancestral
406 isolates exhibiting resistance alleles currently found in European populations of *Z. tritici*. The
407 resistant isolates (R_{SDHI}, R_{DMI} and $R_{SDHI/DMI}$) were isolated from a second-generation progeny of a cross
408 between the susceptible (S) wild-type reference isolate and a field isolate bearing the *sdhC*^{H152R}
409 change and several *cyp51* substitutions (*i.e.* V136A, A379G, I381V, Y461S and S524T; hereafter
410 simplified as *cyp51*^{I381V}). We checked that the resulting changes to the target proteins of SDHI and
411 DMI fungicides, respectively, were associated with the expected phenotypes and calculated their
412 resistance factors (RFs) relative to the S isolate (**Fig. 1A**; **Supplementary Information 1**). We
413 confirmed that the R_{SDHI} and $R_{SDHI/DMI}$ isolates bearing *sdhC*^{H152R} had RFs exceeding 90 for all SDHIs
414 (Dooley et al., 2016b; Yamashita and Fraaije, 2018) and that the R_{DMI} and $R_{SDHI/DMI}$ isolates bearing
415 *cyp51*^{I381V} displayed moderate resistance to all DMIs tested (9<RFs<40), although the RFs for
416 mefentrifluconazole were lower than those for other azoles (Huf et al., 2018; Ishii et al., 2020).
417 Complementary genotyping revealed that the R_{SDHI}, R_{DMI} and $R_{SDHI/DMI}$ isolates had inherited resistance
418 to benzimidazoles from their parental field isolate, precluding any further use of these fungicides in
419 selection regimes (Hawkins and Fraaije, 2016), and that only the multiple-resistant $R_{SDHI/DMI}$ isolate
420 had inherited resistance to QoIs due to the *cytb*^{G143A} change (Torriani et al., 2015). All isolates were
421 equally susceptible to the multisite agent chlorothalonil and to tolnaftate, an inhibitor of squalene
422 epoxidase used to reveal multidrug resistance in *Z. tritici* (Leroux and Walker, 2011).
423 The fitness of the ancestral isolates was assessed by establishing growth curves under the conditions
424 used for experimental evolution and in the absence of fungicide selection (**Fig. 1B**), and the
425 parameters of the logistic model were used to compare isolates (**Fig. 1C**). The time required to reach

426 the half-maximal concentration of spores was significantly greater for the resistant isolates than for
427 the reference isolate (2.94-3.08 days *vs.* 2.5 days), but maximal spore concentration was significantly
428 higher for resistant isolates than for the reference isolate at the end of the experiment (+4.1 -
429 +5.8%). However, in populations from control lines not exposed to fungicide (see below), the
430 resistance alleles initially present at low frequency were not detected at the end of the experimental
431 evolution period, suggesting that either the weak and early penalty associated with resistance
432 observed in our conditions was expressed, and/or that they were lost by chance because of the
433 regular bottlenecks affecting populations during their serial cultivation. As no other genotype
434 replicates were tested, it remains unclear whether this fitness penalty was related to the resistance
435 mutations or to the genetic background of the isolates. Nevertheless, these findings clarify and
436 complete previous observations concerning the fitness cost associated with the *sdhC*^{H152R} variant
437 expressed in culture *in vitro* and in competition experiments (Scalliet et al., 2012; Gutiérrez-Alonso et
438 al., 2017), and restoration of the fitness cost of the I381V variant when associated with a
439 modification to the Y461 codon of *cyp51*, as in R_{DMI} and $R_{SDHI/DMI}$(Hawkins and Fraaije, 2018). We
440 assumed that resistance to benzimidazoles did not bias fitness measurement, as no associated
441 fitness penalty has ever been observed for this resistance in any species, including *Z. tritici* (Hawkins
442 and Fraaije, 2016; Hawkins and Fraaije, 2018). Resistance to QoIs has been reported to decrease *Z.*
443 *tritici* virulence *in planta* (Hawkins and Fraaije, 2018), but we assumed that this cost did not operate
444 *in vitro* for the $R_{SDHI/DMI}$ isolate.
445

446 Population composition affects the performance of resistance management strategies

447 We investigated the effect of population composition on the performance of strategies, by
448 comparing the evolution of three artificial populations containing 0% (PopS) or 5% (PopR and PopRR)
449 mixed resistant individuals, to reproduce the late stages of emergence of resistance as usually
450 detected with current monitoring tools in field situations (**Fig. 1D**). PopR contained only single-
451 resistant isolates (*i.e.* R_{SDHI} and R_{DMI}) and PopRR contained both single-resistant isolates and a small

452 proportion of multiple-resistant individuals (*i.e.* $R_{SDHI/DMI}$), to mimic the results of the sexual
453 reproduction likely to occur in field populations of *Z. tritici* (Singh et al., 2021). These populations
454 were subjected to selection pressure from alternating treatments with benzovindiflupyr (a SDHI;
455 hereafter referred to as B) and prothioconazole-desthio (a DMI; hereafter referred to as P) or a
456 mixture of these treatments, and were then compared with untreated lines (**Fig. 2**; **Supplementary**
457 **Information 2**). We quantified the dynamics of resistance in each line as its mean normalized
458 Malthusian growth, M_i^n, a proxy for the increase in resistance under selection (**Fig. 3A**). The growth
459 of PopS was significantly smaller (82% and 84% smaller, under P and B mixture or alternation,
460 respectively) than that of the control. Significant decreases in growth relative to the control were
461 also observed for the PopR population treated with the P+B mixture (85%), and to a lesser extent,
462 the PopR population subjected to an alternation of P and B (46%). By contrast, the growth of PopRR
463 was much more similar to that of the control population, despite very slight, but nevertheless
464 significant decreases of 3% in alternation and 5% in mixture, revealing that both these strategies
465 performed very poorly once multiple-resistant isolates were present in the population. These
466 observations mirrored the strong and significant selection (increase by a factor of 53 to 157) of the
467 resistant *sdhC*^{H152R} and *cyp51*^{I381V} alleles in all PopRR lines (**Fig. 3B** and **3C**), and, more specifically, of
468 the multiple-resistant isolate $R_{SDHI/DMI}$, as revealed directly by the peak frequencies of *cytb*^{G143A} at the
469 end of the experiment (**Supplementary Information 5**). The more moderate increase (increase by a
470 factor of 0 to 12) in the frequencies of *sdhC*^{H152R} and *cyp51*^{I381V} in PopR lines confirmed that the
471 various strategies, including mixture in particular, were useful for mitigating the selection of single-
472 resistant isolates, at least during the eight cycles of the experiment (*i.e.* ≈ 50-55 generations).

473 We also established phenotype resistance profiles for all the lines at the end of the experiment, to
474 observe the selection of *de novo* resistances other than those introduced *via* the ancestral
475 phenotypes. Principal component analysis confirmed that the evolved lines differed mainly in their
476 ancestral composition rather than in selection regimes (**Fig. 5**). The PopS and PopRR lines retained

477 their initial phenotypes, but one PopR line evolved resistance to tolnaftate, an inhibitor of squalene
478 epoxidase often used to reveal multidrug resistance(Leroux and Walker, 2011).

479

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

482 We investigated the extent to which population composition interfered with the components
483 of strategies and determined their performance, by subjecting PopR and PopRR to 12 selection
484 regimes mimicking various degrees of selection heterogeneity and diversity, and comparing them
485 with untreated controls. We used two SDHI fungicides (B and F) potentially selecting for $sdhC^{H152R}$,
486 and two DMI fungicides (P and M) potentially selecting for $cyp51^{I381V}$ to investigate the effect of the
487 number of AIs (1 *vs.* 2) and of the number of MoAs (1 *vs.* 2) on resistance evolution (**Fig. 2** and
488 **Supplementary Information 2**). In PopRR lines, the maximal concentration of the multiple-resistant
489 isolate was estimated to be the lower of the concentrations of the two alleles. In situations in which
490 $cytb^{G143A}$ was quantified, these two measurements were found to be highly correlated (Pearson's r =
491 0.998; P<0.0001).

492 In strategies including two AIs with the same MoA, resistance was rapidly established, as shown by
493 the maximal Malthusian growth and the increase in resistance allele frequencies, as early as the
494 second cycle, as with the continuous use of a single AI, whatever the ancestral population (**Fig. 6A**).
495 Such strategies even significantly accelerated the selection of resistant alleles in PopR lines relative to
496 the continuous use of a single AI (+17.8% for $sdhC^{H152R}$ on average in strategies combining two SDHIs;
497 +2.3% for $cyp51^{I381V}$ on average in strategies combining two DMIs). The selection of single-resistant
498 isolates was also favored over that of multiple-resistant ones in PopRR lines (a mean allele frequency
499 of ≈97% and ≈86% for $cyp51^{I381V}$ and $sdhC^{H152R}$, respectively, over the experiment, *vs.* a mean
500 frequency of 42% for multiple-resistant individuals) (**Fig. 4**, **Fig. 6A** and **Supplementary Information**
501 **5**). Moreover, the relative ratio of single- *vs.* multiple-resistant isolates for each MoA was 2:1 at the

502 beginning of the experiment. This ratio greatly increased under DMI selection but stayed roughly
503 stable under SDHI selection, at the end of the experiment. This finding suggests a relative resistance
504 cost for the $R_{SDHI/DMI}$ isolate in competition with R_{DMI} isolate under DMI selection pressure although
505 both co-occurred.

506 By contrast, the introduction of a second AI with a different MoA (SDHI or DMI) significantly
507 decreased population growth (by 2 - 85%, with a mean of 39%), whilst reducing the selection of
508 resistant alleles (-49% - -100%, mean -82% for $sdhC^{H152R}$ and -43% - -100%, mean -87% for $cyp51^{I381V}$,
509 relative to selection with a single AI) in lines in which only single-resistant isolates to each of the two
510 MoAs were initially introduced (**Fig. 6B**). The greatest reductions were observed with the P and B
511 fungicides, rather than with the M and F ones. This benefit of combining MoAs was much more
512 limited (from -4% to +4% of M_i^n, mean -0.6%) when the multiple-resistant isolate was introduced,
513 due to its early and rapid generalization (increase by +85% - +596%; mean +241%), at the expense of
514 single-resistant isolates, revealing its greater relative fitness when multiple selection is applied (**Fig.
515 4**, **Fig. 6B** and **Supplementary Information 5**). These findings confirm that diversifying modes of
516 action, whatever the heterogeneity in the timing of their application (as a mixture or in alternation) is
517 beneficial only in the absence of multiple resistance.

518 We investigated whether increasing the number of AIs and of MoAs induced the selection of new
519 resistance profiles, by performing droplet tests on the 99 population lines evolved under one of the
520 31 selection regimes and collected at the end of the experiment, for 10 fungicide treatments and a
521 control. These treatments included the fungicides used for selection but also other independent AIs
522 (**Fig. 5** and **Supplementary Information 3**). Population resistance profiles were generally consistent
523 with allele quantification, with the population displaying the resistance phenotype profile of the
524 predominantly selected ancestral isolate. In particular, in PopRR lines that had evolved under
525 diversified selection pressure from SDHIs combined with DMIs, low phenotypic diversity was
526 observed, confirming the generalization of the multiple-resistant isolate. By contrast, greater

527 diversity was observed after selection by a single MoA, reflecting the variation in resistance
528 frequency of the single-resistant isolates between replicates and selection regimes. In PopR lines, the
529 lack of growth in the presence of DMI and SDHI mixtures of lines evolved under a single selection
530 pressure confirmed the preferential selection of single-resistant isolates. Interestingly, not only did
531 lines evolved under diversified selection regimes grow in the presence of the two MoAs
532 independently, confirming the co-selection of single-resistant isolates, but some also displayed
533 additional resistances, as suggested by their greater diversity in the PCA and original patterns of
534 resistance on heatmaps (**Fig. 5**). In particular, some lines selected under pressure from a combination
535 of M (DMI) and F (SDHI) fungicides displayed resistance to the non-DMI and non-SDHI fungicides
536 azoxystrobin and tolnaftate. Tolnaftate has been shown to reveal multidrug resistance in *Z. tritici*
537 (Leroux and Walker, 2011). We therefore checked for insertions in the promoter of the MFS1
538 transporter gene, which is overexpressed in field isolates (Omrane et al., 2017), in a selection of 20
539 strains isolated from these populations with various scores for tolnaftate resistance. We found no
540 change in the length of the promoter sequence (data not shown). We then measured the expression
541 of *mfs1* in the same isolates after growth in the absence of fungicide. We confirmed that nine of
542 these isolates displayed moderately enhanced (about 3.3 - 10 times higher) fungicide efflux. By
543 contrast, the level of expression of *mfs1* in the MDR field isolate was about 30 times higher,
544 suggesting only moderately enhanced efflux due to MFS1 in some of our isolates, with still unknown
545 determinism. Furthermore, the poor correlation between tolnaftate resistance scores and *mfs1*
546 expression strongly suggests that unidentified generalist resistance mechanisms may be at work in
547 our collection. Finally, this finding suggests that diversified selection pressure mediates a trade-off
548 between the growth rate of the evolving population and the degree of generalism of the evolved
549 isolates.

550

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

553 We further investigated the reasons for the better performance, in terms of effects on
554 population growth, of strategies combining several MoAs and AIs than of continuous use of the same
555 fungicide, by analyzing the effects of the respective intrinsic resistance risks of the MoAs and AIs on
556 resistance evolution. This analysis was made possible by the use of two different MoAs (SDHIs *vs.*
557 DMIs) and two AIs within each chemical class (B and F *vs.* P and M, respectively). It has been claimed
558 that the intrinsic risk of resistance is low-to-moderate for DMIs, but moderate-to-high for SDHIs
559 (Kuck and Russell, 2006). AIs were compared at the same effective dose (EC_{97}) to ensure a fair
560 comparison based on their intrinsic properties.

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

574 The intrinsic resistance risk may also be AI-specific, and was therefore investigated for SDHIs and
575 DMIs. In our conditions, the M_i^n of strategies combining P and B was lower, by a factor of 1.45 - 5.7

576 (mean 2.4), than that of strategies combining M and F in PopR lines. This finding is consistent with
577 the -55% to -100% decrease in the concentrations of *sdhC*^{H152R} or *cyp51*^{I381V} in strategies including P
578 and B (**Fig. 6B**). The poorer control observed in M- and F-based strategies may also explain the
579 emergence of additional generalist resistance mechanisms, as described above, in these lines (**Fig.**
580 **5D**). The benefit of strategies combining P and B over those combining M and F was also significant in
581 PopRR lines, but to a much lesser extent (-2.6% of M_i^n; -1% in content of the multiple-resistant
582 isolate). These findings suggest that it may also be difficult to generalize resistance risk to a whole
583 MoA.

584

585 New MoAs at appropriate doses are relevant partners for enhancing the performance
586 of strategies

587 Fungicide diversity was found to affect the selection of resistance for AIs for which resistance
588 was present at low frequencies in the initial populations. We also explored whether introducing a
589 "new" AI, *i.e.* an AI to which the whole populations were fully susceptible, could improve the
590 performance of strategies, and under which conditions. We therefore combined chlorothalonil (C), a
591 multisite inhibitor with a very low risk of resistance, with the B and P fungicides previously used (**Fig.**
592 **6B**).

593 Introducing C into a combination of B and P in PopR lines induced a slight increase of M_i^n (+46% for
594 the BP/C alternation and +13% for the BPC mixture when compared with the already high-
595 performing BP mixture), its overall value being close to the detection limit over the course of the
596 experiment. This finding can be explained by equal full control of both single-resistant isolates by C
597 that reinforces that of the BP mixture. By contrast, in PopRR, combining C with B and P had a
598 beneficial effect on resistance (-48% and -30% M_i^n respectively, for the BP/C alternation and BPC
599 mixture, compared with the poorly-performing BP mixture) and on the frequency of resistant alleles
600 (-63% for *sdhC*^{H152R} and -63% for *cyp51*^{I381V} average). However, resistance dynamics differed

601 depending on whether C was alternated or mixed with a BP mixture. In the mixture strategy, a half-
602 dose of C was not sufficient to control the increasing proportion of the multiple-resistant isolate in
603 the population and to compensate for the decrease in efficacy of P and B ($M_i^n = 0.66$ and mean
604 double resistant frequency = 38%). In the alternation strategy, C applied at full dose was fully
605 effective against the $R_{SDHI/DMI}$ ancestral isolate, restored efficacy, and selected against multiple
606 resistance every two cycles, enabling a better overall control of the population ($M_i^n = 0.49$ and
607 mean double resistant frequency = 25%). In populations in which resistance has emerged, the
608 performance of strategies involving a new AI may therefore be dependent on its dose and its
609 redundancy in the selection pattern.

610 In strategies combining B, C and P, the increase in MoA diversity due to the introduction of a third
611 MoA could potentially increase the degree of generalism in the evolved populations, as previously
612 described (Ballu et al., 2021; Ballu et al., 2023). This trade-off was not observed (*i.e.* no additional
613 growth on tolnaftate; **Fig. 5**) in PopRR lines where the multiple-resistant isolate was present and
614 fitter, and invaded the population rapidly, at the expense of the susceptible isolate. However, PopR
615 populations subjected to alternation or mixture involving C acquired generalist resistance, as
616 revealed by their growth on tolnaftate, unlike all but one population subjected to a combination of B
617 and P. This emergence and selection of generalist resistance might have been permitted by the
618 maintenance of higher diversity in populations, more likely to experience favourable mutation.
619 Therefore, when a new MoA is introduced and combined with previously used MoAs, attention must
620 be paid to the prior presence or absence of certain resistances, which could strongly influence the
621 risk of generalist resistance emerging, as well as specialist resistance to the new MoA, depending on
622 its own resistance risk.

623

624 Mixtures do not systematically outperform alternation: population composition also
625 matters

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

629 The temporal heterogeneity achieved by alternating instead of mixing AIs with the same MoA had no
630 (for DMIs) or little (-3% to -5%; for SDHIs) effect on M_i^n, whatever the composition of the ancestral
631 population (**Fig. 6A**). Within-MoA alternations and mixtures of SDHI and DMI fungicides led to the
632 rapid generalization of $sdhC^{H152R}$ and $cyp51^{I381V}$, respectively, in a similar manner. The rate of
633 $sdhC^{H152R}$ selection was slightly lower (-9% in PopR and -2% in PopRR) for the alternation of B and F
634 than for their mixture.

635 When SDHIs and DMIs were combined, their mixtures performed significantly but moderately better
636 than their alternation (in average, -34% for M_i^n; -70% for $sdhC^{H152R}$ concentration; -99% for $cyp51^{I381V}$
637 concentration), only when single-resistant isolates were introduced (PopR lines). However, mixtures
638 combining M and F were less effective than those combining B and P, because of the lower level of
639 control exerted by their half-doses or differences in their inherent resistance risks, as explained
640 above (**Fig. 6B**).

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

647 Alternation and mixtures resulted in a diversity of resistance phenotype population profiles,
648 reflecting the variable frequencies of the resistances to SDHIs or DMIs, or their co-occurrence (**Fig. 5**).

No resistances other than those initially introduced from the ancestral strains were observed, with the exception of the generalist resistance profiles of PopR lines, which were also able to grow on tolnaftate and azoxystrobin, as previously described. These generalist profiles were found exclusively in mixture and alternation selection regimes including two or three MoAs; neither alternation nor mixing could be considered more likely to favor generalist resistance. Temporal heterogeneity may 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

672 selection pressure to mitigate the selection of resistance if the initial populations were fully
673 susceptible or included only single-resistant isolates at low frequency. By contrast, the introduction
674 of multiple-resistant isolates abolished the beneficial impact of the best strategy drivers, indicating
675 that population composition is the principal factor to be considered when tailoring anti-resistance
676 strategies.
677

678 **Alternation can be as sustainable as mixture and may reconcile resistance management and**
679 **socioenvironmental expectations**

680 Using an original experimental evolution approach, we show here that maximizing the
681 diversity of selection while smartly combining drivers can effectively delay the evolution of
682 resistance, as previously reported in empirical studies comparing a limited number of field or
683 modeled situations, based on assumptions simplifying the biological reality (REX_Consortium, 2013;
684 van den Bosch et al., 2014a). In particular, our findings confirm that selection was weakest for the
685 combination of several MoAs (*i.e.* when maximizing inter-MoA diversity), and stronger when AIs with
686 the same MoA were combined (*i.e.* when maximizing intra-MoA diversity).
687 Indeed, the exclusive use of DMI or SDHI fungicides, with one or two AIs, rapidly led to the
688 establishment of resistance, associated with a loss of full control. This situation mirrors findings
689 previously obtained with the same approach concerning the emergence of resistance in a naïve
690 population (Ballu et al., 2023). Several studies have documented the poor efficacy of sequences of
691 fungicides with the same MoA for controlling target-site resistance in *Z. tritici,* in the field or in
692 models (Mavroeidi and Shaw, 2006; Hobbelen et al., 2013; Dooley et al., 2016a; Heick et al., 2017b;
693 Hagerty et al., 2021). Indeed, AIs with the same MoA generally display positive cross-resistance, as
694 confirmed by the phenotyping of our ancestral isolates. However, resistance intensity may differ
695 between AIs for the same resistance allele, or between alleles for the same AI, reflecting the
696 variation in AI affinity for the mutated target protein (e.g. Rehfus et al., 2018), ranging from strong to
697 weak or a lack of resistance to some specific AIs. AIs exceptionally not or less affected by cross-

698 resistance may appear of interest from a practical standpoint. However, this benefit is strongly
699 limited by the high diversity of resistance mutations and phenotypes in field populations and by the
700 later emergence of mutations determining full cross-resistance (Leroux and Walker, 2011; Huf et al.,
701 2018; Rehfus et al., 2018; Garnault et al., 2021). Our results suggest that although combinations of
702 fungicides sharing the same MoA involve potentially very diverse AIs, with the entire range of
703 authorized SDHIs and DMIs generally used in agricultural areas, they do not provide sufficiently
704 diverse selection pressure and globally favor the selection of resistance on a long-term perspective.

705 The use of multiple MoAs, rather than AIs, is another option to maximize the diversity of selection,
706 but this can be achieved either in mixture, with all generations of the pathogen submitted to the
707 same combination of selection pressures, or in alternation, in which a few generations are subjected
708 to a single selection pressure different from that imposed on their ancestors or progeny. The efficacy
709 of mixtures is based on redundant killing and the low probability of *de novo* multiple resistance
710 developing, but also possibly on higher dose rates (see below) (Ballu et al., 2021), whereas
711 alternation may delay the build-up of resistance by spreading selection pressure out over longer
712 periods, and by decreasing the rate of evolution of resistance to the fungicides alternated (Ballu et
713 al., 2023). Many studies have compared the efficacy of these strategies for limiting the emergence of
714 resistance and selection in fungi and other taxa (see reviews in REX_Consortium, 2013; Kim et al.,
715 2014; van den Bosch et al., 2014a). Most, but not all, of these studies ranked mixtures above
716 alternation in terms of performance in theoretical and empirical studies and simplicity of
717 implementation, notably due to the availability of commercial ready-to-use mixtures. In most
718 studies, mixtures were often defined as the simultaneous application of two MoAs, both used at
719 their effective dose (van den Bosch et al., 2014a). In the field, this would lead to the quantities of
720 fungicide used almost doubling. In this study, we propose an original and fair comparison of
721 alternation and mixtures at constant environmental impact, *i.e.* with equal cumulative amounts of
722 fungicide. Mixtures were, thus, defined as half the selection dose of each AI calculated for
723 susceptible isolates. In this context, the mixture and alternation of SDHIs and DMIs were equally

724 effective for delaying the selection of resistance and controlling the growth of initially naïve
725 populations. Mixtures moderately outperformed alternation when single-resistant isolates were
726 present in the initial population, because the partial and possibly synergistic efficacies of both
727 partners remained sufficient to control emerging single-resistant isolates and keep them at low
728 frequency in the population. By contrast, in alternation, each single-resistant isolate alternatively
729 escaped control when subjected only to a full dose of the corresponding AI every other cycle,
730 because this strategy allowed to increase frequency of both isolates, alternatively. Performance was
731 also determined by the AIs combined (*e.g.* at the chosen selection doses, M and F performed less
732 well than P and B, whatever the strategy used). Mixtures and alternation were equally poor when
733 the multiple-resistant isolate was present in the initial population and selected, as both AIs were
734 ineffective, which rapidly established itself within the population. Interestingly, in this context, the
735 introduction of a new MoA to which the whole population was susceptible was more valuable in
736 alternation than in mixtures, as alternation allowed maximal efficacy against multiple resistance due
737 to the application of a full dose every other cycle. In situations in which multiple resistance is
738 observed, the benefits of alternation may depend more on the dose, frequency of use and
739 vulnerability to resistance of the new agent introduced, than on the AIs alternated. Here, the new
740 fungicide introduced was a multisite inhibitor with a very low resistance risk. The introduction of a
741 new, previously unused fungicide may be considered with caution for a single-site fungicide with a
742 greater inherent risk of resistance, the efficacy of which may decrease over time. Overall, our
743 findings demonstrate that at similar and constrained total dose, mixtures may not always outperform
744 alternation and are in line with a recent modelling study (Hardy, 2022) that concludes that the
745 relative efficacies of these strategies clearly depend on the composition of the target population.
746 This finding might be highly relevant in a context where the reduction of pesticide use, more
747 achievable by using alternation than by using many current mixtures, constitutes a strong
748 socioenvironmental expectation in an increasing number of countries. It finally represents an exciting
749 perspective for future field investigations, where the unpredictable nature of the wider environment

750 may pose greater challenges and exaggerate some to the inherent differences that may exist
751 between the different MoAs used.

752

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

755 Our analysis of the relevance of drivers of resistance management strategies for delaying the
756 development of resistance unequivocally identifies population composition with regard to resistance
757 as the primary determinant of the performance of strategies, ahead of the diversity of the MoAs
758 applied and temporal heterogeneity. Multiple resistance, in particular, is an aggravating factor, as it
759 is very poorly controlled by the fungicides that contributed to its selection, whether they were used
760 alone or in combination. In strategies combining multiple MoAs, multiple-resistant isolates are
761 preferentially selected over single-resistant ones, because they confer greater relative fitness under
762 multidimensionnal selection (Corkley et al., 2022). This study confirms *in vivo* the results of modeling
763 studies considering multiple resistance (Hobbelen et al., 2013; Tepekule et al., 2017). Finally, this
764 experimental evolution approach also provides evidence that increasing the dimensionality of
765 selection may favor generalist phenotypes over specialist ones, a question rarely explored so far,
766 except for a recent experimental work (White et al., 2022) whose results tend to agree with ours,
767 and that would deserve complementary theoretical investigation (Mérot, 2022).
768 Generalist fungicide resistance is also observed when non target-site resistance is selected, that is,
769 when resistance mechanisms other than those encoded by mutations in the target-encoding genes
770 are selected and confer wide patterns of cross-resistance to unrelated fungicides (Berman and
771 Krysan, 2020; Hu and Chen, 2021). In an experimental design in which resistance is already present,
772 we expected to see the direct selection of the resistance alleles, but at contrasting intensities,
773 depending on the selection regime applied. Surprisingly, the analysis of population phenotypes at the
774 end of the evolution experiment revealed the occurrence of *de novo* phenotypes in some lines. In

775 populations into which only single-resistant isolates were present, additional resistance to
776 azoxystrobin, a QoI not used for selection, and tolnaftate, an inhibitor of sterol-squalene epoxidase
777 generally used to reveal enhanced fungicide efflux (Leroux and Walker, 2011), formerly absent, was
778 detected, exclusively in some lines subjected to alternations or mixtures of SDHIs and DMIs,
779 associated or not to the multisite inhibitor C. This generalist resistance co-existed, within
780 populations, with the initially introduced DMI and SDHI resistances. Generalist resistance can even
781 be associated with target-site resistance allele, as highly probable in lines undergoing M and F
782 selection where *sdhC*^{H152R} was fully established at the end of the experiment. The purification of
783 isolates resistant to tolnaftate made it possible to confirm the slight-to-moderate overexpression of
784 *mfs1*, encoding a MFS transporter responsible for multidrug resistance in *Z. tritici* in the field
785 (Omrane et al., 2017), in some, but not all of the strains tested. The absence of the usual regulatory
786 insertions in the promoter of *mfs1*, and the poor correlation between susceptibility to tolnaftate and
787 *mfs1* expression suggest that unknown mutations and resistance mechanisms probably underlie the
788 generalist resistance of these isolates. This evolutionary trade-off in resistance management, with
789 attempts to reduce selection for specialist resistance traits promoting the evolution of generalist
790 resistance, was observed in a previous study dealing with alternation and effective-dose mixture
791 strategies in naïve populations (Ballu et al., 2021; Ballu et al., 2023). It may apply more generally to
792 any strategy combining multiple MoAs in alternation or mixtures and aiming to mitigate the
793 evolution of resistance in other organisms, such as weeds (Lagator et al., 2013a; Lagator et al.,
794 2013b; Comont et al., 2020) or bacteria (Kim et al., 2014). This trade-off is still little considered in the
795 field, with attention instead focused on more quantitative criteria for measuring strategy
796 performance, such as resistance frequency, or the effective life-time of a fungicide. However, high
797 frequencies of individuals with generalist resistance in the pathogen population may irreversibly
798 decrease the number of effective AIs even more than TSR. In weeds and insects, NTSR and TSR
799 generally occur together and are associated with high RFs and unexpected resistance patterns
800 (Mitchell et al., 2014; Gaines et al., 2020). In *Z. tritici,* MDR *per se* is associated with moderate

801 resistance factors, but its combination with target-site alterations has been found to increase
802 resistance factors to DMIs or SDHIs significantly (Walker and Gazeau, 2019).

803 Given the importance of population composition for strategy performance and the trade-off
804 described above, these findings suggest that the debate should be refocused on the prevention of
805 multiple and, to a lesser extent, generalist resistance, rather than on the relative merits of mixtures
806 and alternation, as summarized in (Corkley et al., 2022).

807

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

809 Multiple resistance occurs in populations for several reasons. First, it reflects the sequential
810 use of MoAs, following their successive discoveries and withdrawals following the development of
811 intensive cropping systems, which began just after World War II. Resistance led to new AIs often
812 being combined with their predecessors to improve residual efficacy, and resistance to new MoAs
813 was successively selected in genetic backgrounds that were already resistant to the older MoAs,
814 which were generally withdrawn once resistance became widely established. Multiple resistance has
815 been described as resulting from a stepwise accumulation of resistances in several plant pathogens
816 (*e.g. Venturia inaequalis* (Koller and Wilcox, 2001), *Monilinia fructicola* (Luo and Schnabel, 2008),
817 *Botrytis cinerea* (Li et al., 2014), and *Cercospora beticola* (Secor et al., 2010)), and it may even favor
818 the development of resistance to new MoAs. *Z. tritici* is no exception, as resistance to
819 benzimidazoles and QoIs remains widespread in populations from Western Europe, despite the
820 withdrawal of these fungicides from the market to control septoria leaf blotch. The ongoing selection
821 of resistance to SDHIs is almost systematically associated with DMI resistance, simply because it is
822 selected within DMI-resistant populations (Dooley et al., 2016b; Garnault et al., 2019).

823 Second, multiple resistance may result from the recombination of resistance alleles during sexual
824 reproduction. The primary inoculum of *Z. tritici,* including ascospores, is never a limiting factor for
825 epidemics and sexual reproduction. Indeed, population-level deep sequencing revealed intense

826 interplay between asexual and sexual reproduction (Singh et al., 2021). In this airborne pathogen, the
827 ascospores resulting from sexual reproduction are dispersed over long distances and have been
828 reported to spread QoI resistance between fields locally (Fraaije et al., 2005) but also at the
829 continental scale in Europe (Torriani et al., 2009).

830 Despite these strong assumptions on the occurrence of multiple resistance in agricultural systems,
831 the performance of strategies has generally, with few exceptions, been modeled from initially
832 susceptible or populations containing single-resistant isolates. Yet, the reality in the field is that new
833 AIs are probably only rarely confronted with the ideal situation in which all the AIs and MoAs used
834 are fully effective. In the real world, multiple resistance is the norm, and the initial frequency of
835 single- and multiple-resistant isolates almost certainly differ at fine scales depending on the historical
836 use of fungicides and resistance management ; strategies need to be adapted accordingly (Taylor and
837 Cunniffe, 2022). In this work, we assessed the performance of strategies in conditions mimicking
838 real-life conditions, while artificially introducing single- and multiple-resistant isolates, to reproduce
839 the combination of resistance alleles likely to occur in the field by sexual reproduction, but hardly
840 achievable in the laboratory. However, the lack of this phenomenon in our experimental system
841 precludes any assessment of the time required for the emergence of multiple resistance by
842 recombination, all the more since preexisting resistances are often established by stepwise
843 accumulation in populations.

844 One of the first conclusions to be drawn from our findings is that the design of resistance
845 management strategies, and the long-term durability of MoAs would greatly benefit from resistance
846 monitoring data (*i.e.* the composition of the local population) being made publicly available at the
847 finest possible scale and detection limit. The high-throughput quantification of multiple resistance
848 genotypes may become possible in the near future with the development of next-generation
849 sequencing techniques for use in diagnostics for plant pathogen populations (Radhakrishnan et al.,
850 2019; Samils et al., 2021). This, in turn, should make it possible to take informed decisions in spraying

851 programs. Second, more attention should be paid to limiting the development of multiple resistance,
852 and of the combination of TSR and NTSR, as both could greatly decrease the efficacy of any strategy.
853 Recombination in airborne pathogens such as *Z. tritici* is an aggravating factor, but it is one that is not
854 really preventable. However, delaying the emergence of single-resistant isolates and limiting the
855 probability of recombination between them by ensuring that their frequencies remain low might be a
856 more realistic option. It has been estimated that *Z. tritic*i produces at least 2.1-9.9 trillion
857 pycnidiospores per hectare (McDonald et al., 2022). The number of *de novo* mutation events
858 associated with resistance occurring in a field may, therefore, be sufficiently high to prevent all of
859 these mutations being lost by chance, even if the mutation rate is low. The time to the emergence of
860 full resistance has been found to decrease monotonically with increasing dose, over realistic ranges
861 of pathogen life-history and fungicide dose-response parameters (Mikaberidze et al., 2017). The
862 maintenance of low frequencies of resistance alleles during the selection phase would involve
863 limitations on the use of all MoAs, especially when the frequency of resistance reaches a critical
864 threshold over a large area. Disease control should more than ever be based on complementarity
865 with other control measures, such as the replacement of synthetic fungicides by biological agents,
866 resistant cultivars, prophylactic measures and agricultural practices, to increase the diversity of
867 selection pressures and limit the emergence of resistance by controlling population size. The overall
868 sustainability of integrated pest management may therefore rely on the vulnerability of each method
869 to fungal adaptation and then on our capacity to predict and monitor these phenomena. This would
870 also lead the community to consider criteria other than the effective life of fungicides when assessing
871 the durability of crop protection. Assessing qualitative changes in evolved population while
872 evaluating the diversity of protection measures used would also help us to work towards current
873 socioenvironmental expectations while better reflecting the multiple evolutionary processes at work.
874 However, although adapting the way we conceive resistance management strategies to focus more
875 on multiple resistance would be a huge step forward in itself, the implementation of these strategies
876 in practice will probably prove an even greater challenge. Indeed, optimal disease control would

877 imply using the best fungicides and at high rates in standardized programs. By contrast, resistance
878 management with limited environmental impact would imply using the whole range of available AIs,
879 whatever their intrinsic efficacy, within parsimonious combinations tailored to the current local
880 composition of the population, associated with other control measures. These approaches are
881 conceived for short (*i.e.* an agricultural season) and long (*i.e.* decades) time scales, respectively, and
882 maximize profit or promote sustainable agriculture, respectively, potentially creating an intractable
883 dilemma for growers. Education about resistance issues and communication between stakeholders
884 and farmers are almost certainly the key to changing the paradigm and initiating a transition towards
885 agroecology.

Data availability

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

Citation : Ballu, A., et al. (2022). Dataset of an experimental evolution on the effect of fungicide-resistance composition of populations on the durability of anti-resistance strategies on the wheat pathogen *Zymoseptoria tritici*, Recherche Data Gouv, https://doi.org/10.15454/DCVDZN.

Supporting Information Captions

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: Molecular quantification of resistance alleles in evolved lines

Supplementary information 5: cytb^{G143A} content of evolved lines for PopRR

Supplementary information 6: Expression of *mfs1* in evolved lines of *Zymoseptoria tritici*

Supplementary information 7: Models used for data analysis

Acknowledgments

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

Funding

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

Author contributions

AB, ASW, FC, AD 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, ASW, FC and AD, with additional contributions from all authors.

Competing interests

The authors declare no competing interests.

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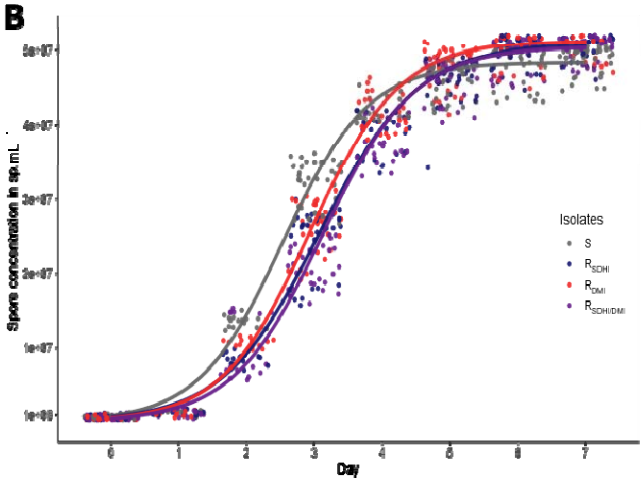
1099 Yamashita, M., and Fraaije, B. (2018) Non-target site SDHI resistance is present as standing genetic
1100 variation in field populations of *Zymoseptoria tritici*. *Pest Management Science* **74**: 672-681.
1101

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)
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

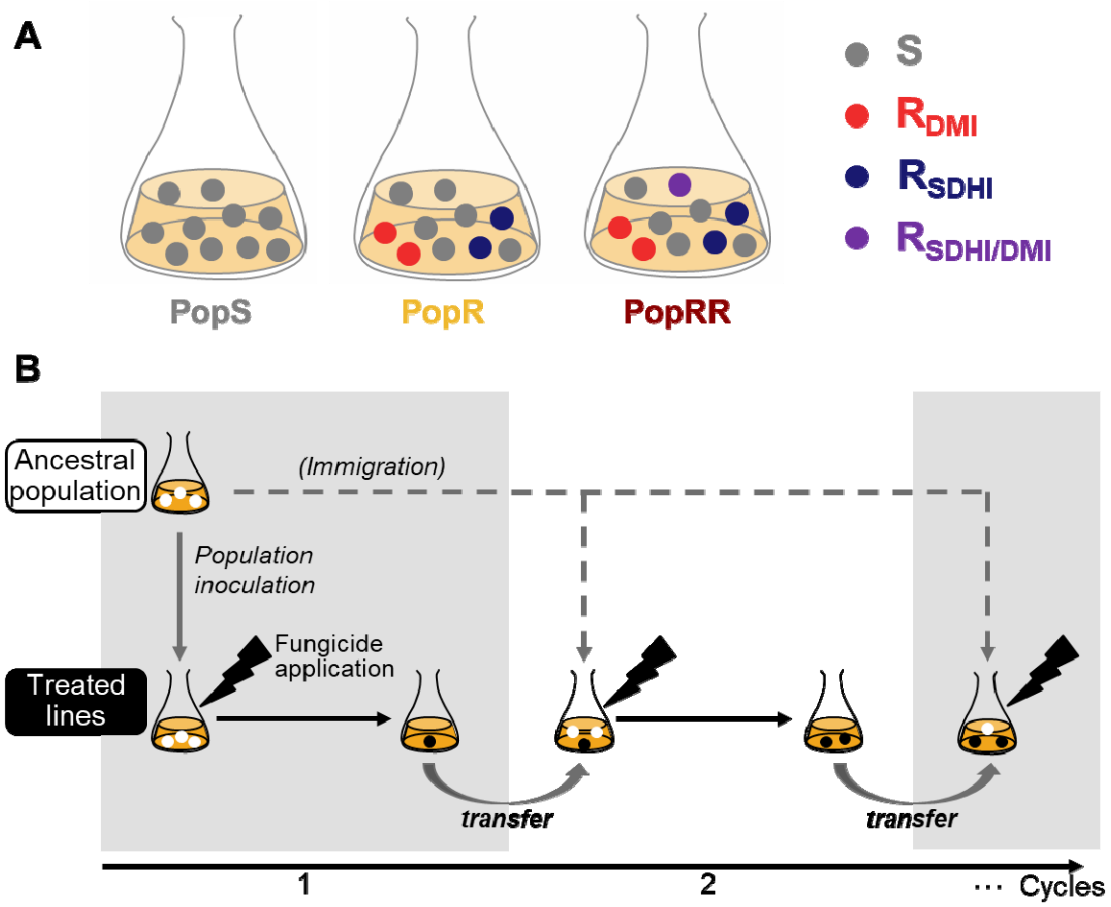
Isolates	xmid	slope	Asym
S	2.54 (0.05) a	1.61 (0.02) a	4.84E+07 (4.37E+05) a
R_{SDHI}	3.08 (0.05) bc	1.41 (0.05) b	5.08E+07 (4.43E+05) b
R_{DMI}	2.94 (0.05) b	1.54 (0.02) ab	5.12E+07 (4.40E+05) b
$R_{SDHI/DMI}$	3.13 (0.05) c	1.49 (0.02) ab	5.04E+07 (4.42E+05) b

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.*** The S, R_{SDHI}, R_{DMI} and $R_{SDHI/DMI}$ isolates are the ancestral isolates used in the experiment fully susceptible, resistant to SDHIs, resistant to DMIs and resistant to both, respectively (Dooley et al., 2016b; Huf et al., 2018). ***A. Profiles of susceptibility to multiple fungicides of ancestral isolates of* Z. tritici.** Estimations of EC_{50} and Resistance Factors (*i.e.* $EC_{50\ R\ isolate}$ / $EC_{50\ S\ isolate}$). The standard error is specified in brackets. ***B. Fitness of ancestral isolates in the conditions used for experimental evolution.*** Growth without fungicide, for cultures of single isolates.

1110 The experiment was performed five times and the data were modeled with a mixed non-linear logistic
1111 regression model. ***C. Estimated parameters of the growth curves for ancestral isolates.*** The standard error is
1112 shown in brackets. *asym* is the asymptote (*i.e.* maximum growth); *xmid* is the time required to decrease the
1113 asymptote by 50%; *scal is* the inverse of the slope . *The group letters (a,b,..) refer to Tukey's pairwise tests;*
1114 *different letters indicate significant differences (P<0.05).* ***D. Artificial populations of* Z. tritici *used for***
1115 ***experimental evolution.*** The evolution of a fully susceptible PopS population was compared to that of the PopR
1116 and PopRR populations containing only single-resistant isolates and single- and multiple-resistant isolates,
1117 respectively.
1118



C Modes of action

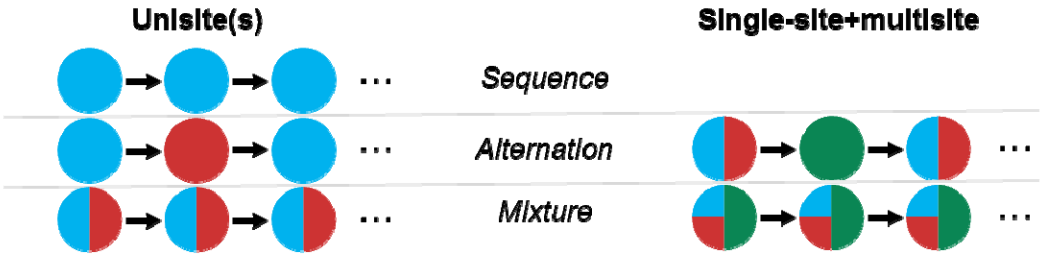
SDHI **DMI**
(concerned by resistance)

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

Multisite
(naive)

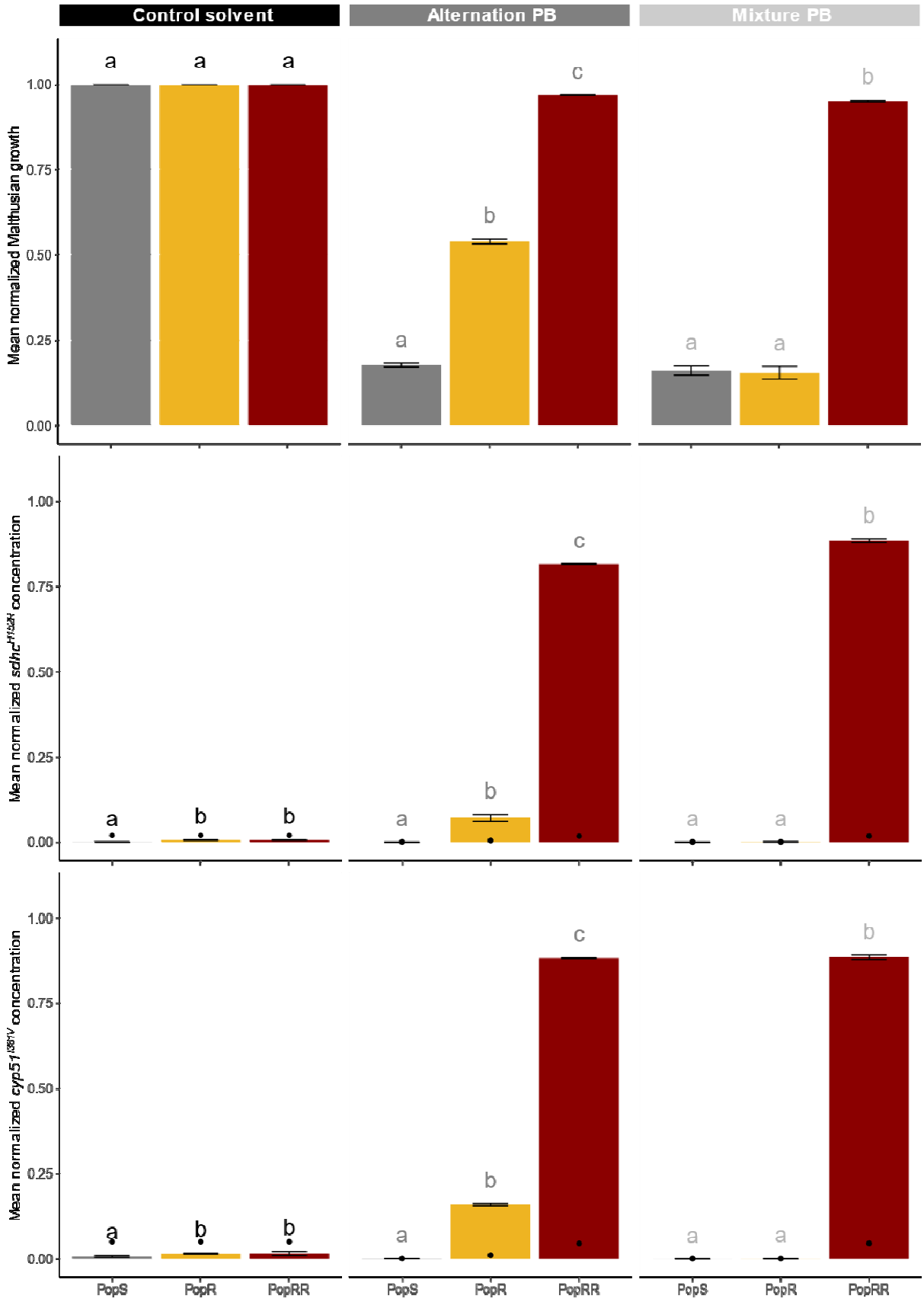
- C — Chlorothalonil

Selection regimes (4-8 cycles)



1119

1120 **Figure 2: Experimental evolution design.** ***A. Ancestral populations.*** Isolate S (susceptible) in gray; R_{SDHI}
1121 (resistant to SDHIs) in blue; R_{DMI} (resistant to DMIs) in red; $R_{SDHI/DMI}$ (resistant to SDHIs and DMIs) in purple. ***B.***
1122 ***General design of the experimental evolution.*** The three ancestral populations were used to found 10^7 spores
1123 lines. Fungicides were added to the treated lines to mimic distinct patterns of selection. After seven days, 2% of
1124 the population was used to inoculate fresh medium to start the next cycle, supplemented, if necessary, by
1125 immigration from the untreated control line from the same cycle to achieve a total of 10^7 spores. ***C.***
1126 ***Fungicides and selection regimes.*** Five fungicides (indicated by specific labels and colors) were studied: two
1127 SDHIs, benzovindiflupyr and fluxapyroxad (B and F; shades of blue; moderate resistance risk); two DMIs,
1128 prothioconazole-desthio and mefentrifluconazole (P and M; shades of red; low resistance risk); and the
1129 multisite inhibitor chlorothalonil (C; green; no risk of resistance). Fungicides were used in sequence, alternation
1130 or in mixture (see **Supplementary Information 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

1131 **Figure 3: Performance of management strategies according to the resistance composition of the ancestral**
1132 **population of Z. *tritici*.** Selection regimes included the mixture or alternation of benzovindiflupyr (B) and
1133 prothio-desthio (P), contrasted with a control amended only with 0.5% solvent. Ancestral populations (PopS,
1134 PopR and PopRR,) respectively in gray, yellow and dark red. The group letters refer to Tukey's pairwise tests;
1135 different letters indicate significant differences (P<0.05). ***A: Mean normalized Malthusian growth. B: Mean***
1136 ***normalized concentration of sdhc^{H152R}.*** This allele confers high-level resistance to B and was quantified by
1137 qPCR. Black dots indicate the detection limit of this allele. ***C. Mean normalized concentration of cyp51^{I381V}.***
1138 This allele confers moderate resistance to P and was quantified by pyrosequencing. Black dots represent the
1139 detection limit of this allele. Statistics as for ***B*** (MODV0).

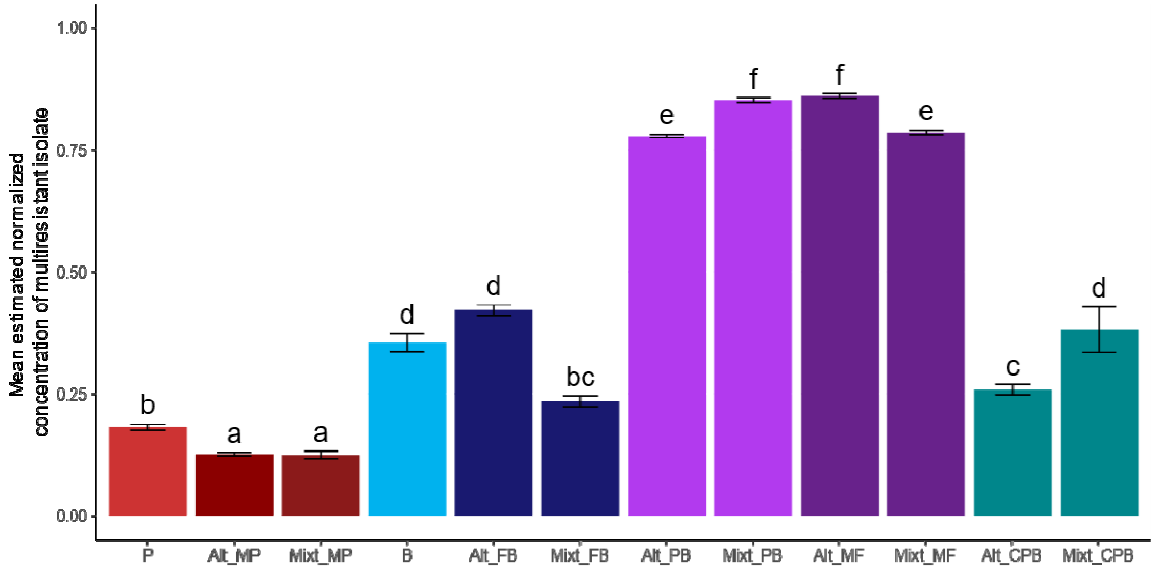


Figure 4: Mean maximal concentration of the multiresistant isolate in lines evolved from the PopRR ancestral population. Molecular methods used to quantify allele content could not distinguish combinations of sdhC^{H152R} *and cyp51*^{I381V} within the same individual (i.e. as in $R_{SDHI/DMI}$) from co-occurrence of the same alleles in different individuals (i.e. as in the R_{SDHI} and R_{DMI} isolates). Group letters refer to Tukey's pairwise tests; different letters indicate significant differences ($P<0.05$).

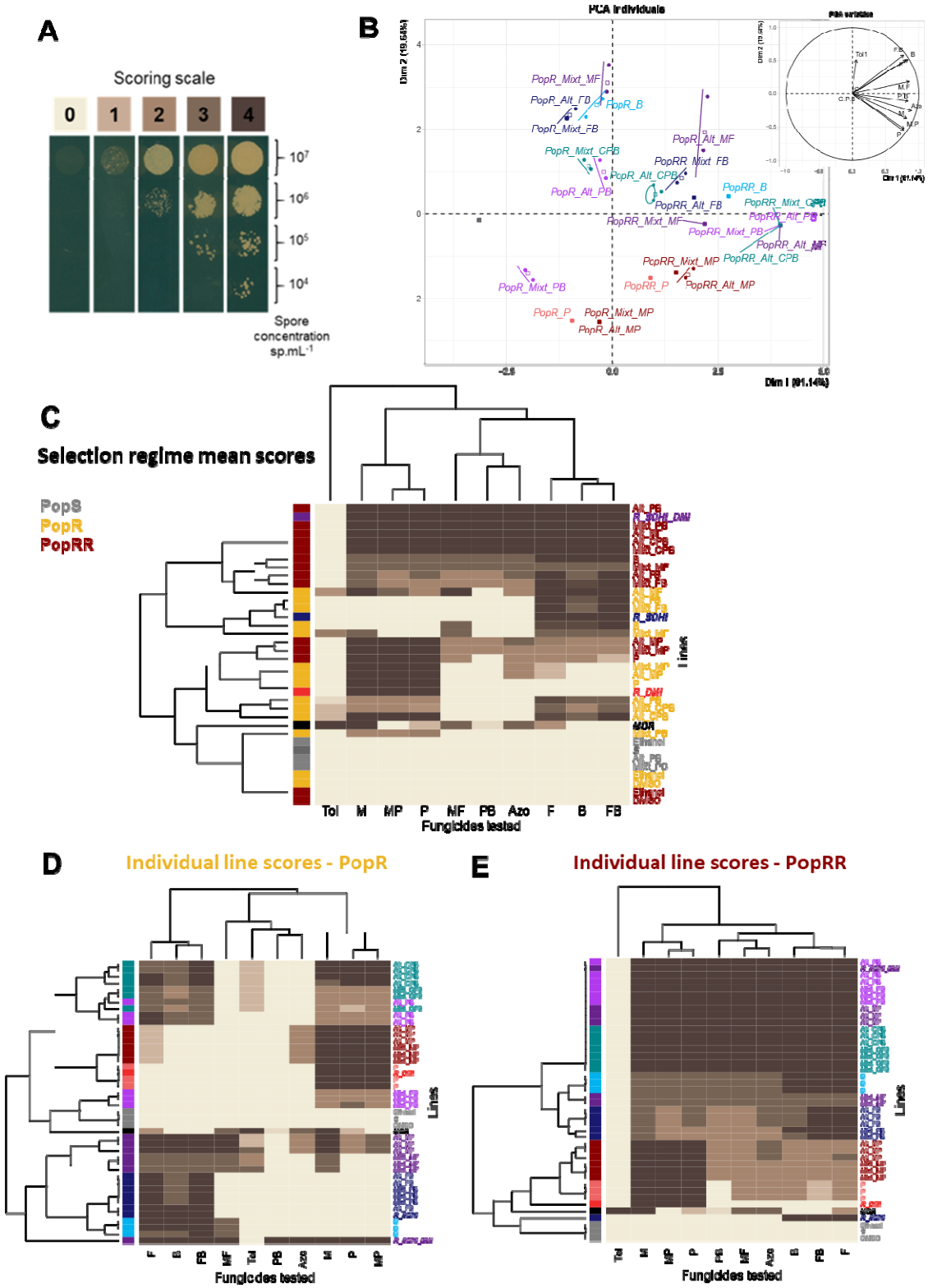
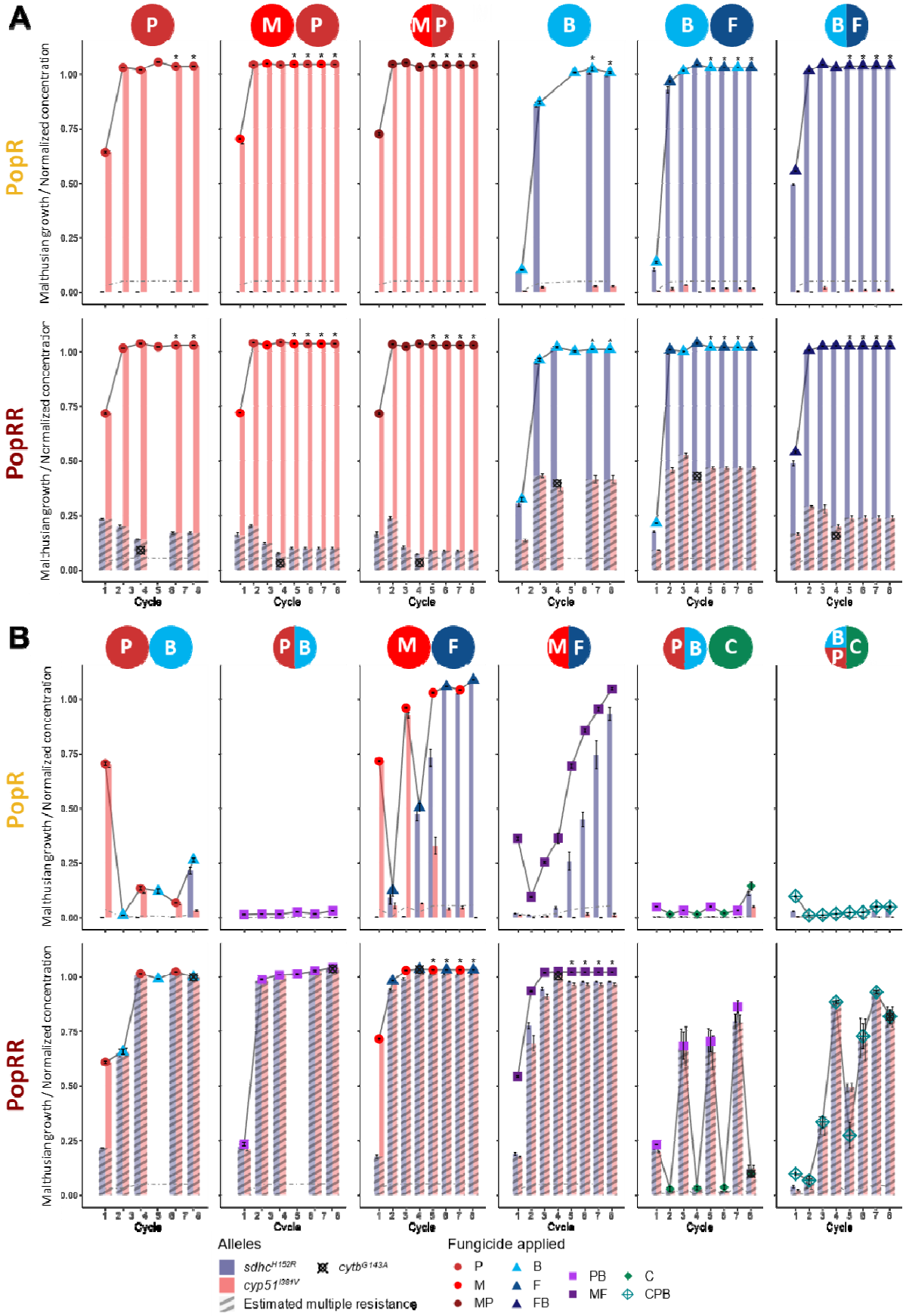


Figure 5: Phenotype resistance profiles of *Z. tritici* observed at the end of experimental evolution. The names of the ancestral isolates and selection regimes are as in **Fig. 1** and **Supplementary Information 2**. The

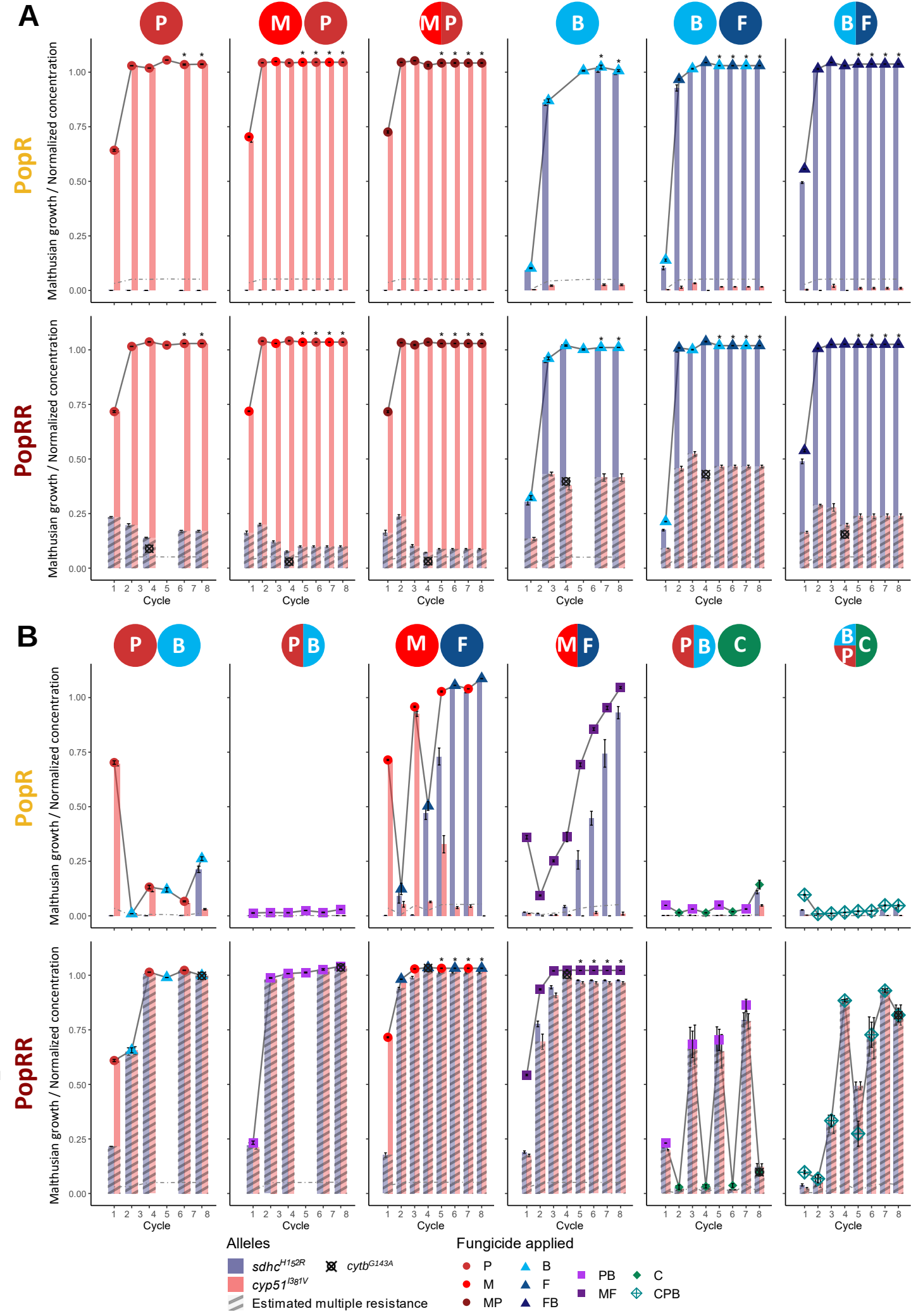
1149 fungicides tested in droplet tests included benzovindiflupyr (B), fluxapyroxad (F), prothioconazole-desthio (P)
1150 and mefentrifluconazole (M) at their selection doses (EC_{97}) and in mixture (1/2 EC_{97} of each), as well as
1151 azoxystrobin (Azo) and tolnaftate (Tol) which were not used for experimental evolution, at the minimal dose
1152 controlling the ancestral susceptible ancestral isolate. MDR: MDR isolate collected in the field (Leroux and
1153 Walker, 2011) serving as a reference for generalist resistance and exhibiting high resistant to tolnaftate. Scoring
1154 after the 4th or 6th cycle, according to selection regime. ***A. Scoring scale used to assess the growth of lines on***
1155 ***solid medium supplemented with discriminatory doses of fungicides (droplet tests).*** For each population, four
1156 spore concentrations were tested on the same plate. 0: no growth (susceptible isolate). 1-4: growth varying
1157 with spore concentration (resistant isolate). ***B: Individual graph from the principal component analysis***
1158 ***performed on the phenotype resistance profiles of the evolved lines***. Colors according to selection regimes
1159 (ellipses for each ancestral population line x selection regime). The inset graphic shows the PCA variables. The
1160 first axis of the PCA discriminated the evolved lines according to their ancestral populations, based on their
1161 resistance phenotype profiles. ***C: Heatmaps of average resistance phenotype profile scores for all selection***
1162 ***regimes and ancestral populations.*** Mean of the scores established for the three population replicates
1163 subjected to the same selection regime. ***D: Heatmaps of phenotype resistance profile scores for individual***
1164 ***PopR lines***. Ancestral or field strains are shown in bold italics. Profiles of lines presenting no or few variability
1165 between repeats are shown as their mean in order to improve heatmap visibility. Individual scores for the
1166 three replicates of each selection regime are shown otherwise.. ***E: Heatmaps of phenotype resistance profile***
1167 ***scores for individual PopRR lines***. Ancestral or field strains are shown in bold italics. Profiles of lines presenting
1168 no or few variability between repeats are shown as their mean in order to improve heatmap visibility.
1169 Individual scores for the three replicates of each selection regime are shown otherwise.


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

1170

1171 **Figure 6: Performance of management strategies according to the selection regime applied to the ancestral**
1172 **population of *Z. tritici*.** Selection regimes are indicated at the top of the panels and refer to the sequence (one
1173 fungicide dot), alternation (two successive fungicide dots) or mixture (one shared dot) of fungicides applied.
1174 The color and shape of the symbols in the graphs indicate the selecting fungicide used at a given cycle. Light
1175 gray lines show the change in Malthusian growth in evolved lines over the eight cycles of experimental
1176 evolution. Bars show the change in the concentration of resistance alleles ($sdhC^{H152R}$ in blue and $cyp5^{I381V}$ in
1177 red). The dashed portion of these bars corresponds to the maximum possible proportion of the multiresistant
1178 ancestral strain $R_{SDHI/DMI}$. The final concentration of $cytb^{G143A}$ measured in PopRR lines is indicated by a black
1179 crossed circle. Dashed gray lines represent the detection limit of the alleles. Statistical models are detailed in
1180 **Supplementary Information 7**. ***A. Performance of strategies with poorly diverse selection pressure.*** Increasing
1181 the number of AIs within a strategy did not significantly improve Malthusian growth (MODM1, *P*=0.5464),
1182 normalized resistance allele concentration (MODR1, *P*=0.0706; MODV1, *P*=0.1384) or the normalized
1183 concentration of the multiple mutant (MODDM1, *P*=0.1155). In particular, combining AIs sharing the same MoA
1184 (SDHI or DMI) did not reduce the growth of the population, actually even increasing it (MODM1.1, *P*=0.9358 for
1185 DMIs and *P*<0.0001 for SDHIs), whilst also accelerating the selection of single resistance alleles (MODR1.1,
1186 *P*<0.0001; MODV1.1, *P*=0.0236) in PopR lines, and limiting the selection of multiple resistance in PopRR lines,
1187 particularly under DMI exposure (MODDM1.1, *P*=0.3022). DMI-based strategies resulted in 1.04 times higher
1188 mean normalized Malthusian growth than SDHI-based strategies, whatever the initial population composition
1189 (PopR and PopRR, MODM1.1, *P*<0.0001), the number of AIs (1 or 2, MODM1.1, P<0.0001) or the temporal
1190 pattern of fungicide application (alternation and mixture, MODM2.1, *P*<0.0001). In PopRR lines, SDHIs controlled
1191 the multiple-resistant isolate less efficiently than DMIs (MODDM1.1, *P*<0.0001). The temporal pattern of
1192 fungicide application had little or no effect on Malthusian growth when AIs with the same MoA were combined
1193 (MODM2.1, *P*<0.0001 for SDHIs only, *P*=0.7783 for DMIs only) and on the selection of single resistance alleles
1194 (MODR2.1, in the presence of SDHIs P=0.7833, in the presence of DMIs only *P*=0.9597; MODV2.1, in the presence
1195 of DMIs only *P*=0.8505). ***B. Performance of strategies with highly diverse selection pressure.*** Combining AIs
1196 with different MoAs (SDHIs and DMIs) significantly reduced growth (modM1, *P*<0.0001) and the selection of
1197 resistance alleles (MODR1, *P*<0.0001; MODV1, *P*<0.0001) when only single-resistant isolates were introduced into
1198 the initial population. This benefit was not observed in PopRR lines (MODM1, *P*=0.2287), due to the early and
1199 rapid selection of the multiple resistant ancestral strain as opposed to its counterselection in the presence of a

1200 single MoA (MODDM1, *P*<0.0001; MODG143A1, *P*<0.0001). Strategies combining P and B were more efficient
1201 than those combining M and F for decreasing Malthusian growth (MODM2.2, *Ps*<0.0001) or the selection of
1202 single resistance alleles (MODR2.2 or MODV2.2, *Ps*<0.001) in PopR lines, and, to a much lesser extent, in PopRR
1203 lines (MODDM2.2, *P*= 0.0296). The addition of a previously unused AI (C) maintained (PopR lines; MODM3,
1204 *P*<0.0001) or restored (PopRR lines; MODM3, *P*<0.0001) the performance of strategies, but the utility of this
1205 driver depended strongly on its dose and redundancy in the strategy in which both B and P lost efficacy due to
1206 the establishment of multiple resistance (PopRR lines). Mixtures outperformed alternation when SDHIs and
1207 DMIs were combined (Malthusian growth MODM2.2, P<.001) but only if exclusively single-resistant isolates
1208 were present in the initial population. Mixture and alternation strategies performed similarly otherwise, but
1209 with alternation limiting the Malthusian growth of the multiple-resistant isolate more efficiently when a third
1210 previously unused MoA was added to SDHIs and DMIs (MOD3, *P*<0.001).


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

A

Chemical class	Fungicide	EC_{50} (mg.L^{-1})	Resistance factor (RF)		
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SDHIs	Benzovindiflupyr	0.028 (0.003)	92.61 (18.4)	3.73 (0.53)	247.8 (191.6)
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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)
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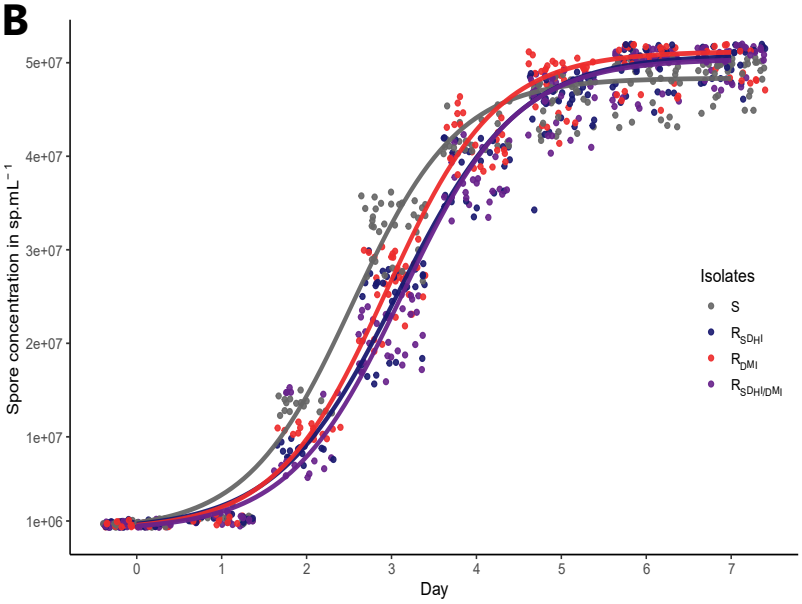
Benzimidazoles	Carbendazim	0.115 (0.037)	> 430	> 430	> 430
QoIs	Azoxystrobin	0.047 (0.004)	0.61 (0.11)	0.7 (0.11)	454.1 (787)
Multisites	Chlorothalonil	1.185 (0.104)	0.42 (0.04)	0.67 (0.08)	0.78 (0.09)
SBIs class IV	Tolnaftate	0.23 (0.022)	1.47 (0.33)	1.34 (0.2)	0.5 (0.07)

B

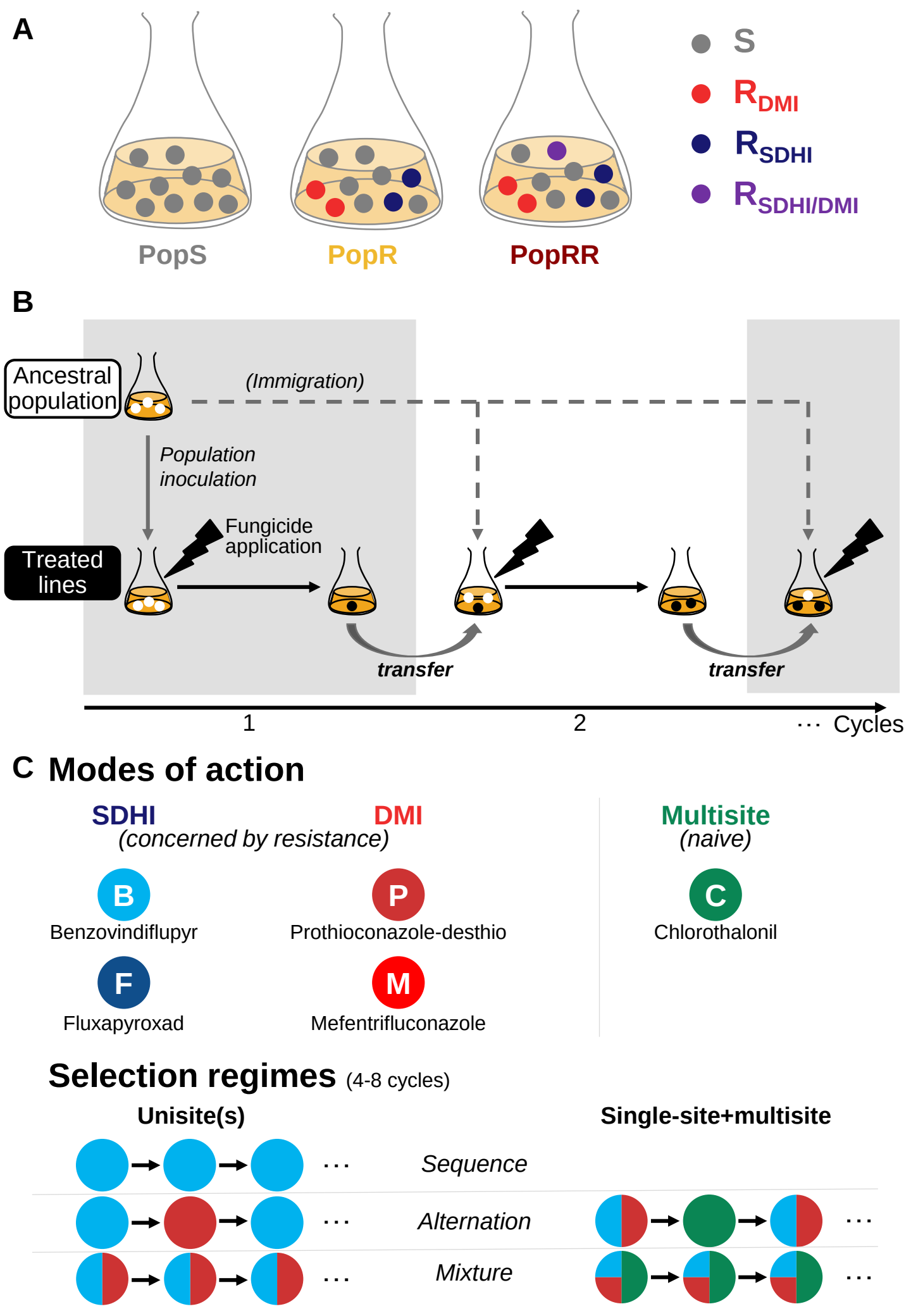


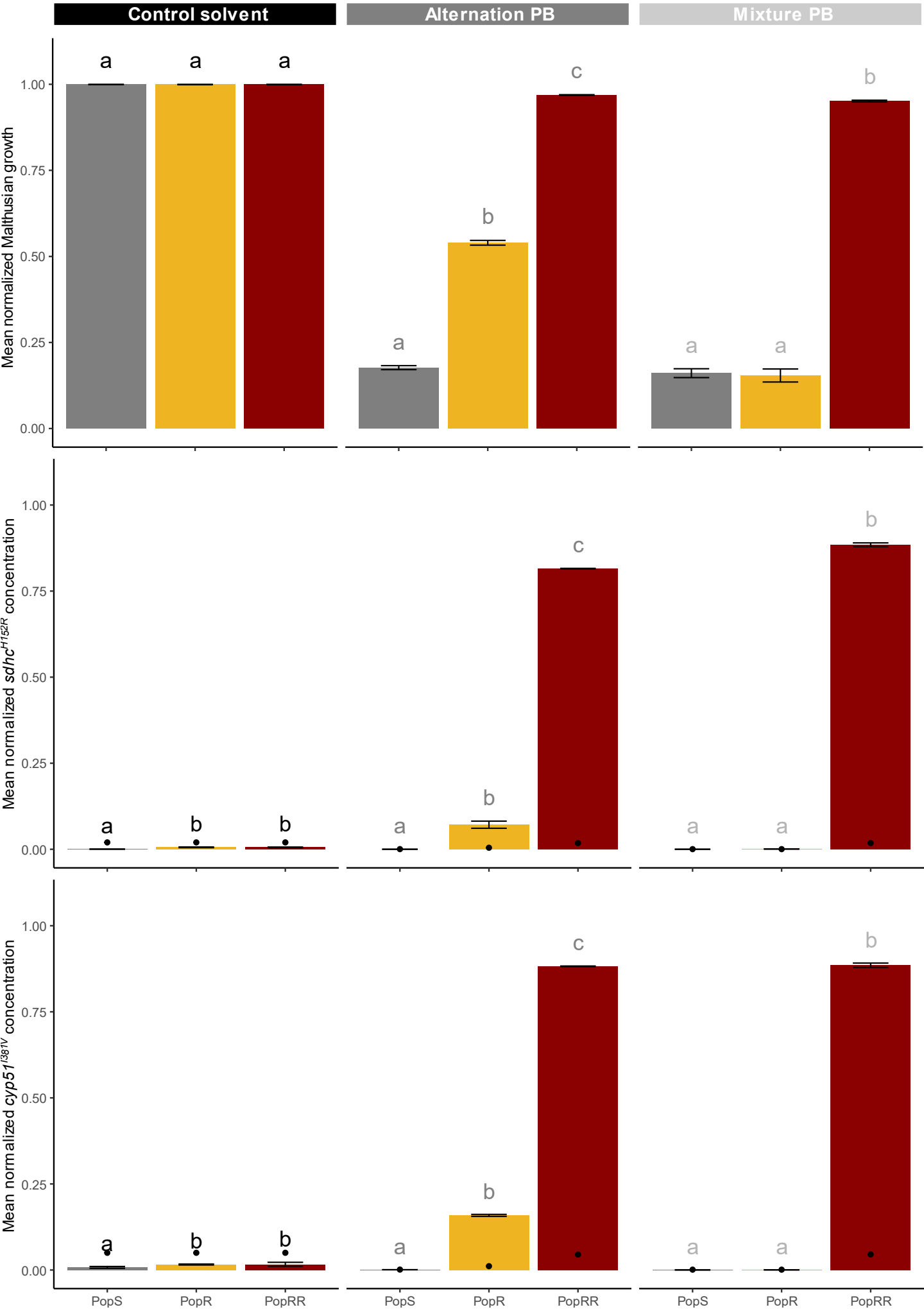
C

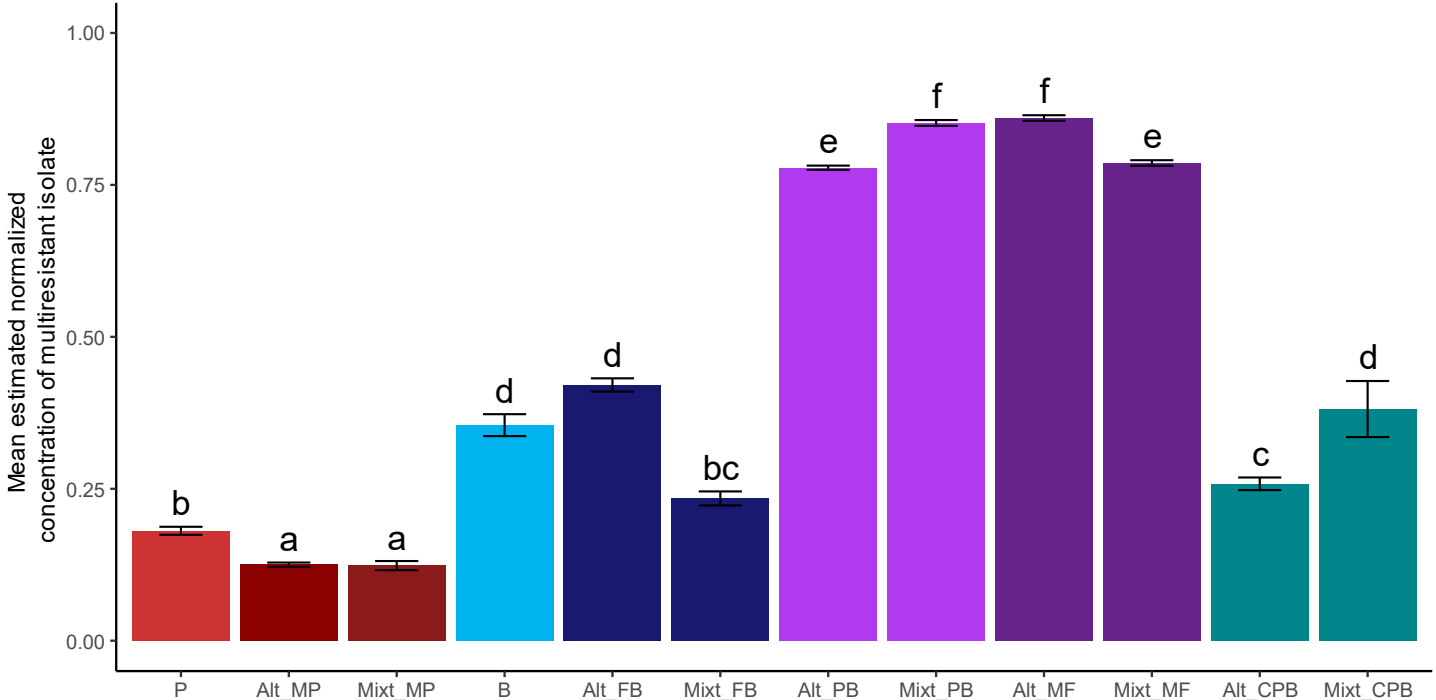
Isolates	xmid	slope	Asym
S	2.54 (0.05) **a**	1.61 (0.02) **a**	4.84E+07 (4.37E+05) **a**
R_{SDHI}	3.08 (0.05) **bc**	1.41 (0.05) **b**	5.08E+07 (4.43E+05) **b**
R_{DMI}	2.94 (0.05) **b**	1.54 (0.02) **ab**	5.12E+07 (4.40E+05) **b**
$R_{SDHI/DMI}$	3.13 (0.05) **c**	1.49 (0.02) **ab**	5.04E+07 (4.42E+05)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

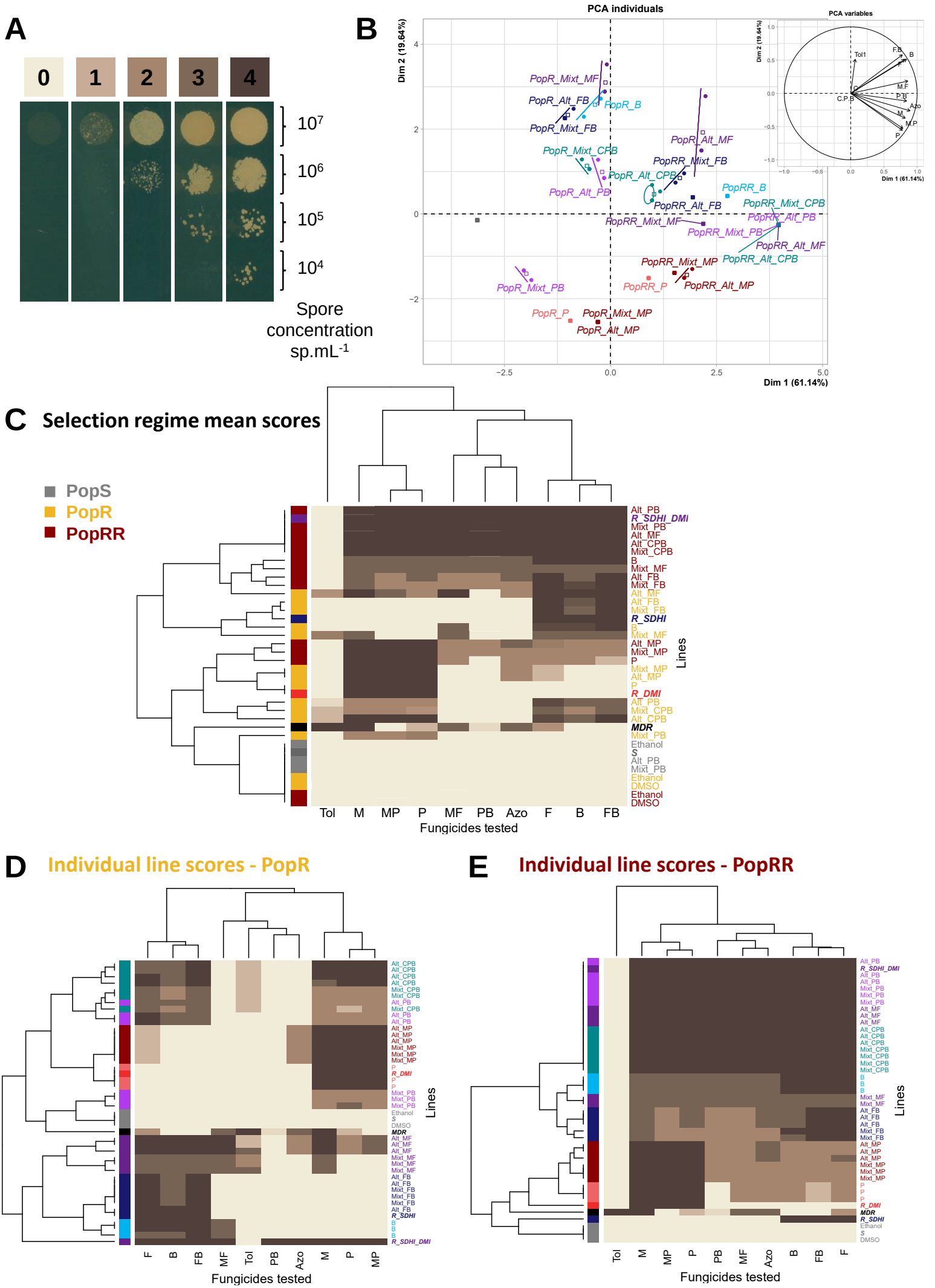
D

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%


A
S
R_{DMI}
R_{SDHI}
$R_{SDHI/DMI}$
PopS
PopR
PopRR
B
Ancestral population
(Immigration)
Population inoculation
Treated lines
Fungicide application
transfer
transfer
1
2
⋯ Cycles
C Modes of action
SDHI
(concerned by resistance)
DMI
Multisite
(naive)
B
Benzovindiflupyr
P
Prothioconazole-desthio
C
Chlorothalonil
F
Fluxapyroxad
M
Mefentrifluconazole
Selection regimes (4-8 cycles)
Unisite(s)
Single-site+multisite
Sequence
Alternation
Mixture


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


Mean estimated normalized concentration of multiresistant isolate
1.00
0.75
0.50
0.25
0.00
b
a
a
d
d
bc
e
f
f
e
c
d
P
Alt_MP
Mixt_MP
B
Alt_FB
Mixt_FB
Alt_PB
Mixt_PB
Alt_MF
Mixt_MF
Alt_CPB
Mixt_CPB


A
0
1
2
3
4
10^7
10^6
10^5
10^4
Spore concentration sp.mL-1
B
PCA individuals
Dim 2 (19.64%)
Dim 1 (61.14%)
PopR_Mixt_MF
PopR_Alt_FB
PopR_B
PopR_Mixt_FB
PopR_Mixt_CPB
PopR_Alt_CPB
PopR_Alt_PB
PopR_Alt_MF
PopRR_Mixt_FB
PopRR_B
PopRR_Alt_FB
PopRR_Mixt_CPB
PopRR_Alt_PB
PopRR_Mixt_MF
PopRR_Mixt_PB
PopRR_Alt_MF
PopRR_Alt_CPB
PopRR_Mixt_MP
PopRR_P
PopRR_Alt_MP
PopR_Mixt_PB
PopR_P
PopR_Mixt_MP
PopR_Alt_MP
PCA variables
Tol1
F.B
B
F
M.F
P.B
C.P.B
Azo
M
M.P
P
C
C
Dim 2 (19.64%)
Dim 1 (61.14%)
C Selection regime mean scores
PopS
PopR
PopRR
Alt_PB
R_SDHI_DMI
Mixt_PB
Alt_MF
Alt_CPB
Mixt_CPB
B
Mixt_MF
Alt_FB
Mixt_FB
Alt_MF
Alt_FB
Mixt_FB
R_SDHI
B
Mixt_MF
Alt_MP
Mixt_MP
P
Mixt_MP
Alt_MP
P
R_DMI
Alt_PB
Mixt_CPB
Alt_CPB
MDR
Mixt_PB
Ethanol
S
Alt_PB
Mixt_PB
Ethanol
DMSO
Ethanol
DMSO
Lines
Tol
M
MP
P
MF
PB
Azo
F
B
FB
Fungicides tested
D Individual line scores - PopR
Lines
F
B
FB
MF
Tol
PB
Azo
M
P
MP
Fungicides tested
E Individual line scores - PopRR
Lines
Tol
M
MP
P
PB
MF
Azo
B
FB
F
Fungicides tested