Why do fungicide mixtures delay the evolution of resistance?

An experimental evolutionary approach

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Abstract

Pesticide resistance poses a critical threat to agriculture, human health and biodiversity. Mixtures of fungicides are recommended and widely used in resistance management strategies. However, the components of the efficiency of such mixtures remain unclear. We performed an experimental evolution study on the fungal pathogen *Z. tritici,* to determine how mixtures managed resistance. We compared the effect of the continuous use of single active ingredients to that of mixtures, at the minimal dose providing full control of the disease, which we refer to as the "efficient" dose. We found that the performance of efficient-dose mixtures against an initially susceptible population depended strongly on the components of the mixture. Such mixtures were either as durable as the best mixture component used alone, or worse than all components used alone. Moreover, efficient-dose mixture regimes probably select for generalist resistance profiles as a result of the combination of selection pressures exerted by the various components and their lower doses. Our results indicate that mixtures should not be considered a universal strategy. Experimental evaluations of specificities for the pathogens targeted, their interactions with fungicides and the interactions between fungicides are crucial for the design of sustainable resistance management strategies.

Keywords

experimental evolution; fungicide resistance; selection drivers; generalism, ecological specialization; environmental variation; selection heterogeneity; mixture; dose variation; *Zymoseptoria tritici*

Introduction

The widespread use of pesticides and drugs has led to the rapid evolution of resistance, which reduces or even abolishes their efficacy in some situations [1]. Resistance management is therefore crucial, to prevent the overuse of pesticides, which would be deleterious to human health and biodiversity, and to maintain sufficient levels of high-quality agricultural production, in a context in which the number of new modes of action (MoA) discovered is dwindling and agricultural practices favour the emergence and spread of resistance [2]. Management strategies aim to slow resistance build-up by maximising the heterogeneity of selection pressure. This may involve dose reduction and/or combinations of different MoAs in space and time [3].

Fungicide mixtures (*i.e.* the combination of two or more fungicides within the same treatment) are the most widely used, studied and recommended strategy for controlling plant pathogens (FRAC recommendations for fungicide mixtures 2010; REX Consortium 2013). The efficacy of such strategies for delaying the development of resistance and maintaining disease control has been demonstrated in both empirical and modelling studies [4–6]. The adoption of this strategy is also driven by practical concerns, as many manufacturers offer ready-to-use commercial mixtures, and it is possible to design tank mixtures with the same active ingredients (AIs) [7]. Finally, one side benefit of mixtures is that they can be used to control multiple pathogens with a single spray (*i.e.* they broaden the activity spectrum).

Several non-exclusive processes can account for the efficacy of mixtures. First, mixtures expose pathogens simultaneously to several fungicides (*i.e.* multiple intragenerational killing (REX Consortium 2013)), and the evolution of specific resistance to each of the mixture components

(*i.e.* multiple resistance) is less likely than the evolution of resistance to a single fungicide [8]. Second, according to the established "governing principles" of resistance management, the growth rate of individuals with single resistances (*i.e.* resistant to one AI) is decreased by the use of mixtures of fungicides [4,9]. The AIs mixed can control both resistant and susceptible strains, resulting in decreases in the growth rates of both resistant and susceptible strains, and a decrease in the selection coefficient, defined as the difference between these growth rates.

Dose reduction can also be used to control resistance; this strategy acts by reducing the growth rate of resistant individuals [4]. Most of the available empirical and theoretical evidence indicates high doses increase selection once resistance has emerged, although there are counter-examples that can be explained by the convergence of the dose-response curves of resistant and susceptible strains at high doses [10]. During the emergence phase, the effect of dose is highly specific to the interaction between the fungicide and the pathogen, with high doses having either a beneficial or a deleterious influence on resistance. The use of high doses to keep the pathogen population small limits the mutation load but accelerates the selection of any mutations that do emerge [11]. Theoretical studies have shown that, for an overwhelming majority of realistic parameters of fungicide-pathogen combinations, low-dose strategies better limit the emergence of qualitative resistance [11,12].

The combination of mixtures with dose reduction in "efficient-dose mixtures" (*i.e.* mixtures of reduced doses of AI but providing a similar level of disease control to that provided by these components used alone at their full authorised rate) may decrease the rate at which resistant individuals are selected, thereby increasing fungicide durability [4]. The socio-environmental benefits of reducing the rates of fungicides in mixtures are obvious, but, in practice, commercial mixtures nevertheless include fungicide components at or close to their full rate for use on

86 their own (*e.g.* in commercial products used on wheat to control septoria leaf blotch; Table S1).
87 Efficient-dose mixtures are thus rarely used, possibly due to the difficulties of evaluating their
88 potential advantages. First, such mixtures may not display the beneficial effects of high-dose
89 strategies, long advocated as a means of reducing the occurrence of mutations and,
90 particularly, the selection of partially resistant mutants, putative mutational stepping stones to
91 high-level resistance. Second, the efficacy of efficient-dose mixtures may be equivocal because
92 it may depend on the biology of the pathogen (*e.g.* its ploidy and mode of reproduction; [3,13]),
93 fungicide performance [14], the interaction between mixture components (antagonism or
94 synergism; [13,15,16]) and resistance costs [17]. Third, most studies on mixture durability have
95 focused on the evolution of specific resistance to the fungicide considered most at risk of
96 resistance development, rather than the durability of the mixture itself. Finally, the assessment
97 of mixture strategies usually focuses on their performance during the selection phase rather
98 than the emergence phase of resistance dynamics [3,12].

99 We performed an experimental evolution study to determine how an efficient-dose mixture
100 could be used to manage resistance, with a view to improving comparisons with strategies
101 based on single AIs. In particular, we analysed how mixture components drove the quantitative
102 and qualitative performance of this strategy. We studied *Zymoseptoria tritici*, an ascomycete
103 responsible for septoria leaf blotch (STB), a major disease of winter wheat [18]. STB accounts
104 for up to 70% of fungicide use in Western Europe [19]. Various degrees of resistance to all
105 authorised single-site inhibitors (*i.e.* with a single biochemical mode of action) — inhibitors of
106 the polymerization of β-tubulin or benzimidazoles, inhibitors of cytochrome *b* of mitochondrial
107 complex III or QoIs, inhibitors of succinate dehydrogenase (a component of mitochondrial
108 complex II of respiration or SDHIs, and inhibitors of sterol 14α-demethylase or DMIs — have

been observed in *Z. triciti* in France [20]. Resistance results from mutations affecting the target sites for these four MoAs. Target overexpression has also been demonstrated for DMIs. Overexpression of the MFS1 transporter causes enhanced efflux [21], a generalist mechanism causing multidrug resistance (MDR) affecting all MoAs but with a limited impact on the susceptibility of isolates.

Using an approach previously developed for the study of resistance selection in alternation strategies [22], we observed the evolution of resistance in a haploid yeast-like easily cultured form of a fully susceptible strain of *Z. tritici*. We first compared the rates of resistance evolution under single or mixed fungicide treatments, for three AIs with different modes of action applied in amounts resulting in similar efficacy (*i.e.* EC_{90}). We then determined the cross-resistance profiles of the evolved lines, assessing whether the efficacy of fungicide mixtures was counterbalanced by an increase in the occurrence of generalist resistance profiles. Finally, we investigated how the heterogeneity of selection pressure associated with efficient-dose mixtures determined the cross-resistance profiles in evolved strains, relative to strains exposed to a single fungicide at a similarly effective or lower dose.

Materials and methods

General design

The protocol of the experimental evolution was adapted from that of a previous study [22].

The ancestral *Z. tritici* isolate used was IPO323, which is susceptible to all fungicides. Cultures on YPD plates (20 $g.L^{-1}$ dextrose, 20 $g.L^{-1}$ peptone, 10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ agar; USBiological) incubated at 18°C in the dark for seven days were used to prepare a founding culture in 25 mL liquid YPD (composition as above, but without agar) in a 50 mL Erlenmeyer flask plugged with cotton wool. This primary culture was incubated in similar conditions for seven days, with shaking at 50 rpm, and was used to establish all the other lines.

The various lines were cultured as described above, in in 25 mL liquid YPD medium in 50 mL Erlenmeyer flasks. Each fungicide treatment was repeated on four independent populations (*i.e.* lines). Each Erlenmeyer flask was inoculated with 10^7 spores (500 µL of the primary culture). Control lines were not treated with fungicides and contained the same amount of solvent as was introduced for the treated lines. Experimental evolution was allowed to occur over seven-day cycles (*i.e.* roughly six to seven generations per cycle). This cycle duration made it possible to keep cultures in the exponential growth phase (without reaching stationary phase). At the each end of cycle, 2% of the evolved culture was transferred to a new Erlenmeyer flask containing fresh medium. We ensured that population sizes were equivalent at the start of each cycle by mimicking immigration from external populations through the addition of spores from the untreated line to reach a total of 10^7 spores for each line. OD_{405} was measured at the end of each cycle and used to calculate population size (see [22] for details). Malthusian growth was calculated for each line as previously described [23]:

$$m = \ln\left(\frac{\text{cell density at the end of the cycle, day 7}}{\text{cell density at the beginning of the cycle, day 0}}\right)$$

Spore concentration and Malthusian growth were normalized against the concentration and Malthusian growth, respectively, of the control line.

Selection regimes and selection doses

We designed selection regimes for studies of the influence of three different factors on resistance evolution. First, selection regimes differed in the number of AIs used (from 1 = direct use to 2-3 = mixtures). Second, the AIs chosen corresponded to different modes of action: prothioconazole-desthio (P; a DMI), benzovindiflupyr (B; a SDHI) and carbendazim (C; a benzimidazole). Finally, each AI was applied at several concentrations: an efficient dose and reduced doses (indicated by line names including a subscript r). All single fungicides were applied at the full efficient dose and at reduced doses, continuously, over the course of the experiment. All combinations of AIs were applied at the full efficient dose. We observed 16x4 = 64 independent lines (Table 1). The experiment was conducted over 10 cycles for all but six of the lines (BP, BCP, Br2, Cr1, Cr2, Pr2) for which it was conducted over only nine cycles (calibration problem for the first cycle).

Table 1: Doses of fungicides B, C and P and of their mixtures used to select resistance in the various experimental evolution regimes. The proportion of the reference dose applied refers to the efficient dose of the mixture. For example, the selection dose of the CP mixture was EC_{90}(CP)=0.082 $mg.L^{-1}$ of C + 0.00205 $mg.L^{-1}$ of P, *i.e.* 0.41×(EC_{90} (C) + EC_{90} (P)). The interaction between AIs was calculated with the Wadley formula (*Wadley, 1945*). Each selection regime is associated to a specific colour, as used in the results figures, in the first column.

Selection regime name	Proportion of the reference dose applied per cycle	Interaction between AIs	Observed efficacy in the 1st cycle	Benzovindiflupyr mg.L^{-1}	Carbendazim mg.L^{-1}	Prothioconazole-desthio mg.L^{-1}
B	1.00		0.92	0.5		
Br1	0.53		0.62	0.263		
Br2	0.50		0.73	0.25		
C	1.00		0.90		0.2	
Cr1	0.45		0.92		0.09	
Cr2	0.40		0.95		0.08	
P	1.00		0.89			0.005
Pr1	0.80		0.86			0.004
Pr2	0.60		0.54			0.003
BC	0.68	0.74	0.90	0.34	0.136	
CP	0.41	1.22	0.90		0.082	0.00205
BP	0.60	0.83	0.92	0.3		0.003
BCP	0.42	0.79	0.89	0.21	0.084	0.0021

171

172 Efficient doses were chosen so that each treatment, whether a mixture or a fungicide alone,
173 exerted a selection pressure of similar intensity on a naive population. Dose-response curves
174 were established for the three AIs: B, C and P. EC_{90} values were established as the fungicide
175 concentration inhibiting 90% of growth relative to untreated lines after seven days. For each
176 selection regime, we used the EC_{90} as the reference dose because it was not possible to
177 determine the MIC (*i.e.* the minimal inhibitory concentration) experimentally. Fungicide

178 mixtures were prepared with the same proportion of the EC_{90} for each AI, to ensure a similar
179 contribution of each fungicide to overall efficacy. Dose-response curves were also established
180 for each of the three possible pairs of AIs with a range of proportions of the EC_{90} (*i.e.* from
181 roughly 0.41 to 0.68 times the EC_{90} of each AI; Table 1). Table 1 details the final doses used in
182 the different selection regimes. We calculated their interaction R, as R = $EC_{90}^{theo}/EC_{90}^{obs}$, with
183 the Wadley formula,

184 $$i.e.\ EC_{90}^{theo} = \frac{1}{\sum_{i \in M} f_i \ EC_{90}^{i}}$$

185 where *M* is the mixture of AIs, f_i is the proportion of AI *i* in the mixture (calculated from AI
186 concentrations) and EC_{90}^{i} is the EC_{90} of AI *i*. EC_{90}^{obs} is the sum of AI concentrations in the mixture
187 [24]. By definition, additive interactions were positive. Synergism was considered to occur if R
188 exceeded 1 and negative interactions were considered to result in antagonism if R was lower
189 than 1.

190

191 *Establishment of resistance phenotype profiles at the end of the experiment*

192 At the end of the evolution experiment, we performed droplet tests on each of the lines
193 that had gone through nine cycles (*i.e.* the last cycle common to all lines) of selection, to
194 characterize their resistance profiles.

195 For each line, four droplets with spore densities adjusted to 10^7, 10^6, 10^5 and 10^4 spores.mL^{-1}
196 were deposited on solid YPD medium to which a discriminatory dose of fungicide had been
197 added in a square Petri dish. The discriminatory doses were validated in preliminary
198 experiments and were designed to prevent the growth of the susceptible ancestral IPO323

199 isolate but to allow the growth of reference resistant isolates from our collections. The ancestral
200 isolate IPO323 and a negative control were included in each test. Lines evolved under efficient
201 doses were subjected to eight different conditions: the efficient doses of each of the single AIs,
202 the efficient doses of each of the four AI combinations and tolnaftate at 2 $mg.L^{-1}$. We used
203 tolnaftate as a marker of generalist resistance. Lines exposed to reduced doses were subjected
204 to the same set of discriminatory doses and to nine additional discriminatory doses,
205 corresponding to the selection dose of each AI in mixtures (Table 1).

206 Each test was scored according to the rank of the droplet with the lowest concentration of
207 spores allowing growth (*e.g.* a score of 2 was attributed if growth was observed for both the
208 first and second dilution, but not for the third or fourth spore dilution).

209

210 ## *Statistical analysis*

211 We compared the mean growth of lines over the course of the experiment by one-way
212 ANOVA with line as a factor. Four ANOVAs were performed, one per mixture. Pairwise
213 comparisons between lines were performed with Tukey *post-hoc* correction. Resistance
214 dynamics analyses were performed with a non-parametric permutation test (10^4 permutations)
215 for repeated measures, with spore concentration as the dependent variable, selection regime
216 and cycle as explanatory variables and line as a repeated unit of observation. Multiple pairwise
217 *P* values were obtained after Bonferroni correction. The number of selection regimes against
218 which a line was resistant, and its mean resistance score, were calculated as the number and
219 mean of scores strictly greater than zero in its resistance profile, respectively. Linear models
220 were used for the analysis, with the number of resistances modelled with a quasi-Poisson

distribution and the mean resistance score modelled with a logGaussian distribution, with the type of selection regime (a single AI or two-or-three-AI mixture) and the selection regime nested within selection regime type as the explanatory variables.

The structuration of the resistance profiles of lines exposed to single AIs or efficient-dose mixtures was represented by a heatmap of the resistance phenotype profiles detected at the end of the experiment, after nine cycles. The Euclidean pairwise distance was used for the hierarchical clustering of these profiles, with dendrograms for the rows and columns. We also performed a principal component analysis (PCA). The effect of dose is represented by three heatmaps of the resistance phenotype profiles of lines exposed to a single fungicide at efficient or reduced doses.

The effects of AI number, alternation partner (C or P) and their interaction with reduced dose exposure (single fungicide or mixture) on tolnaftate resistance score were investigated with a linear model (quasi-Poisson GLM model determined by stepwise variable selection from a Poisson GLM), with exclusion of the lines in which no resistance emerged (*i.e.* the control lines and B and BP lines).

All analyses and figures were produced with R 4.0.4 and the packages CAR, EMMEANS, FACTOEXTRA, EZ, GGPLOT2, GGPUBR, COWPLOT, GRIDEXTRA, MULTCOMP and FACTOMINER.

Results

Mixture durability strongly depends on mixture components

241 In this experiment, all selection regimes, whether a mixture or a single AI, were designed
242 to have the same efficacy (90% efficacy) relative to the untreated control. The selection doses
243 were therefore fixed at the EC_{90} (hereafter referred to as the "efficient dose") after the
244 establishment of dose-response curves for each AI and their four possible mixtures. For the CP
245 mixture, the level of interaction was R=1.22 with the IPO-323 isolate, which is greater than one
246 and, therefore, suggestive of some synergism. R values were below 1 for the other mixtures
247 applied on the same isolate, suggesting antagonism (BC: 0.74, BP: 0.83 and BCP: 0.79) (Table
248 1). These interactions (synergy or antagonism) were considered non-significant as R<1.5 for
249 synergy and R>0.5 for antagonism, according to the criteria proposed in a previous study [24].

250 We observed the dynamics of *Z. tritici* after experimental evolution in independent lines
251 subjected to treatment with single fungicides or mixtures of fungicides designed to be 90%
252 effective, for three fungicides with different modes of action: benzovindiflupyr (B), carbendazim
253 (C) and prothioconazole-desthio (P) (Figure 1A). Variability was generally low between the four
254 lines exposed to the same treatment. For lines under continuous exposure to a single AI at its
255 efficient dose, resistance emerged first in lines exposed to C and P: the normalised spore
256 concentration (hereafter referred to simply as the spore concentration) of the C and P lines
257 exceeded 20% (double the initial concentration) after five cycles, and resistance was
258 generalised (spore concentration above 90%) after eight and nine cycles for C and P,
259 respectively. For lines exposed to B, no clear emergence of resistance was emerged, with spore
260 concentration remaining below 20% after 10 cycles.

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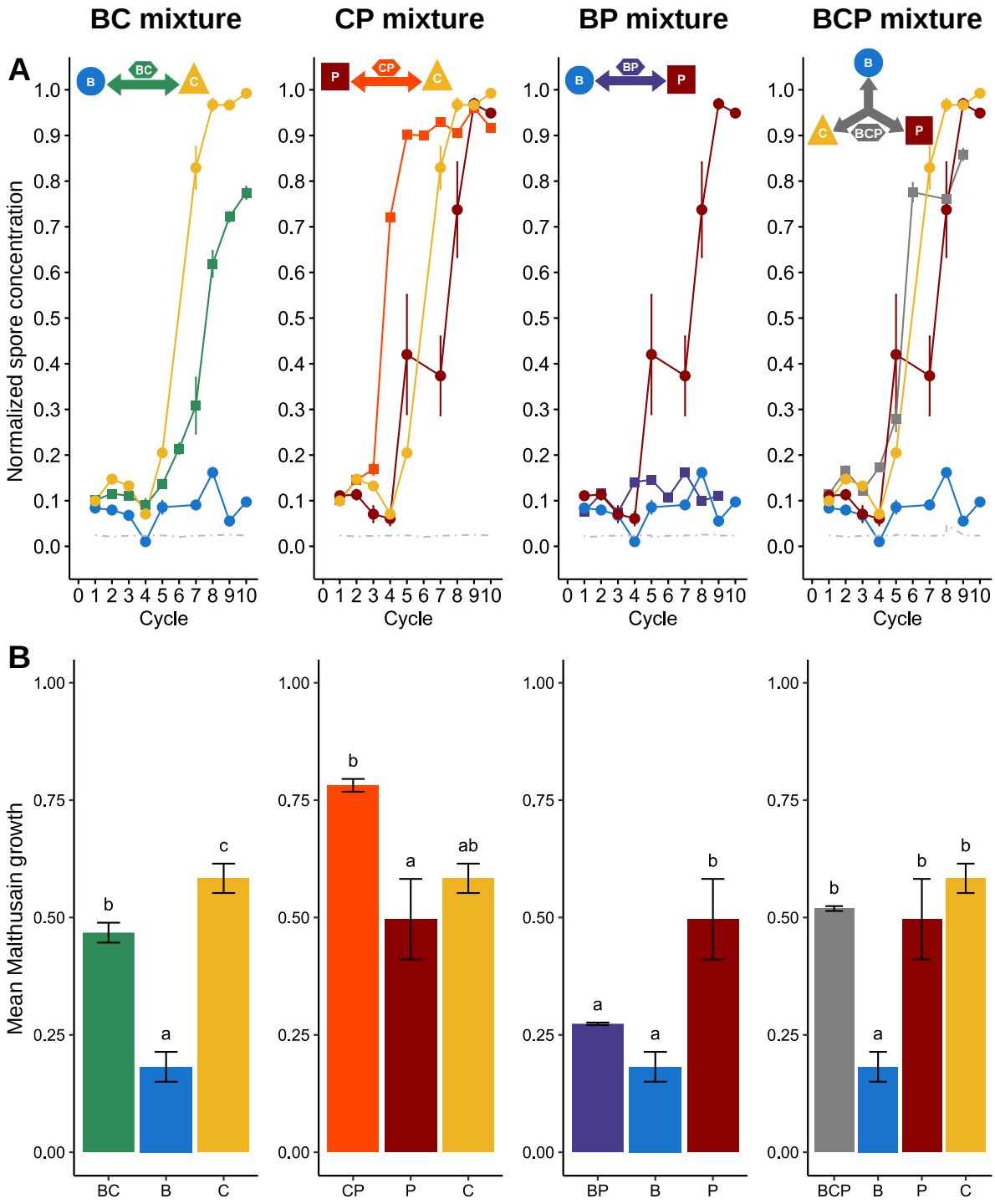

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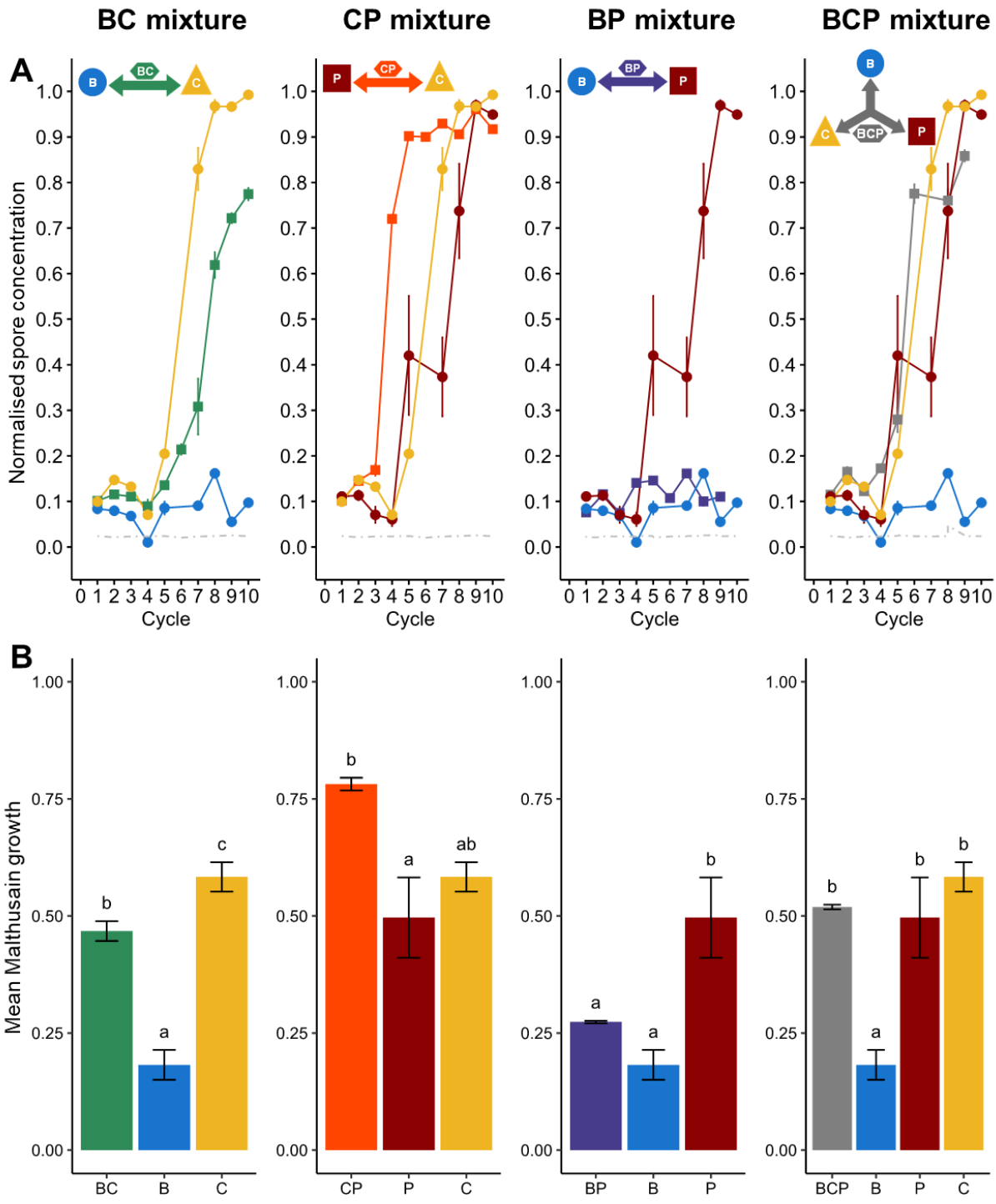


Figure 1: Dynamics of resistance evolution in the lines selected at 90% treatment efficacy. Each column represents the results for a pair of fungicides used alone or as a mixture, at their efficient dose, as explained in the pictograms at the top. B: benzovindiflupyr (SDHI), C: carbendazim (benzimidazole) and P: prothioconazole-desthio (DMI). **(A)** The normalised spore concentration is the spore concentration observed at the end of a cycle relative to that in the control line (*i.e.* a susceptible

population not exposed to fungicides). **(B)** Mean Malthusian growth. Results are normalised against the Malthusian growth of the control (histogram bars) and are presented with their standard deviations (upper and lower lines). Different letters indicate significant differences between groups ($P<0.05$).

The evolution of lines exposed to efficient-dose mixtures was highly heterogeneous. The BP mixture fully delayed resistance, as no resistance emerged in these lines after 10 cycles, as for the B lines. Dynamics differed highly significantly between BP and P ($P<1e^{-3}$) but dynamics between BP and B were similar ($P=0.56$). The BC mixture had an intermediate performance, significantly different from those of B and C ($P<1e^{-3}$ for both), with resistance emerging after six cycles (*i.e.* one cycle later than for direct exposure to C but before that for direct exposure to B) and a normalised spore concentration that reached 80% by cycle 10, when resistance was generalised in C lines. The CP mixture was not sustainable, as the emergence and generalisation of resistance at cycles 3 and 5, respectively, occurred more rapidly than in lines exposed to C or P alone (emergence of resistance at cycle 5 and generalisation at cycles 8 and 9, respectively) and resistance dynamics differed significantly from those for P and C alone ($P<1e^{-3}$ for both). The three-way mixture (BCP) yielded intermediate results, with resistance emerging and generalising more slowly than in lines exposed to the least durable mixture, CP (but this difference was not significant, $P=0.20$) although resistance did emerge eventually, by contrast to the BP mixture ($P<1e^{-3}$).

We compared the global increase in resistance, based on cycle-averaged Malthusian growth rates, which produced a similar ranking of these strategies (Figure 1B). The increase in resistance in BC lines was intermediate, significantly higher than that in B lines but lower than that in C lines ($P<0.05$). The increase in resistance in CP lines was similar to or significantly

292 greater than that in the corresponding single-fungicide lines. The performance of BP lines was
293 not significantly different from that of B lines, which displayed the highest level of resistance
294 durability. BCP lines were intermediate, with a performance not significantly different from that
295 of the two least durable AI treatments.

296 CP, the least "durable" mixture, was the only mixture to display any evidence of synergism
297 (non-significant) and was applied with an efficient dose lower than the sum of half the efficient
298 doses of each component.

299

300 *Efficient-dose fungicide mixtures select for generalist and/or multiple resistance*

301 We determined the phenotypic resistance profile of each population in droplet tests
302 performed at cycle 9 (Figure 2). As expected, the control lines displayed no resistance to any
303 of the fungicide treatments tested in the droplet test. The lines exposed to single fungicides
304 presented contrasting patterns of resistance. Those exposed to C had a unique, narrow
305 resistance profile characterised by strong resistance to C (mean resistance score of 4, *i.e.* the
306 maximal score) and moderate resistance to the BCP mixture (mean resistance score of 2). By
307 contrast, lines exposed to P had specific profiles in each of the four repeats, all broader than
308 that for lines exposed to C (on average, P lines were resistant to 3.25 of 8 discriminatory doses,
309 whereas C lines were resistant to 2) and including various degrees of resistance to P and to CP,
310 but also to tolnaftate (for 3 of 4 lines). Tolnaftate resistance is considered an indicator of
311 multidrug resistance due to enhanced efflux in *Z. tritici* [21,25]. Such patterns are consistent
312 with the evolution of multiple and/or generalist resistance mechanisms. Lines exposed to B, in

which no resistance had emerged, displayed no resistance in any of the modalities of the droplet test.

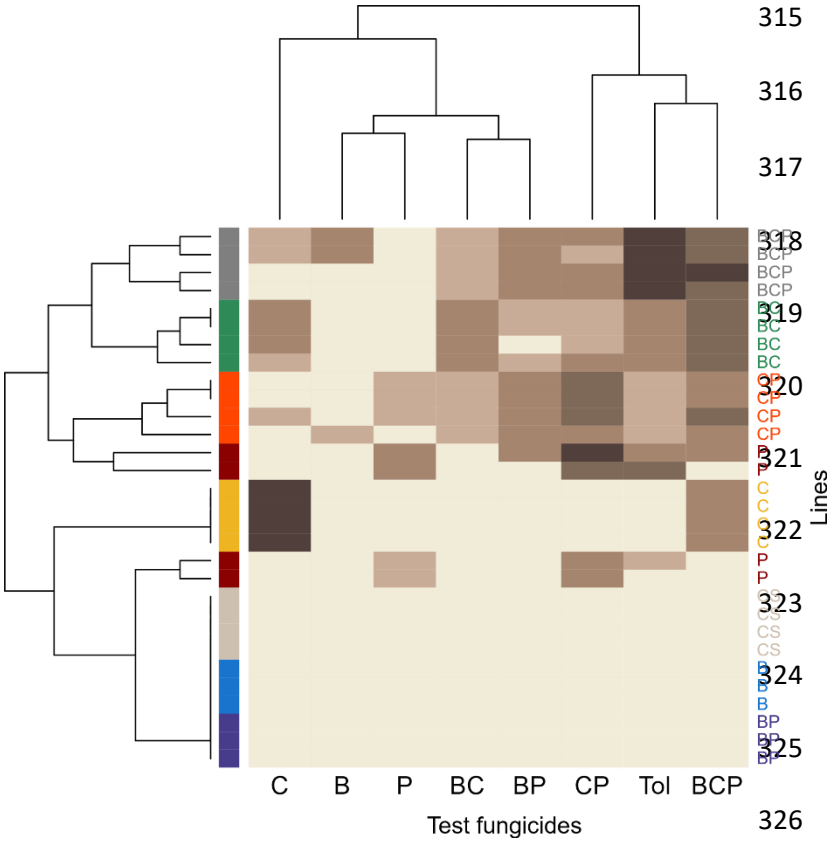


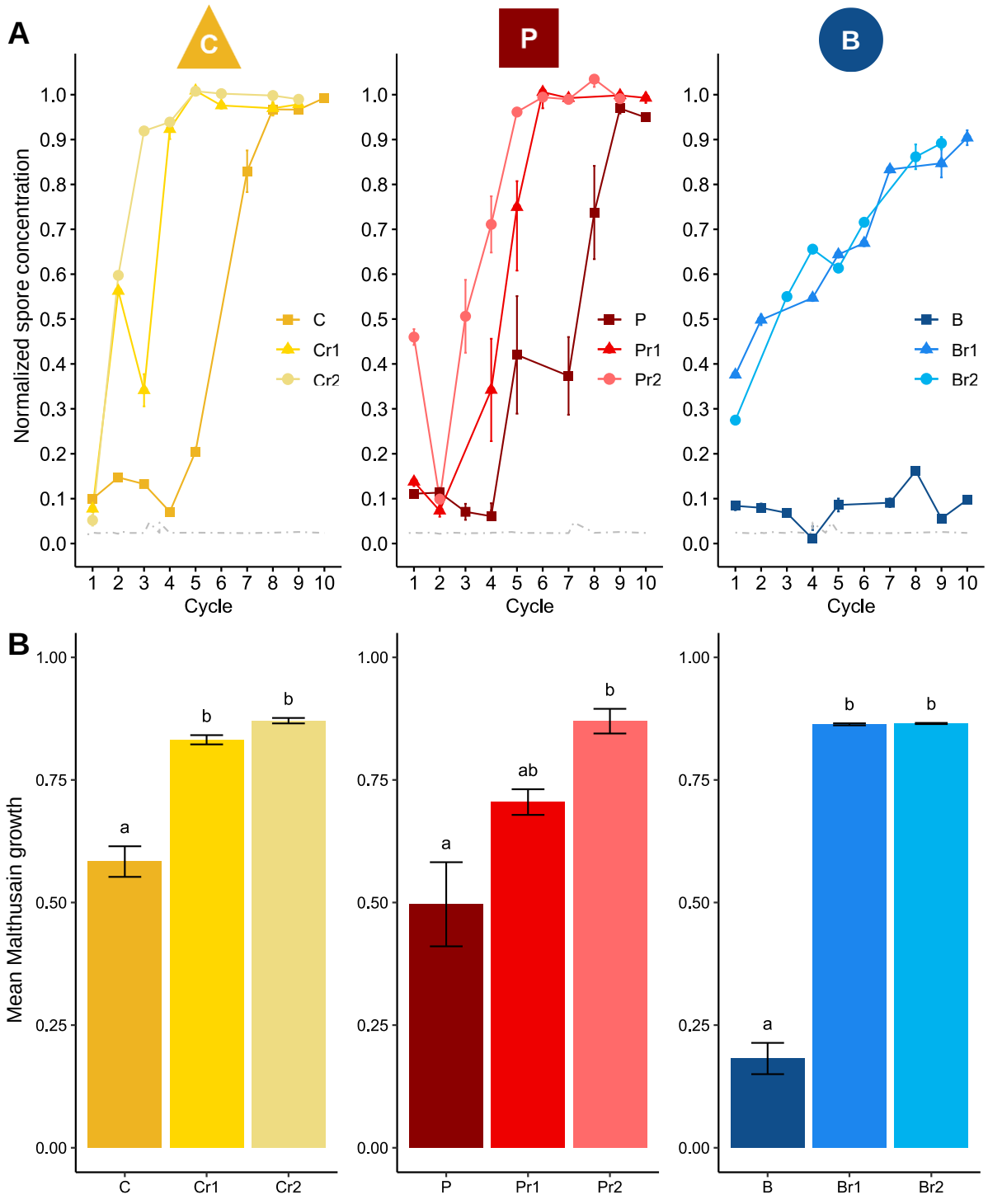
Figure 2: Heatmap of phenotypic resistance profiles at cycle 9. Resistance scores (0-4; on the brown scale) are shown for each of the 12 populations evolved under one of the eight selection regimes (four or three replicate lines per regime; regimes represented by the rainbow scale, as described in Figure 1) and for each fungicide or mixture tested. Heatmaps were established on the basis of pairwise Euclidean distance.

The lines exposed to efficient-dose fungicide mixtures in which resistance had emerged (BC, CP and BCP) had broader resistance profiles than those exposed to a single AI, even P. Indeed, they were, on average, resistant to 2.3 times more testing modalities than those exposed to a single AI ($P<1e^{-4}$), but to a lesser extent, with scores 0.8 times lower for selection regimes against which they were resistant. These lines were resistant to their selection mixture, to various degrees, but also to the other three mixtures and to tolnaftate, especially for BCP lines, which had the highest possible score for resistance to tolnaftate. This, again, suggests that multiple and/or generalist resistance was evolving in these lines. However, these lines were not

337 necessarily resistant to the efficient dose of the components of the selection mixture used
338 alone: BC lines were resistant to C but not B; CP lines were mostly resistant to P but remained
339 susceptible to C; and half the BCP population displayed resistance to B and C whereas the other
340 half presented no resistance to any single AI. The lines exposed to BP, in which no resistance
341 had emerged, also displayed no resistance in the droplet tests.
342

343 ## *Reduced doses of single AIs still select for resistance*

344 As expected, over the course of the experiment, the control of *Z. tritici* was weaker in
345 the lines exposed to reduced doses than in those exposed to the efficient dose of the same
346 fungicide (Figure 3). In particular, resistance to B emerged in populations subjected to
347 treatment with reduced doses of this fungicide, whereas the emergence of such resistance was
348 prevented by use of the efficient dose. For each AI, mean Malthusian growth was significantly
349 greater in reduced-dose lines than in efficient-dose lines (P=0.04 and P=0.003, for P_r1 and P_r2,
350 respectively, versus P, and $P<1e^{-4}$, for all pairwise comparisons between efficient and reduced
351 doses of B and C). Surprisingly, C_r lines exposed to reduced doses of C (*i.e.* 0.4 and 0.45 of the
352 efficient dose in the preliminary data), initially displayed a similar level of control to lines
353 exposed to the full efficient dose (Table 1). Nevertheless, control of the fungus was weaker in
354 these lines, as expected, from the second cycle (Figure 3). The greater continuous increase in
355 spore concentration over time cycles indicates that reduced-dose regimes select for resistance,
356 in addition to providing poorer control over fungal populations. However, it was not possible
357 to test the effect of dose reduction on resistance selection, because lines exposed to full or
358 reduced doses were not subject to the same treatment intensity, making it impossible to
359 dissociate resistance selection from growth control.



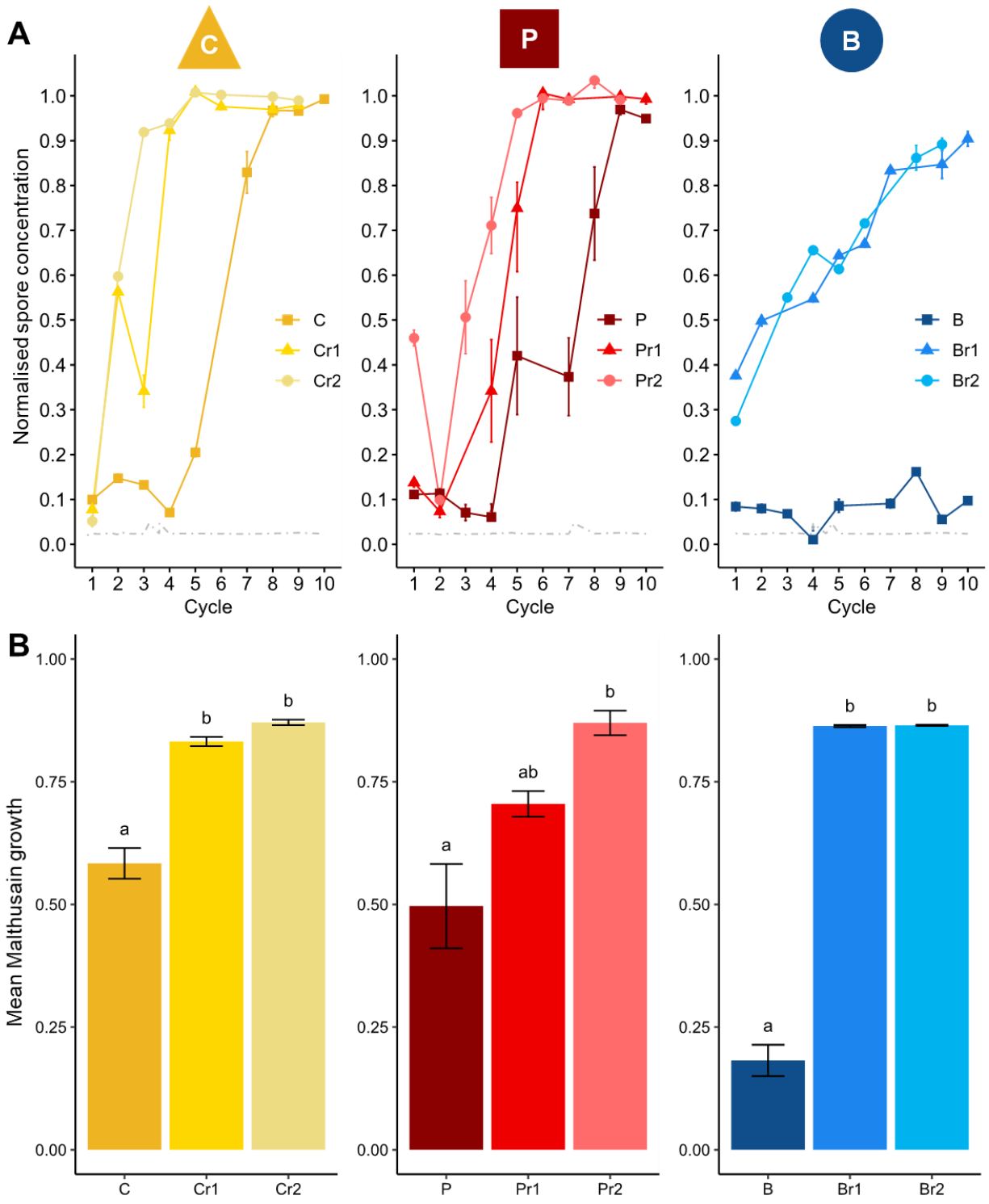


Figure 3: Dynamics of resistance evolution in the lines exposed to a single fungicide at the full efficient dose or a reduced dose. Each column represents results for an AI used at its EC_{90} selection dose or at two reduced doses, corresponding to a fraction of this EC_{90} (Table 1). B: benzovindiflupyr (SDHI), C: carbendazim (benzimidazole) and P: prothioconazole-desthio (DMI). **(A)** The normalised spore concentration is the spore concentration observed at the end of a cycle divided by the

spore concentration in the control line (*i.e.* a susceptible population not exposed to fungicides). **(B)** Mean Malthusian growth. Results are normalised against the Malthusian growth of the control (histogram bars) and are presented with their standard deviations (upper and lower lines). Different letters indicate significant differences between groups ($P<0.05$).

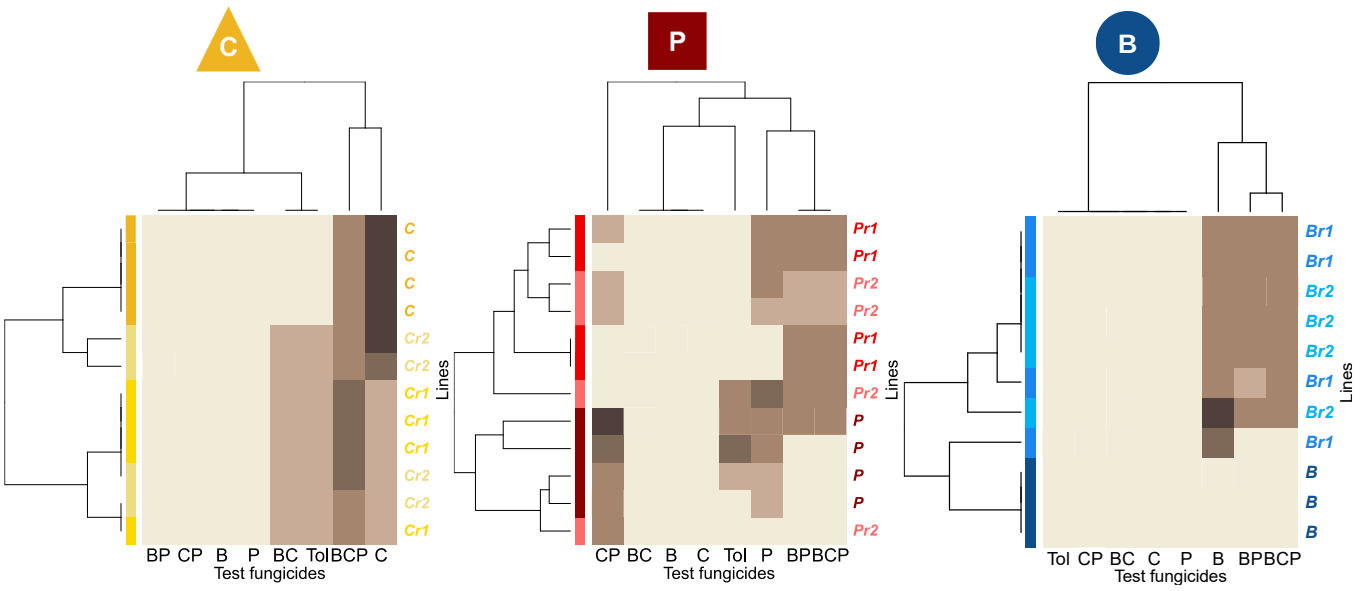
Reduced doses of fungicides also select for generalist phenotypes

Heatmaps of the phenotypic resistance profiles confirmed that reduced doses of B, C or P selected for resistance (Figure 4). Lines subjected to selection with reduced doses of B or C and more than half of those exposed to reduced doses of P (five of eight) were resistant to the fungicide used for selection at its efficient dose. The resistance profiles selected at reduced doses were broader than or different from those selected at the efficient dose of the same fungicide. For C, the efficient-dose regime selected a unique resistance profile with high resistance to C and moderate resistance to BCP, whereas the reduced-dose regime selected for generally weaker resistance, but with additional resistance to tolnaftate. For P, the efficient-dose regime selected for resistance to P and CP, and also to tolnaftate, in three of four lines. The reduced-dose P regime selected for BP and BCP resistance (except for one line), but only half the lines were resistant to CP or P and all lines were susceptible to tolnaftate. For fungicide B, the reduced-dose regime mostly selected for resistances to B, BP and BCP that we were unable to compare with efficient-dose regime-induced resistance, because no resistance emerged under efficient-dose treatment.

Resistance profiles are determined by the balance between selection heterogeneity and reduction of the dose of single AIs in efficient-dose mixtures

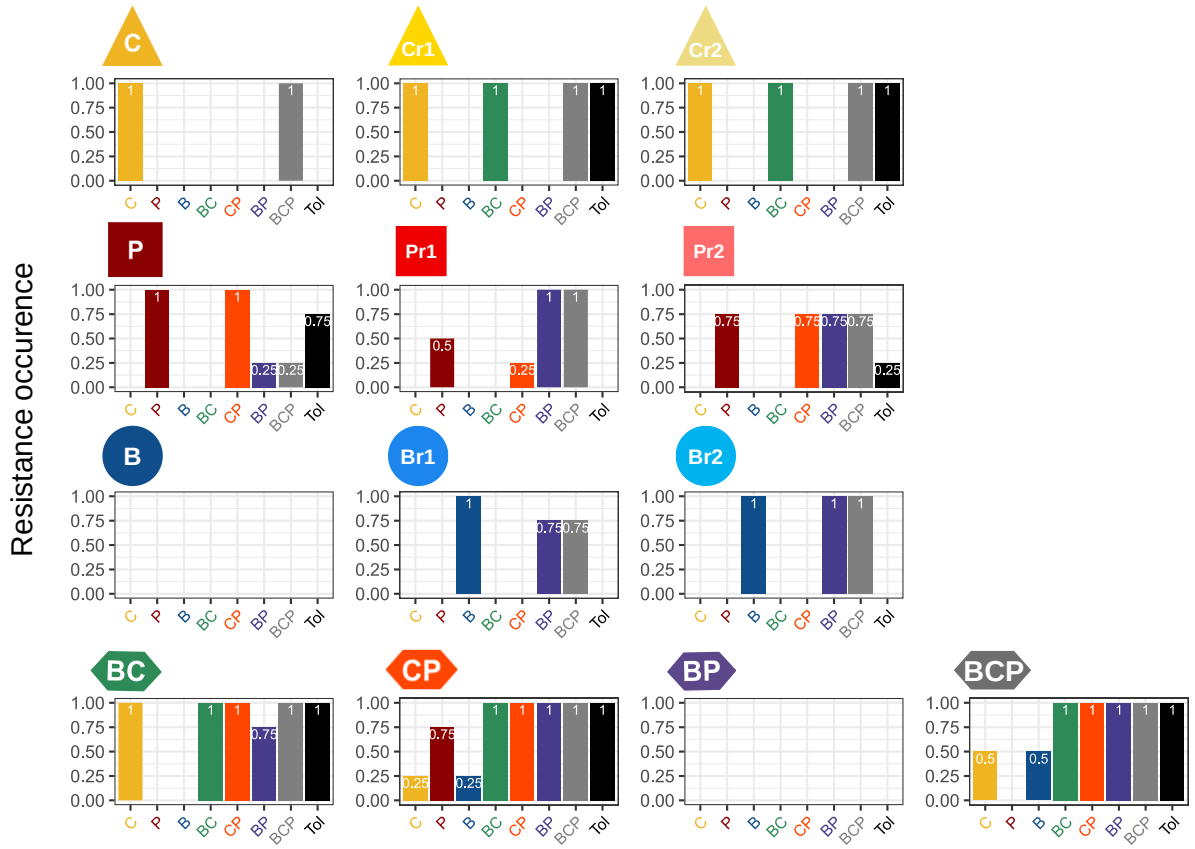
Resistance spectra differed in terms of the number of fungicides for which resistance was detected and the occurrence of these resistances in the replicates of the different selection regimes (Figure 5). The resistance spectrum of BC lines, including six resistances, corresponded almost exactly to the union of the resistance spectra of B_r and C_r (with an extra resistance to CP and an absent resistance to B). By contrast, the cumulative resistance spectra of B and C included only two resistances. The CP lines had a similar profile, because the CP resistance spectrum included a common resistance to BC and BP observed only for reduced-dose regimens of C and P but not for efficient-dose regimes. The resistance spectrum of BCP lines was also better explained by the spectra of the reduced-dose B, C and P regimes, which contained more resistances to BC, BP and B than the efficient-dose regime spectra.

401



402

403 **Figure 4: Heatmaps of phenotypic resistance profiles at cycle 9.** The resistance rating scores
404 (0-4; represented by the brown scale) are shown for each of the 12 lines evolved under 3 possible
405 selection doses of single-AI treatments (4 replicate lines per dose) and for each fungicide or mixture
406 tested. From left to right, the single AI used is B (benzovindiflupyr; SDHI), C (carbendazim;
407 benzimidazoles) and P (prothioconazole-desthio; P). Heatmaps were established with the pairwise
408 Euclidean distance.



409

410 **Figure 6: Occurrence of resistance during evolution under each selection regime**. The
411 histograms show the occurrence of resistance within a line for each modality in the droplet test. For
412 example, a score of 0.25 means that one of the four replicated lines of this selection regime had a
413 resistance score above zero.

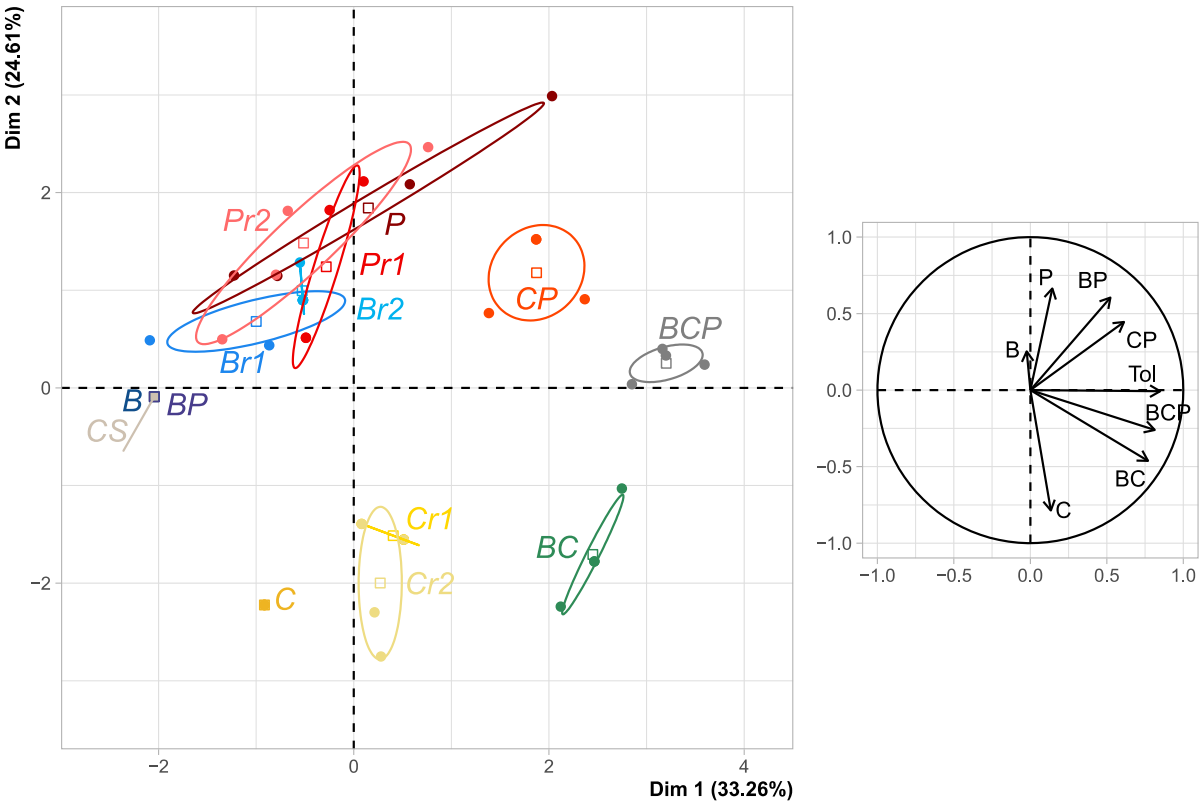
414

415 In PCA of the resistance profiles established for each line, the first axis corresponded principally
416 to resistance to tolnaftate and BCP, and secondarily to resistance to the two-compound
417 mixtures (Figure 6). This first axis showed that efficient-dose mixtures often selected higher
418 intensity generalist resistance. Indeed, to the left of this axis were lines with narrower resistance
419 spectra (*i.e.* selected with efficient-dose single-AI regimes). Towards the centre of the PCA were
420 lines with low resistance to tolnaftate and BCP (*e.g.* C_r1, C_r2), and, to the right, were lines with
421 higher rates of resistance to tolnaftate and BCP (all treated with effective-dose mixtures). An

422 analysis of the occurrence of tolnaftate resistance revealed a significant effect of mixture on
423 the selection of resistance to this fungicide, with significantly higher scores for two- and three-
424 way mixtures than for the corresponding AIs used alone (P=0.19 and P=0.002, respectively).
425 This analysis also revealed a positive significant effect on the selection of generalist resistance
426 for lines exposed to reduced doses of C (P=0.0059). No negative or highly positive cross-
427 resistance was observed between the different MoAs (*i.e.* the correlations between scores for
428 different fungicide testing modalities ranged between 0.14 and 0.66; SI Figure 1).

429 The generalist resistance profiles selected in efficient-dose mixtures thus result from both the
430 multiplicity of selection pressures exerted by the mixtures and the reduction of the dose of
431 each of their components.

432



433

Figure 6: Phenotypic resistance profiles for all lines at the end of the experiment. The PCA was structured by generalist resistance, detected on the basis of resistance to tolnaftate and the BCP mixture.

DISCUSSION

We investigated the effect of efficient-dose mixtures on the emergence and selection of fungicide resistance, by subjecting multiple lines of a susceptible isolate of *Z. tritici* to fungicides representative of three modes of action, applied either singly at the efficient dose or at a fraction of this dose (EC_{50}), or as two- or three-component mixtures. Efficient-dose applications of single AIs or mixtures resulted in the same treatment efficacy (EC_{90}). The effect of efficient-dose mixtures on resistance dynamics differed considerably between mixtures, according to their components: such mixtures were either as durable as the best mixture component used alone, or worse than all AIs used alone. Moreover, efficient-dose mixtures favoured generalist resistance phenotype profiles, with all lines subjected to such regimes displaying resistance to all mixtures, but also to tolnaftate, an indicator of multidrug resistance (MDR), a generalist resistance mechanism already described in field strains of *Z. tritici*. The resistance profiles characterised in lines treated with efficient-dose mixtures resulted from the combined selection pressures exerted by each of the components of the mixture at their reduced doses. Indeed, these profiles were similar to the union of profiles obtained after exposure to reduced-doses of the corresponding single AIs, but with higher scores recorded for modalities associated with generalist resistance (*i.e.* resistance to tolnaftate and mixtures).

The design of this experiment was similar to that used in a previous study [22] using the same AIs but addressing the issue of the sustainability of alternation strategies. Here, the ranking of

457 times to resistance emergence did not reflect the assumed hierarchy of the intrinsic risks of
458 resistance associated with benzimidazoles (high; C), SDHIs (moderate to high; B) and DMIs
459 (moderate; P) [26]. Indeed, resistance emerged first in C lines and later in P lines, but was never
460 selected in B lines. This discrepancy may reflect differences in temperature and humidity
461 between the two evolution experiments, or most probably differences in treatment efficacy
462 (particularly in the use of EC_{90} rather than EC_{95}, leading to a substantial difference in the
463 selection doses for B and C). We therefore considered that the lines in this experiment, which
464 evolved in the same environment, were comparable, but we focused our conclusions on the
465 effects of the C and P AIs and did not interpret our results in terms of intrinsic risks.

466

467 *Mixtures were no more durable than single fungicides applied at the efficient*
468 *dose.*

469 We observed highly contrasting resistance dynamics, despite similar initial disease
470 control, depending on the strategy (single or two- or three-way mixtures) and the components
471 of mixtures. Our findings demonstrate that mixture-based strategies do not systematically
472 provide better resistance control than single-fungicide treatments. This result is contrary to the
473 prevailing view and recommendations concerning mixtures [3,4,27]. Indeed, previous studies
474 have reported an ability of mixture-based strategies to delay the emergence [11] and selection
475 [6] of resistance to a high-resistance risk fungicide, increasing the effective life of this fungicide.
476 However, significant differences between this and previous studies may account for the
477 divergent conclusions.

First, we studied efficient-dose mixtures, as suggested in a previous study [28], based on the argument that mixtures could be used at lower doses, and at the minimal dose still giving effective control in particular, to decrease the selection of resistance. Little attention has, as yet, focused on half-dose mixtures [13], and almost all the studies to date on mixtures have considered full-dose mixtures (but see [14] for an exception). We studied the reported "redundant-killing" effect of mixtures and disentangled it from any additive or synergistic effects of combinations of AIs, by exposing all lines to treatments of similar efficacy. We then modified the fraction of the efficient dose of each component. The CP selection regime included the two fungicides, each at 0.4 times their EC_{90}, whereas the doses of the other mixtures included components at more than half the EC_{90} of their component (or one third of the dose for BCP). Considering half-doses might have modified the ranking of mixture strategies. For example, the CP selection regime, which was the least sustainable for the efficient-dose mixture (0.4 x EC_{90}- dose mixture) would have included higher doses, possibly resulting in greater durability, whereas the other mixtures would have included lower doses, possibly resulting in lower durability.

Second, we used a naive ancestral population, susceptible to all fungicides, whereas most studies have focused on the selection phase of resistance dynamics, *i.e.* after resistance to at least one of the components has already emerged.

Third, most studies have focused on the evolution of resistance to only one of the components of the mixture, generally the fungicide considered to be at the highest risk of resistance development. Resistance to the other components of the mixture is often assumed to be insignificant, despite its probable contribution to the gradual growth of the population, and generalist mechanisms are neglected. A previous review [3] identified only four papers

501 considering resistance to both components of two-compound mixtures. Our findings can, thus,
502 be interpreted in terms of the overall durability of the mixture, rather than just the effect of the
503 mixture in delaying a specific resistance phenotype. Finally, we performed an experiment in
504 which it was possible to study resistance dynamics without making *a priori* assumptions about
505 resistance phenotypes or the mechanisms likely to be selected [29–31], whereas previous
506 theoretical studies were limited to the consideration of one or a few resistance phenotypes.
507 Our results support the conclusions of the empirical study by Mavroeidi and Shaw (2006)
508 suggesting a strong dependence of the benefit of mixtures on the specific combinations of
509 their components, which required experimental demonstration.

510

511 ## *Mixtures favour generalist resistance in a phytopathogenic fungus*

512 We found that mixtures favoured the selection of broad resistance phenotype profiles,
513 consistent with multiple resistance and/or generalist mechanisms. Indeed, lines evolved under
514 mixture regimes often displayed broad resistance spectra than those exposed to a single AI,
515 with lower resistance intensity, and growth on tolnaftate. As tolnaftate resistance is considered
516 to be an indicator of MDR [25], we assume that generalist resistance was more likely to occur
517 than multiple specific resistances, although we cannot rule out the possibility of such specialist
518 resistance. Indeed, both types of resistance may coexist within an individual or within a
519 population, as previously described [33] in the "bet-hedging" hypothesis, according to which,
520 in an isogenic population, differently specialized phenotypes with fitnesses varying between
521 conditions, may co-exist in a dynamic equilibrium in a heterogeneous environment. Genetic
522 analysis (*e.g.* of the promoter of the *mfs1* gene, variants of which are associated with MDR in

523 field isolates of *Z. tritici;* [34]) could be performed to determine the resistance structure of
524 evolved populations, although non-target-site resistance could also be acquired by epigenetic
525 mechanisms [35].

526 Our findings, indicating that the use of mixtures favours generalist resistance, is consistent with
527 the findings of at least two other studies, [36] and [15], for herbicide mixtures and another
528 study, [37], on combinations of antibiotics. MDR is an increasing problem worldwide [38].
529 Greater attention should, therefore, be paid to this trade-off in the design of resistance
530 management strategies, by including considerations relating to the management of non-target
531 site resistance, for example, as suggested in two previous studies on SDHI fungicides, [39] and
532 [40].

533

534 ## *Resistance profiles are shaped by dose variation and should therefore be*
535 ## *considered in management strategies*

536 In resistance management strategies for fungi, the question of dose rate has generally
537 focused on variation in resistance dynamics: the time to resistance emergence or the selection
538 rate [3,6,11,12]. Our experiment did not resolve this debate, because the growth of susceptible
539 and resistant variants was confounded in observations of fungal growth, and because the
540 reduced doses considered here were too low for any realistic description of resistance
541 management strategies with sufficient disease control. However, it did tackle the question of
542 the dose rate from a new standpoint, by considering the qualitative outcome of selection rather
543 than just the dynamics of resistance.

544 We observed that strains resistant to the efficient dose of B, C or P could be selected with
545 reduced doses of the same fungicides, even for the lines exposed to benzovindiflupyr (B), for
546 which resistance never emerged at full dose. This is consistent with previous observations for
547 antibiotics [31,41,42] and herbicides [43]. Indeed, low-dose treatment leads to the higher
548 frequency selection of resistance mutations with a small effect size, resulting in high-level
549 resistance [43].
550
551 The presence of specific resistances in lines treated with reduced-dose regimes suggests that
552 dose mitigation also favours selection for generalist mechanisms. Indeed, resistances to
553 tolnaftate and the BCP mixture were found in lines exposed to reduced doses of B and P,
554 respectively, but not in lines treated with full efficient doses of the same fungicides. These
555 results are consistent with those of many previous studies, in domains other than plant
556 pathology, in which low doses have been shown to select for off-target mutations [44–46] and
557 for polygenic resistance mechanisms [44,47] more likely to result in multiple or generalist
558 resistance (see Raymond (2019) for a review).
559
560 The selection exerted by reduced doses of fungicides may also shape the resistance profiles of
561 lines exposed to efficient-dose mixtures, which are more similar to the union of resistance
562 profiles of lines exposed to reduced doses of the components of mixture than to the union of
563 resistance profiles for lines exposed to efficient doses. In particular, resistance to tolnaftate was
564 observed in lines exposed to reduced doses of C (but not in lines exposed to the efficient dose)
565 and in all lines exposed to efficient-dose mixtures including C. As highlighted in a previous
566 study [16] on antibiotics, low doses should be considered with caution in resistance strategy

567 management, as they do not prevent resistance and could lead to the evolution of generalist
568 resistance, even in mixtures.
569

570 ## *Experimental evolution: a useful tool for comparing strategies*

571 The use of an experimental evolution framework made it possible to subject
572 populations to resistance management strategies with various degrees of selection
573 heterogeneity and to compare the performance of different strategies in standardised
574 conditions. In this controlled environment, it was possible to untangle and assess the
575 performance of several drivers of mixture and dose-reduction strategies, which would have
576 been difficult to achieve in field experiments. The observation of selected resistance profiles
577 was also an advantage over model studies. Despite these multiple advantages, the experiment
578 remained tricky to handle, resulting in the study of only a limited number of strategies. Further
579 studies testing other AIs, different dose ranges for fungicides used alone or in mixtures and
580 double the efficient dose are required to consolidate our conclusions, particularly as concerns
581 the effect of dose in mixtures. In terms of applications, a better understanding of the predictive
582 capacities of such experiments (*e.g.* by relating growth dynamics and resistance profiles to
583 disease control and in-field resistance frequency) is likely to be the key to designing resistance
584 strategies tailored to the intrinsic properties of pathogens and fungicides. Finally, we tested
585 our strategies on naive populations, susceptible to all fungicides. Applying this approach to
586 populations in which initial resistance is present might make it possible to offer farmers
587 additional advice, as contrasting resistance statuses have been reported in monitoring studies
588 [20].

589

590 # Conclusion

591 Our results demonstrate that the use of mixtures cannot be considered a universal
592 strategy for resistance management. At the minimal dose able to control the disease, the use
593 of a mixture against a naive population may decrease durability and increase generalist
594 resistance relative to single fungicide treatments of similar efficacy. However, efficient-dose
595 mixtures, provided that they have appropriate components, could potentially provide disease
596 and resistance control as effective as that achieved with single-fungicide treatments, at a lower
597 environmental and economic cost. It is therefore essential to take into account the specificities
598 of the targeted pathogens, their interactions with fungicides and the interactions between
599 fungicides, as demonstrated here, together with the frequency and type of resistance already
600 present in the population, in the design of sustainable resistance management strategies.
601 Resistance management remains a key challenge for the development of a more sustainable
602 agriculture. Experimental evolution is a highly promising tool that can help us to achieve this
603 goal, as a useful complement to theoretical studies and field monitoring.

604

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Author contributions

ASW and FC conceived and designed the study, with contributions from AD and AB. AB performed the experimental evolution experiment. AB and FC performed the statistical analysis, with contributions from ASW and AD. The paper was written by AB and FC, with significant contributions from ASW and AD.

Conflicts of Interest

The authors declare no conflicts of interest.

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Table S1: Commercial mixtures and single-fungicide formulations used to control Septoria leaf blotch on wheat in France. For each commercial product, the composition, recommended rate and use are detailed. Percentages indicate the fraction of each AI used in the mixture, relative to the commercial product including the same AI used alone, and the total line is the equivalent amount of fungicide in a mixture.

Use as a single compound / Use in mixture						**Commercial Product**	Aviator Xpro Oceor Xpro SDH2 Pro	Karosse Xpro Skyway Xpro	Cavando Korema Korema Star Osiris Star Osiris Win Epomet	Adexar Tenax XM SDH1	Librax SDH-CO	Ceratavo era Elatus era Velogy era	Kestrel Onnel Piano Prosaro Prosafort Prosatop	Ampera Diams Epopée Galactica Nebraska
						Composition g.L^{-1}	75+150	75+100+100	56.25+41.25 (or 37.5+27.5)	62.5+62.5	62.5+45	150+75	160+80 (or 125+125)	132.5+267.1
						Recommended rate l.ha^{-1}	1.25	1	2 (or 3)	2	2	1	1	1.5
						Recommended use g.ha^{-1}	93.75+156.25	75+100+100	112.5+82.5	125+125	125+90	150+75	160+80 (or 125+125)	198.75+400.65
						Fungicides	Bixafen + prothioconazole	Bixafen + prothioconazole + tebuconazole	Epoxiconazole+ metconazole	Fluxapyroxad + epoxiconazole	Fluxapyroxad + metconazole	Prothioconazole + benzovindiflupyr	Prothioconazole + tebuconazole	Tebuconazole + prochloraz
Fungicide	**Chemical class**	**Commercial product**	**Composition g.L^{-1}**	**Recommended rate l.ha^{-1}**	**Recommended use g.ha^{-1}**									
Benzovindiflupyr	SDHI	Elatus plus	100	0.75	75							100%		
Bixafen		Thore	125	1	125		75%	60%						
Epoxiconazole		Rubric	83	1.5	124.5				90%	100%				
Fluxapyroxad		Imtrex	62.5	2	125					100%	100%			

		Syrex Fluxatop												
Metconazole	DMI	Sirena	90	1	90				92%		100%			
Prochloraz		Eyetak Proca Prochloflash Pro Plex 450 Faxer Fujara Saranta Sporaz Septoraz	450	1	450									89%
Prothioconazole		Joao Protioline	250	0.8	200		94%	50%				75%	80% (or 62.5%)	
Tebuconazole		Illiade Mystic Ew Fezan Colnago Rivazon Erasmus Spekfree Curzol Ulysses	430 (or 250)	0.6 (or 1)	258 (or 250)			39-40%					31-32% (or 48-50%)	77-79.5%
						Total	**169%**	**149-150%**	**182%**	**200%**	**200%**	**175%**	**111-112% (or 110.5- 112.5)**	**166-168.5%**

Figure S2: Correlation between susceptibilities to test fungicides in droplet tests.