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

Short title: Delayed but generalist resistance in alternation

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

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

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

[3]AgroParisTech, 75005 Paris, France

[¶]These authors contributed equally to this work

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

ORCID numbers:

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

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

Abstract

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

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

Introduction

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

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

components of the strategy are the most relevant for delaying the evolution of resistance. Indeed, the heterogeneity of selection in an alternation strategy depends on how many AIs are alternated, the nature of the AIs concerned, and the pattern of alternation adopted.

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

resistance has also been shown to be more frequently selected in situations in which antibiotics are alternated [29]. Overall, these studies highlight the importance of treatment frequency relative to the pathogen life cycle [30, 31]. The question of the efficacy of alternation strategies thus remains unresolved for pesticides, as treatment frequency may result in the interval between treatments being much longer than the generation times of pests, particularly for some pathogens.

Here, we use the ascomycete fungus *Zymoseptoria tritici* — the cause of septoria leaf blotch (STB), the most devastating foliar disease of wheat in Europe [32, 33] — as a general model to determine how the components of fungicide alternation drive the quantitative and qualitative performance of this strategy. This highly adaptive pathogen, with large and diverse populations and dual reproduction mode, has evolved resistance in the field to four different unisite modes of action in Western Europe, resulting in contrasting decreases in field efficacy depending on AIs and their combination[34, 35, 36, 37, 38, 39, 40]. Resistance dynamics also differs according to MoAs, as demonstrated quantitatively for France[41, 42]. *Z. tritici* exhibits TSR, determined by various specific target-site mutations, that can be associated to target overexpression [40, 43, 44, 45] but also to NTSR *via*enhanced efflux. This latter generalist mechanism causes weak multidrug resistance (MDR) when not associated to TSR and affects at least three modes of action [43, 46]. These biological features and field background make *Z. tritici* a textbook model for dissecting the adaptation of populations under contrasting selection pressures and reasoning about general principles. We made use of the haploid yeast-like easily-culturable form of this pathogen (*i.e.* blastospores)[47] in liquid medium, in an experimental evolution approach [48] based on the selection of resistance under regimes mimicking alternation. The selection patterns were designed such that three components of alternation — the intrinsic risk of resistance to AIs, the number of AIs alternated and the alternation rhythm — could be disentangled, to assess their respective impacts on resistance dynamics and on the resistance mechanisms selected. We found that the combination and interplay between alternation drivers clearly guided the evolutionary trade-off between the quantitative (*i.e*. the overall and fungicide-specific resistance evolution rates) and qualitative (*i.e.* the degree of generalism of evolved strains) performances of alternation, opening up

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

Results

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

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

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

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

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

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

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

Changes in the frequency of resistance in a population typically follow sigmoid curve, with an emergence phase of low resistance (<15%; here, the lowest frequency reliably detectable by measuring the change in OD_{405} in our experimental conditions), a selection phase (here, resistance rates between 15% and 90%, chosen as a convention) and an established-resistance phase (here, over 90%)[13]. Cox survival analysis was used to investigate the effect of alternation components on the exposure time (number of cycles with a given fungicide) required to reach a given threshold of mean normalized Malthusian growth. Fig **2b** shows the number of cycles of fungicide exposure gained or lost to reach critical values of M_{it}^{n} depending on alternation components relatively to the lines continuously exposed to the same fungicide. Alternation decreased the resistance evolution rate of the high-risk fungicide mostly towards the end of the selection phase and during the established-resistance phase, rather than during the early phases (delaying the established-resistance phase by 0.77 and 1.31 cycles for alternations of two and three AIs, respectively). For the medium- and low-risk fungicides, this beneficial delaying of the established-resistance phase was observed only in the case of alternation with the partner of lowest possible risk (delayed by 1.70 and 1.01 exposure cycles, respectively), with the high-risk partner decreasing the time required to reach the established-resistance phase (by 1.22 and 2.42 exposure cycles, respectively). By contrast, alternation with a partner with a higher resistance

risk generally increased to the rate of selection of resistance to the low-risk fungicide, particularly during the emergence and early selection phases (by 1.52 to 2.13 exposure cycles).

The relative selection pressure exerted by each fungicide during alternation therefore varied not only according to the selection regime and its components, but also according to the phase of resistance dynamics. This contrasting pattern of selection over time was consistent with the exploration of adaptive landscapes [49], *i.e.* the successive selection of different mutations increasing fitness over the course of the experiment, to adjust to the overall selection pressure. We therefore analyzed the change in population composition over time in regimes under continuous exposure (lines 0B, 0C and 0P; Fig **3**), to obtain proof-of-concept. As expected, resistance profiles were generally narrow and mostly focused on resistance to the fungicide used for continuous selection. However, phenotypic resistance profiles differed significantly between different resistance dynamics stages. In AMOVA analyses, within-selection regime diversity was always greater than between-selection regime diversity, but was concentrated throughout evolution, in a manner that varied with the fungicide exerting the selection pressure. The high-risk fungicide (C) ultimately drove the selection of four highly specialized profiles (resistant to a mean of 1.25 AIs). By contrast, the medium- and low-risk fungicides (B and P) selected multiple resistance phenotypes, differing in terms of both cross-resistance and resistance intensity. The medium-risk fungicide selected patterns of resistance to a mean of 2 AIs. The patterns selected by the low-risk fungicide were the most generalist (resistance to a mean of 4 AIs) and often included resistance to tolnaftate, an indicator of multidrug resistance in several pathogens [43]. Even more complex trajectories could be expected in lines undergoing fungicide alternation, where these contrasting patterns of adaptation described for continuous exposure of AIs follow each other over the generations.

Fungicide alternation preferentially selected for multiple or generalist resistance mechanisms

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

The broader resistance profiles of strains subjected to alternation regimes is potentially consistent with the selection of multiple resistance (*i.e.* the co-selection, in the same strain, of several mutations, each conferring resistance to a single fungicide) and/or generalist resistance (*i.e.* the selection of pleiotropic mutations, associated with more general resistance to fungicides). We initially tested these hypotheses by sequencing the genes encoding the targets of carbendazim, prothioconazole and benzovindiflupyr in 141 isolates corresponding to the various phenotypic resistance profiles. Only the E198K substitution in β-tubulin was detected in a few isolates evolving under direct carbendazim selection. This target site mutation has been reported to confer a high degree of resistance to benzimidazoles in field isolates of 12 fungal species, and in nematodes [50, 51]. No other change was

recorded relative to the 330 full-gene sequences established for *tub2, cyp51, sdhB, sdhC* or *sdhD.* The systematic genotyping of the promoter regions of *tub2, sdhC* and *cyp51* did not reveal any indels for these strains by comparison to the ancestral isolate. The normalized expression of these genes, quantified by qRT-PCR after cultivation without fungicide induction, varied roughly between 0.5 to 4 comparatively to that of the ancestral isolates (Fig. **S2**). As these weak variations included a susceptible isolate collected from the control line, we assumed that overexpression of these three target genes did not operate in the dataset or was rather weak. Enhanced efflux is a non-target site resistance mechanism associated to low RFs *per se* but causing cross-resistance to unrelated compounds in *Z. tritici* [43]. In field strains, it is determined by insertions in the promoter region of the MFS1 transporter gene [52]. Systematic genotyping of 249 isolates collected in this experiment revealed no changes in the length of the *mfs1* promoter, particularly in strains displaying tolnaftate resistance. However, qRT-PCR revealed approximately 5-, 11- and 30- fold overexpression of *mfs1*, compared to the ancestral strain, in three out of eight isolates cultivated without fungicide induction and collected from lines subjected to alternation regimes (Fig. **S2**). These isolates exhibited moderate to low scores of tolnaftate resistance. Overall, these findings suggest that alternation components favour generalist phenotypes, with enhanced efflux due to MFS1 transporter, regulated from genomic changes differing from those occurring in the field in some isolates, occurring in some but not all isolates. The low correlation observed between scores of tolnaftate sensitivity and *mfs1* expression implies that unknown transporters are also frequently observed in this context, possibly in addition to other unknown non-target site resistance mechanisms.

Discussion

This study confirms the value of experimental evolution as an approach for investigating conceptual issues in temporal adaptation, such as the factors affecting the adaptive differentiation of local

populations [48, 53, 54]. Here, we used an experimental evolution approach and the economically important wheat pathogen *Z. tritici* as a model for general principles to demonstrate that temporal heterogeneity can mitigate the evolution of resistance to fungicides in lines subjected to treatment with fungicide alternation regimes. These experiments clearly highlight the relationships between the components of the temporal heterogeneity of fungicide selection and both resistance dynamics and the generalism or specialism of the evolved isolates. With this experimental design, we were able to validate, in a filamentous plant pathogen *in vivo*, the theoretical assumptions that temporal heterogeneity of selection can quantitatively modulate adaptation [10, 17] and mediate generalism in evolved individuals [27, 55], as previously demonstrated for weeds, insects and bacteria [12, 29, 56, 57, 58, 59]. Specifically, using three different fungicides, we found that alternation was either neutral or beneficial in terms of delaying the overall resistance evolution rate, relative to the continuous use of a single fungicide, whatever its resistance risk. In all cases, alternation strategies performed at least as well as the continuous use of the lower risk fungicide. Alternation may delay the build-up of resistance to a particular fungicide because the selection pressure exerted by the fungicide is spread over a longer period than in a sequence strategy[21]. However, this would suggest that fungicide-specific resistance evolution rate (ρ_f) is similar for continuous-use and alternation strategies, and independent of alternation partner and alternation rhythm. By contrast, during our dissection of the impact of alternation components, we obtained variable ρ_f values and revealed the primary influence of the fungicide resistance risk, determined by its mode of action, relative to alternation partners, on selection and the overall performance of a strategy. Thus, increasing the number of AIs in the alternation may either increase or decrease the fungicide-specific resistance evolution rate, depending on the intrinsic risk of resistance for the added AI relative to those already present in the alternation. This may reflect the balance between the relative fitness costs and levels of resistance of the multiple genotypes selected by a given AI in a cycle, which finally determines the composition of the population at the beginning of the next cycle, before contrasting selection with a different AI. Our observation then also reflects the relative effectiveness of each partner over time in relation to the current

population composition. When needed to prevent line extinction, in the early cycles, immigration of susceptible isolates from the control lines contributed to slow down resistance but promoting the relative effectiveness of both partners similarly. Finally, the temporal heterogeneity of environments may determine the direction of evolution, with more fine-grained temporal environments (in which the environment varies over time scales shorter than the generation time) being more efficient at delaying adaptation while maintaining population diversity[60, 61]. In our design, even the one-week alternation rhythm greatly exceeded the generation time of *Z. tritici* (estimated at 6-7 generations per cycle), so all the temporal environments tested were coarse-grained, particularly the regimes with a three-week alternation. However, alternation rhythm had a much lesser impact than other components on the resistance evolution rate of a given fungicide. This implies that some flexibility is possible in the design of field strategies, as the frequency of fungicide sprays is likely to create coarse-grained environments in many agrosystems. The respective relevance of these selection drivers for mixture strategies, most commonly used in the field, could be interestingly compared in further studies.

We confirm here, in a plant pathogenic fungus, that temporal variation in fungicide application may be correlated with a higher degree of generalism in resistant strains. An analysis of resistance phenotype profiles in the course of direct selection (continuous use of the same fungicide) revealed high within-line phenotype diversity (data not shown) and the concentration of this diversity over time, culminating in specialist profiles, for which the degree of specialism depended on the AI. This dynamics is also consistent with the continual change in population composition, with the rise, selection, fixation or extinction of beneficial mutations, depending on their relative fitness costs and the degree of resistance in the local population [62]. High-risk fungicides can thus explore smooth fitness landscapes, such as those generated by the selection of a unique change underlying high resistance (*e.g.* the tub2 E198K variant conferring high resistance to C). By contrast, medium- and low-risk fungicides can explore less smooth landscapes. According to theory, the global fitness optimum is less likely to be achieved in variable environments [63]. Combining the respective paths associated with AIs not

exhibiting cross-resistance, and considering the population dynamics as previously discussed, we would then expect lines evolving under alternation regimes to explore rugged landscapes, constraining resistance mutations to one fungicide with trade-offs in resistance to other fungicides or to favour genotypes with a higher average fitness over time[29, 64]. This scenario is consistent with the fixation of multiple discrete resistance mechanisms, together with the accumulation of relative fitness costs associated with each resistant trait, as suggested by the wider patterns of cross-resistance (including resistance to new AIs), lower resistance scores and higher frequency of resistance to tolnaftate (associated with enhanced efflux) observed in isolates collected from lines subjected to alternating treatments. The molecular analysis of a representative subsample of resistant isolates hardly detected mutations in genes encoding the targets of selecting fungicides and indicated that enhanced efflux due to the MFS1 transporter was at work in a few - but not all - resistant strains. Our collection therefore provides a good opportunity to make use of the progress in genomics to elucidate unknown genetic and non-genetic bases of adaptation mostly at work in our dataset. These unknown resistance mechanisms (*i.e.* other than target alteration or overexpression), may also operate *in natura*, without our suspecting it and may, therefore, have an overlooked impact on resistant traits [65, 66, 67]. Indeed, a genomic analysis has shown that not only SNPs but also indels, copy number variants, transposable element insertions, chromosome duplications and aneuploidy occur in fungal pathogens exposed to a sublethal dose of fungicides [68]. Our observation of unknown resistance mechanisms in isolates from alternation as well as sequence strategies, is consistent with this pan-genome characterization of fungal adaptation to fungicides, without *a priori* on resistance mechanisms. This finding brings the proof of concept that *Z. tritici* is also a relevant model to explore the various shades of non-target site adaptation, in addition to inform on the general principles to manage resistance, whatever mutations were selected. We also found that alternation components had different impacts on the rate of resistance evolution over the course of the experiment. Alternation significantly delayed the establishment phase of the dynamics of resistance to the high-risk fungicide, suggesting that lower-risk fungicides better perform after the emergence phase. By contrast, alternation with high-risk

fungicides decreased the time required for the early stages of resistance to be reached for a low-risk fungicide, demonstrating that the high-risk fungicide did not counter-select mutations with a low resistance intensity. Finally, experimental evolution has proposed a simplified model for evolution *in natura* [54] and enriched our understanding of the general principles of resistance management by highlighting the relative impact of selection components. Due to the laboratory environment, our experimental design assumes a finite population size, low ancestral diversity, limited time scale and asexual reproduction. However, many plant pathogens, including *Z. tritici,* have large population sizes, high levels of genetic diversity and both sexual and asexual reproduction, and have to adapt simultaneously to their host and abiotic environment [69]. Gene flow is also expected to occur between evolving metapopulations. These differences may explain why adaptive mutations distinct than those occurring in the field were selected in this experiment without limiting the relevance of this study to reasoning about strategies of antifungal management. Beyond these limitations, our study clearly demonstrates that not all alternation strategies guarantee the same performance for preventing resistance, but in no instance did these strategies increase the risk of resistance relative to that associated with the continuous use of individual fungicides. It might then offer an alternative to the mixture strategy to delay resistance, while reducing pesticide load, a growing socio-environmental demand. Indeed, as an example, commercial mixtures used on wheat to control Septoria leaf blotch combine fungicide components at, or close to, their full rate for use on their own [70]. Efforts to educate stakeholders about alternation strategies should, therefore, focus on optimizing the interplay between the various components of alternation. The results of this study pave the way for the effective tailoring of resistance management for plant pathogens based on temporal heterogeneity, especially in perennial crops where multiple sprays are often applied yearly. We would also argue that resistance management *via* temporal heterogeneity is possible provided that the environmental grain is appropriate [61]. The environment should generally be fine-grained, requiring alternating generations to be exposed to compounds with a different mode of action, but we found that coarse-grained temporal fluctuations were potentially acceptable. Furthermore, the order in which the AIs are cycled may affect

the trajectory of evolution. Modeling suggests that the most effective AI should be used first [21], to control emerging mutations as efficiently as possible, but further explorations in experimental evolution experiments would be required to confirm this. The overall performance of alternation approaches including AIs with different inherent resistance risks is achieved at the expense of the AIs with the lowest risk of resistance, suggesting that the use of high-risk AIs in alternation sequences should be limited and/or that the alternation of AIs of similar risk should be preferred. The relative risk between AIs might also be adjusted in the field by using different doses or alternation rhythms of each partner, or by mixing some of the alternated AIs. The efficacy of alternation strategies, as well as of mixtures, should also be confronted to the real-world case in which populations are not naïve and the initial levels of resistance to each fungicide differ, not to mention multiple resistance[71]. Finally, the design of rational strategies with optimized components should take into account the evolutionary trade-off between the rate of resistance evolution and the degree of generalism of the evolved individuals, which may call into question the efficacy of future modes of action. In this respect, the future control of weeds, pests and diseases should be based not only on a diversity of modes of action, but should consider integrated pest management including non-chemical strategies to mitigate overall adaptation [72].

Methods

Ancestral population, culture conditions and assessment

We used IPO-323, as an ancestral isolate, to found all lines, *i.e.* similar ancestral populations further evolving under fungicide selection. This isolate is susceptible to all fungicides and was the source of the high-quality reference genome for *Z. tritici* (Goodwin et al., 2011). The stock spore suspension was kept at -80°C in 20% glycerol and gently thawed at room temperature for the inoculation of solid YPD plates (20 $g.L^{-1}$ dextrose, 20 $g.L^{-1}$ peptone, 10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ agar; USBiological), which were

then incubated at 18°C in the dark for seven days. These plates were used to prepare precultures in liquid YPD (as previously described, but without agar), which were shaken at 150 rpm in similar conditions for seven days before the start of the experiment.

The culture medium used for experimental evolution was liquid YPD supplemented with 100 $mg.L^{-1}$ streptomycin and penicillin to prevent contamination of this long-term experiment. The culture vessels were 50 mL borosilicate Erlenmeyer flasks containing 25 mL medium, with carded cotton wool inserted into the neck. Cultures were grown in the dark at 18°C, with a RH of 70% and shaking at 150 rpm during the duration of the cycle. In these conditions, *Z. tritici* can be kept in its yeast-like form, making it possible to obtain homogeneous liquid cultures [73].

Population size was routinely determined by measuring optical density at 405 nm (or OD_{405}) for two 200 µL replicates of the fungal culture in a 96-well microtiter plate (Sarstedt) sealed with a gas-permeable membrane (Breathe Easy®; Diversified Biotech). The detection threshold of the spectrophotometer (SpectraMax M2, Molecular Devices) was 3.5×10^5 $spores.mL^{-1}$ in the experimental conditions used. The OD_{405} of the fungal cultures was normalized against that of the non-amended medium. Spore concentration was estimated from the OD_{405} with a mathematical model calibrated on the basis of preliminary experiments in our conditions with serial dilutions and hemocytometer-based spore quantification, and cross-validated (R^2=0.91). Population size in $spores.mL^{-1}$ was used in further statistical analyses.

Fungicides and selection regimes

In the experimental evolution experiments, we selected resistance to three fungicides representative of different modes of action: carbendazim (C; benzimidazole, interfering with β-tubulin assembly in mitosis), benzovindiflupyr (B; pyrazole carboxamide, inhibiting complex II, succinate dehydrogenase, in the mitochondrial respiratory chain) and prothioconazole-desthio (P; triazole, inhibiting the sterol 14α-demethylase during sterol biosynthesis), the active metabolite of prothioconazole. These modes

of action are associated with contrasting resistance risk. The fungicides were dissolved in 80% ethanol. The concentration of this solvent never exceeded 0.5% of the final culture volumes.

The selection doses were chosen by examination of dose-response curves for the ancestral strain. For each fungicide dose, 10^7 spores of IPO-323 were used to inoculate YPD in an Erlenmeyer flask, which was then incubated for seven days before OD_{405} measurement. OD_{405} data were modelled by logistic regression, to calculate, for each fungicide, the EC_{50} (concentration inhibiting 50% of growth), EC_{95} (concentration inhibiting 95% of growth) and MIC (minimum inhibitory concentration) values. We finally chose to use the EC_{95} as the selection dose, as 99% inhibition was not achieved for some fungicides, for which the MIC would exceed fungicide solubility in the solvent. The selection doses used were 0.5 $mg.L^{-1}$ for benzovindiflupyr, 0.1 $mg.L^{-1}$ for carbendazim and 0.005 $mg.L^{-1}$ for prothioconazole-desthio.

Selection regimes were organized for analysis of the respective impacts of three components of alternation on resistance evolution, as described in Fig **1b**. First, in treated lines, the number of AIs in the alternation regime ranged from one (direct selection) to three different fungicides alternated over time. Second, the intrinsic risk of resistance differed between MoAs and was predicted to be high for benzimidazoles (represented by C), moderate to high for SDHIs (represented by B) and moderate for DMIs (represented by P) by agrochemical companies[74]. In practice, as we observed similar relative ranking of these fungicides in our experiments, we referred to high-, medium- and low-risk fungicides, respectively, throughout this work, for the sake of simplicity. Finally, the duration of exposure to the same fungicide (or alternation rhythm) was continuous for direct selection (0, no alternation) and ranged from 1 (fungicide changed every cycle) to 3 (fungicide changed every three cycles) cycles. All combinations of these three possible components were tested, except for the three-fungicide alternation, which was tested only for an alternation rhythm of one cycle.

Design of the experimental evolution experiment

The ancestral population was founded from the seven-day liquid YPD preculture of IPO-323. The density of this spore suspension was adjusted to 2 x 10^7 spores.mL^{-1} and each Erlenmeyer flask was inoculated with 10^7 spores (500 µL). The resulting starting concentration of 4 x 10^5 spores.mL^{-1} offered a compromise between large populations in which mutations were likely to occur and not exceeding the culture carrying capacity in the experimental conditions used, based on the findings of preliminary experiments. Each of the 14 selection regimes (1 control and 13 different treatments) was repeated four times (four lines per selection regime). Control solvent lines received only 0.5% solvent, whereas treated lines were treated as shown in Fig **1b**, giving rise to 56 independent lines. Control lines without fungicides nor solvent were grown only to provide susceptible cells for immigration (see below) and were not included in statistical analysis comparing strategies.

Experimental evolution was conducted over 12 cycles of seven days each (about six to seven generations per cycle). This cycle duration corresponds approximately to the time for which a control line remained in the exponential growth phase before reaching a plateau. OD_{405} was measured at the end of each cycle and transformed into population size, as described above. The OD_{405} of the treated lines was normalized with the mean OD_{405} of the control solvent lines. At each transfer, 500 µL of the evolving culture (*i.e.* 2% of the total volume of medium) was transferred to a new Erlenmeyer flask containing fresh medium. If the number of spores in the 500 µL of culture medium was less than 10^7, which would be the case before the evolution of resistance, then the appropriate number of spores from one of the untreated populations was added to make the number of spores present up to 10^7. The starting population size was equalized between the lines at the beginning of each new cycle, and the process mimicked the immigration occurring in field situations. For each of the four replicates, the same source population was used for immigration throughout the experiment. In preliminary experiments, immigration was found to be useful for preventing population extinction after weekly bottlenecks and to minimize genetic drift.

At the end of each cycle, 2 mL of each line was mixed with glycerol (25%), frozen and stored at -80°C for further analyses.

Statistical analysis of resistance evolution rate

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

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

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

For each selection regime, the mean Malthusian growth ("overall resistance evolution rate" or ρ) is the mean of M_{it}^{n} calculated over the 12 cycles, and constitutes a quantitative summary of the increase in resistance of the four replicates over the entire experiment. Similarly, ρ_f ("fungicide-specific resistance evolution rate") reflects the increase in resistance associated with a fungicide *f* during the alternation regime, taking into account only the time segments during which this fungicide was used. This approach made it possible to compare selection due to the same number of fungicide applications (6 applications, except for the BCP regime for which there were only 4 applications), but in the context of different selection regimes. ρ and ρ_f were explained in ANOVA linear models, initially considering the alternation partners as qualitative fixed factors, and then the alternation rhythm for each alternation partner. The reference used was the continuous application of the fungicide.

The exposure time (*i.e.* the number of cycles exposed to a given fungicide) required for the normalized Malthusian growth M_{it}^{n} to reach certain thresholds (15%, 25%, 50%, 75%, 90%) was investigated in a Cox survival analysis taking into account the number of AIs, the alternation partner and the alternation rhythm.

All analyses and figures were produced with R 4.0.4 and the additional packages listed in **S1** Methods.

Isolation and phenotyping of evolved individuals

We assessed the change in resistance phenotypes during the course of selection, by isolating pure strains from direct selection regimes, at critical points in the resistance dynamics, *i.e.* when normalized Malthusian growth reached 15% (early resistance), 75% (intermediate resistance frequency) and 98% (established resistance) relative to that of the control line, and at the end of the experiment. We isolated 12 individuals (3 per replicate) per selection regime from cycles 1, 5, 6, 12 for 0B lines, from cycles 2, 3 and 12 for 0C lines, and from cycles 5, 6 and 12 for 0P lines. This resulted in the isolation of 120 strains in total. We also explored phenotype diversity within and between selection regimes, by systematically isolating 12 strains (3 per replicate) at the end of the final cycle, for all selection regimes. This resulted in the isolation of 129 additional strains. Isolates were retrieved from glycerol population stocks collected at the end of each cycle. Two successive isolations were performed on YPD solid medium supplemented with 100 $mg.L^{-1}$ streptomycin and penicillin. Single isolates were stored at -80°C in 25% glycerol.

The cross-resistance patterns of the 249 isolates were established in droplet tests. We deposited 10^7, 10^6, 10^5, and 10^4 $spores.mL^{-1}$ suspensions as drops organized into columns, in 12 cm square Petri dishes. These plates contained YPD supplemented with discriminatory doses of fungicides. The 15 treatments tested encompassed a control with 0.5% solvent and fungicides B, C and P at their selection doses (0.1, 0.5 and 0.005 $mg.L^{-1}$, respectively) and twice their selection doses (0.2, 1 and 0.01 $mg.L^{-1}$, respectively). Mixtures of pairs of the three fungicides were also included (each fungicide used at 0.8 times its selection dose, as this was found to be the lowest discriminatory dose in mixtures of the reference resistant and susceptible strains). Finally, for the detection of generalist resistance, we also included fungicides not used for experimental evolution (0.5 $mg.L^{-1}$ azoxystrobin, 2 $mg.L^{-1}$ boscalid and 2 $mg.L^{-1}$ tolnaftate). Tolnaftate, in particular, identified individuals displaying enhanced fungicide efflux leading to non-specific multidrug resistance (MDR), as described in field isolates of *Z. tritici* [43]. The ancestral strain IPO-323, and three field strains, each resistant to one or several of the fungicides used for selection, were included as control strains. After seven days of incubation, drops were scored 0 or 1, according to the presence or absence of fungal growth. A total score was established for each strain,

ranging between 0 (susceptible) and 4 (resistant if >0), according to the number of dilution droplets for which growth was observed. For each strain, the resistance phenotype profile was determined as the concatenation of scores established for the 14 sets of fungicide conditions. In total, 132 different resistance phenotype profiles were identified.

Statistical analysis of resistance phenotype profiles

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

Diversity within and between selection regimes, measured at the end of the experiment, was quantified by an analysis of molecular variance (AMOVA) performed with the Arlequin program [76]. AMOVA is generally used for molecular data. Here, we assumed that different scores for a particular test corresponded to different alleles or combination of alleles.

Finally, the number of AIs to which a line is resistant and the intensity of resistance (*i.e.* in each test and for each line, the mean resistance score calculated from individual droplet scores greater than 0) were calculated from the resistance phenotype profiles recorded at the end of the experiment. The effects of AI number, alternation rhythm, alternation partner (and their interaction) on resistance to tolnaftate, and the number of AIs to which a line was resistant and the intensity of resistance were investigated with linear models (quasiPoisson GLM model, Poisson GLM and log-transformed linear models, respectively).

All analyses and figures were produced with R 4.0.4 and the packages listed in **S1** Methods.

Sequencing, genotyping and gene expression of evolved isolates

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

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

Data availibity

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

Supporting Information

S1 Methods: Statistical programs used for data analysis

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

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

S4 Methods: Quantifying the expression of *tub2*, *cyp51*, *sdhC*, *mfs1* in *Z. tritici* by qRT-PCRS1 Figure: Variation of resistance evolution in selection regimes

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

Acknowledgments

We thank Fabrice Blanc for facilitating the administrative organization of this PhD studentship. We would also like to thank Dr. Stefano Torriani, Dr. Stephanie Bedhomme and Dr. Mato Lagator for their sound comments about our findings. We are very grateful to Alicia Noly for help with the analysis of the expression of target genes in evolved isolates of *Z. tritici.*

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

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

Competing interests

The authors declare no competing interests.

References

1. Gould F, Brown ZS, Kuzma J. Wicked evolution: Can we address the sociobiological dilemma of pesticide resistance? *Science* **360**, 728-732 (2018).

2. Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science* **360**, 739-742 (2018).

3. Tabashnik BE, Mota-Sanchez D, Whalon ME, Hollingworth RM, Carrière Y. Defining terms for proactive management of resistance to Bt crops and pesticides. *Journal of Economic Entomology* **107**, 496-507 (2014).

4. Frisvold GB, Bagavathiannan MV, Norsworthy JK. Positive and normative modeling for Palmer amaranth control and herbicide resistance management. *Pest Management Science* **73**, 1110-1120 (2017).

5. Palumbi SR. Humans as the world's greatest evolutionary force. *Science* **293**, 1786-1790 (2001).

6. Bourguet D, Guillemaud T. The hidden and external costs of pesticide use. In: *Sustainable Agriculture Reviews: Volume 19* (ed Lichtfouse E). Springer International Publishing (2016).

7. Goulson D, Nicholls E, Botías C, Rotheray EL. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* **347**, 1255957 (2015).

8. Jepson PC, Murray K, Bach O, Bonilla MA, Neumeister L. Selection of pesticides to reduce human and environmental health risks: a global guideline and minimum pesticides list. *The Lancet Planetary Health* **4**, e56-e63 (2020).

9. Raymond B. Five rules for resistance management in the antibiotic apocalypse, a road map for integrated microbial management. *Evol Appl* **12**, 1079-1091 (2019).

10. REX_Consortium. Heterogeneity of selection and the evolution of resistance. *Trends in Ecology & Evolution* **28**, 110-118 (2013).

11. Vacher C, Bourguet D, Rousset F, Chevillon C, Hochberg ME. Modelling the spatial configuration of refuges for a sustainable control of pests: a case study of Bt cotton. *Journal of Evolutionary Biology* **16**, 378-387 (2003).

12. Lagator M, Vogwill T, Colegrave N, Neve P. Herbicide cycling has diverse effects on evolution of resistance in *Chlamydomonas reinhardtii*. *Evol Appl* **6**, 197-206 (2013).

13. van den Bosch F, Paveley N, Shaw M, Hobbelen P, Oliver R. The dose rate debate: does the risk of fungicide resistance increase or decrease with dose? *Plant Pathology* **60**, 597-606 (2011).

14. van den Bosch F, Paveley N, van den Berg F, Hobbelen P, Oliver R. Mixtures as a fungicide resistance management tactic. *Phytopathology* **104**, 1264-1273 (2014).

15. Baym M, Stone LK, Kishony R. Multidrug evolutionary strategies to reverse antibiotic resistance. *Science* **351**, aad3292 (2016).

16. Coyne FP. Proper use of insecticides. *Br Med J* **2**, 911-912 (1951).

17. Hobbelen PHF, Paveley ND, Oliver RP, van den Bosch F. The usefulness of fungicide mixtures and alternation for delaying the selection for resistance in populations of *Mycosphaerella graminicola* on winter wheat: A modeling analysis. *Phytopathology* **103**, 690-707 (2013).

18. van den Bosch F, Oliver R, van den Berg F, Paveley N. Governing principles can guide fungicide-resistance management tactics. *Annual Review of Phytopathology* **52**, 175-195 (2014).

19. Blanquart F. Evolutionary epidemiology models to predict the dynamics of antibiotic resistance. *Evol Appl* **12**, 365-383 (2019).

20. Birkegård AC, Halasa T, Toft N, Folkesson A, Græsbøll K. Send more data: a systematic review of mathematical models of antimicrobial resistance. *Antimicrobial Resistance & Infection Control* **7**, 117 (2018).

21. van den Bosch F, Gilligan CA. Models of fungicide resistance dynamics. In: *Annual Review of Phytopathology*) (2008).

22. Hawkins NJ, Bass C, Dixon A, Neve P. The evolutionary origins of pesticide resistance. *Biological Reviews* **94**, 135-155 (2019).

23. R4P_Network. Trends and challenges in pesticide resistance detection. *Trends in Plant Science* **21**, 834-853 (2016).

24. Berman J, Krysan DJ. Drug resistance and tolerance in fungi. *Nature Reviews Microbiology* **18**, 319-331 (2020).

25. Corbel V, N'Guessan R. Distribution, mechanisms, impact and management of insecticide resistance in malaria vectors: pragmatic reviews. In: *Anopheles, Mosquitoes - new insights into malaria vectors* (ed Manguin S). In Tech (2013).

26. Délye C. Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. *Pest Management Science* **69**, 176-187 (2013).

27. Kassen R. The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of Evolutionary Biology* **15**, 173-190 (2002).

28. Comont D*, et al.* Evolution of generalist resistance to herbicide mixtures reveals a trade-off in resistance management. *Nature Communications* **11**, 3086 (2020).

29. Kim S, Lieberman TD, Kishony R. Alternating antibiotic treatments constrain evolutionary paths to multidrug resistance. *Proceedings of the National Academy of Sciences* **111**, 14494-14499 (2014).

30. Abel zur Wiesch P, Kouyos R, Abel S, Viechtbauer W, Bonhoeffer S. Cycling empirical antibiotic therapy in hospitals: Meta-analysis and models. *PLoS Pathogens* **10**, e1004225 (2014).

31. Roemhild R, Barbosa C, Beardmore RE, Jansen G, Schulenburg H. Temporal variation in antibiotic environments slows down resistance evolution in pathogenic *Pseudomonas aeruginosa*. *Evol Appl* **8**, 945-955 (2015).

32. Torriani SFF, Melichar JPE, Mills C, Pain N, Sierotzki H, Courbot M. *Zymoseptoria tritici*: A major threat to wheat production, integrated approaches to control. *Fungal Genetics and Biology* **79**, 8-12 (2015).

33. Fones H, Gurr S. The impact of Septoria tritici blotch disease on wheat: An EU perspective. *Fungal Genetics and Biology* **79**, 3-7 (2015).

34. Huf A, Rehfus A, Lorenz KH, Bryson R, Voegele RT, Stammler G. Proposal for a new nomenclature for CYP51 haplotypes in *Zymoseptoria tritici* and analysis of their distribution in Europe. *Plant Pathology* **67**, 1706-1712 (2018).

35. Heick TM, Justesen AF, Jorgensen LN. Resistance of wheat pathogen *Zymoseptoria tritici* to DMI and QoI fungicides in the Nordic-Baltic region - a status. *European Journal of Plant Pathology* **149**, 669-682 (2017).

36. Heick TM, Matzen N, Jørgensen LN. Reduced field efficacy and sensitivity of demethylation inhibitors in the Danish and Swedish Zymoseptoria tritici populations. *European Journal of Plant Pathology* **157**, 625-636 (2020).

37. Hellin P*, et al.* Multiplex qPCR assay for simultaneous quantification of CYP51-S524T and SdhC-H152R substitutions in European populations of Zymoseptoria tritici. *Plant Pathology* **n/a**.

38. Jørgensen LN*, et al.* Decreasing azole sensitivity of *Z. tritici* in Europe contributes to reduced and varying field efficacy. *Journal of Plant Diseases and Protection*, 287-301 (2021).

39. Dooley H, Shaw MW, Mehenni-Ciz J, Spink J, Kildea S. Detection of *Zymoseptoria tritici* SDHI-insensitive field isolates carrying the SdhC-H152R and SdhD-R47W substitutions. *Pest Management Science* **72**, 2203-2207 (2016).

40. Rehfus A, Strobel D, Bryson R, Stammler G. Mutations in *sdh* genes in field isolates of *Zymoseptoria tritici* and impact on the sensitivity to various succinate dehydrogenase inhibitors. *Plant Pathology* **67**, 175-180 (2018).

41. Garnault M*, et al.* Spatiotemporal dynamics of fungicide resistance in the wheat pathogen *Zymoseptoria tritici* in France. *Pest Management Science* **75**, 1794-1807 (2019).

42. Garnault M*, et al.* Large-scale study validates that regional fungicide applications are major determinants of resistance evolution in the wheat pathogen *Zymoseptoria tritici* in France. *New Phytologist* **229**, 3508-3521 (2021).

43. Leroux P, Walker A. Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Management Science* **67**, 44-59 (2011).

44. Cools HJ, Bayon C, Atkins S, Lucas JA, Fraaije BA. Overexpression of the sterol 14α-demethylase gene (MgCYP51) in *Mycosphaerella graminicola* isolates confers a novel azole fungicide sensitivity phenotype. *Pest Management Science* **68**, 1034-1040 (2012).

45. Cools HJ, Hawkins NJ, Fraaije BA. Constraints on the evolution of azole resistance in plant pathogenic fungi. *Plant Pathology* **62**, 36-42 (2013).

46. Omrane S*, et al.* Fungicide efflux and the MgMFS1 transporter contribute to the multidrug resistance phenotype in *Zymoseptoria tritici* field isolates. *Environmental Microbiology* **17**, 2805-2823 (2015).

47. Quaedvlieg W*, et al. Zymoseptoria* gen. nov.: a new genus to accommodate *Septoria*-like species occurring on graminicolous hosts. *Persoonia* **26**, 57-69 (2011).

48. Van den Bergh B, Swings T, Fauvart M, Michiels J. Experimental design, population dynamics, and diversity in microbial experimental evolution. *Microbiology and Molecular Biology Reviews* **82**, (2018).

49. Poelwijk FJ, Kiviet DJ, Weinreich DM, Tans SJ. Empirical fitness landscapes reveal accessible evolutionary paths. *Nature* **445**, 383-386 (2007).

50. Dilks CM, Hahnel SR, Sheng QC, Long LJ, McGrath PT, Andersen EC. Quantitative benzimidazole resistance and fitness effects of parasitic nematode beta-tubulin alleles. *International Journal for Parasitology-Drugs and Drug Resistance* **14**, 28-36 (2020).

51. Hawkins NJ, Fraaije BA. Predicting resistance by mutagenesis: Lessons from 45 years of MBC resistance. *Frontiers in Microbiology* **7**, e1814 (2016).

52. Omrane S*, et al.* Plasticity of the *MFS1* promoter leads to multidrug resistance in the wheat pathogen *Zymoseptoria tritici*. *mSphere* **2**, (2017).

53. Kawecki TJ, Ebert D. Conceptual issues in local adaptation. *Ecology Letters* **7**, 1225-1241 (2004).

54. Kawecki TJ, Lenski RE, Ebert D, Hollis B, Olivieri I, Whitlock MC. Experimental evolution. *Trends in Ecology & Evolution* **27**, 547-560 (2012).

55. Levins R. *Evolution in changing environments* (1968).

56. Fardisi M, Gondhalekar AD, Ashbrook AR, Scharf ME. Rapid evolutionary responses to insecticide resistance management interventions by the German cockroach (*Blattella germanica* L.). *Scientific Reports* **9**, 8292 (2019).

57. Wang A, Singh A, Huang YH, Agrawal AF. Ecological specialization in populations adapted to constant versus heterogeneous environments. *Evolution* **73**, 1309-1317 (2019).

58. Santos-Lopez A, Marshall CW, Scribner MR, Snyder DJ, Cooper VS. Evolutionary pathways to antibiotic resistance are dependent upon environmental structure and bacterial lifestyle. *eLife* **8**, e47612 (2019).

59. Fitzgerald DM. The road to resistance. *eLife* **8**, e52092 (2019).

60. Roux F, Paris M, Reboud X. Delaying weed adaptation to herbicide by environmental heterogeneity: a simulation approach. *Pest Management Science* **64**, 16-29 (2008).

61. Venail PA, Kaltz O, Olivieri I, Pommier T, Mouquet N. Diversification in temporally heterogeneous environments: effect of the grain in experimental bacterial populations. *Journal of Evolutionary Biology* **24**, 2485-2495 (2011).

62. Hawkins N, Fraaije B. Fitness penalties in the evolution of fungicide resistance. *Annual Review of Phytopathology* **56**, 339-360 (2018).

63. Collins S. Many Possible Worlds: Expanding the Ecological Scenarios in Experimental Evolution. *Evolutionary Biology* **38**, 3-14 (2011).

64. Obolski U, Ram Y, Hadany L. Key issues review: evolution on rugged adaptive landscapes. *Reports on Progress in Physics* **81**, 012602 (2017).

65. Bailey SF, Bataillon T. Can the experimental evolution programme help us elucidate the genetic basis of adaptation in nature? *Mol Ecol* **25**, 203-218 (2016).

66. Hu M, Chen S. Non-target site mechanisms of fungicide resistance in crop pathogens: A review. *Microorganisms* **9**, 502 (2021).

67. Remigi P, Masson-Boivin C, Rocha EPC. Experimental evolution as a tool to investigate natural processes and molecular functions. *Trends Microbiol* **27**, 623-634 (2019).

68. Gambhir N, Kamvar ZN, Higgins R, Amaradasa BS, Everhart SE. Spontaneous and fungicide-induced genomic variation in *Sclerotinia sclerotiorum*. *Phytopathology* **111**, 160-169 (2021).

69. Stukenbrock EH, McDonald BA. The origins of plant pathogens in agro-ecosystems. *Annual Review of Phytopathology* **46**, 75-100 (2008).

70. Ballu A, Deredec A, Walker A-S, Carpentier F. Are efficient-dose mixtures a solution to reduce fungicide load and delay evolution of resistance? An experimental evolutionary approach. *Microorganisms* **9**, 2324 (2021).

71. Taylor N, Cunniffe NJ. Optimal resistance management for mixtures of high-risk fungicides: robustness to the initial frequency of resistance and pathogen sexual reproduction. *Phytopathology®* **0**, null (2022).

72. REX_Consortium*, et al.* Combining selective pressures to enhance the durability of disease resistance genes. *Frontiers in Plant Science* **7**, (2016).

73. Francisco CS, Ma X, Zwyssig MM, McDonald BA, Palma-Guerrero J. Morphological changes in response to environmental stresses in the fungal plant pathogen *Zymoseptoria tritici*. *Scientific Reports* **9**, 9642 (2019).

74. Brent JK, Hollomon DW. Fungicide resistance: the assessment of risk. In: *FRAC Monograph n°2*). Croplife (2007).

75. Fisher RA. *The genetical theory of natural selection. A complete variorum edition.* Oxford University press (1999).

76. Excoffier L, Lischer HEL. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**, 564-567 (2010).

1

2

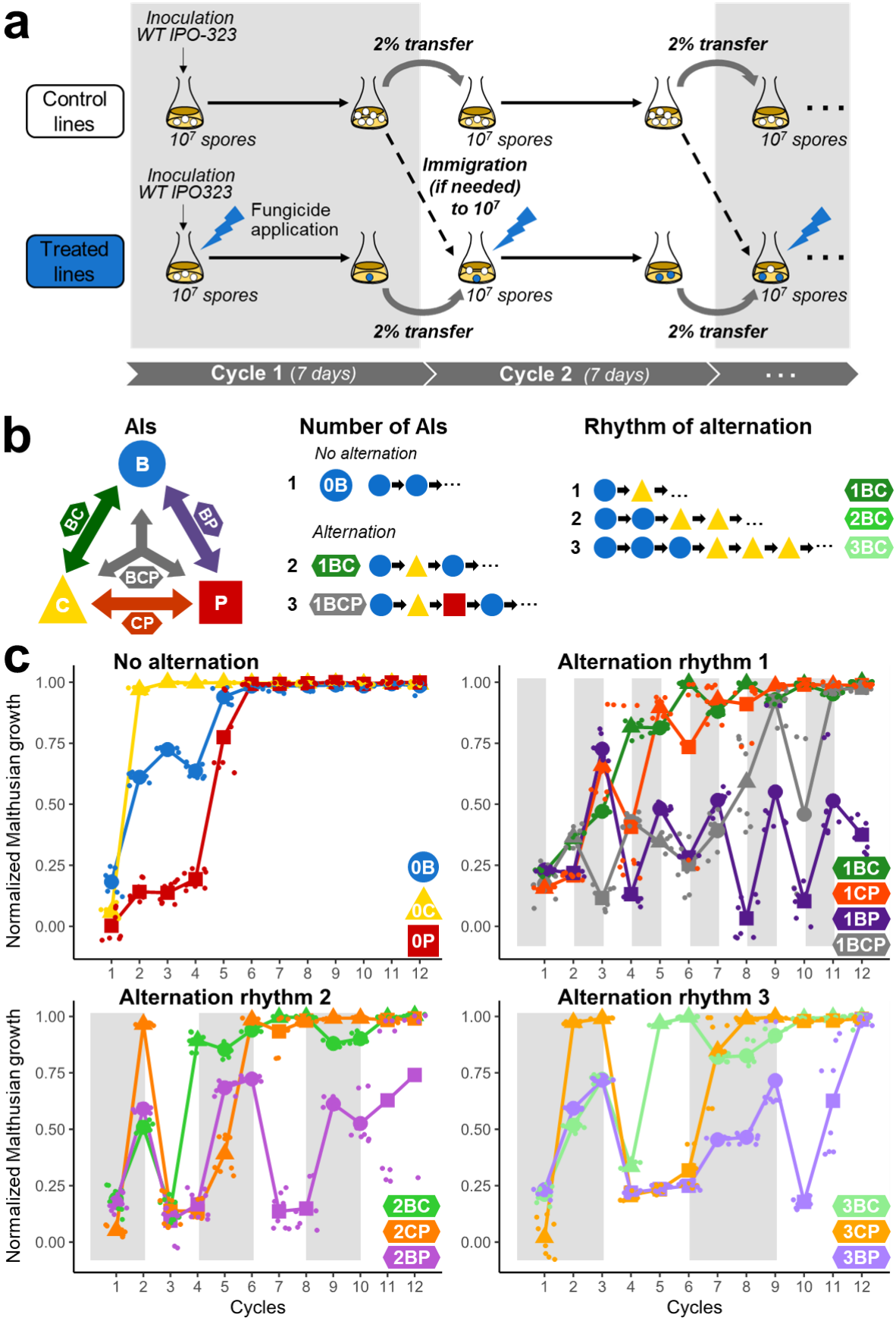

a
Inoculation
WT IPO-323
2% transfer
2% transfer
Control lines
10^7 spores
10^7 spores
10^7 spores
Immigration
(if needed)
to 10^7
Inoculation
WT IPO323
Fungicide application
Treated lines
10^7 spores
10^7 spores
10^7 spores
2% transfer
2% transfer
Cycle 1 (7 days)
Cycle 2 (7 days)
b
AIs
B
BC
BP
BCP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

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

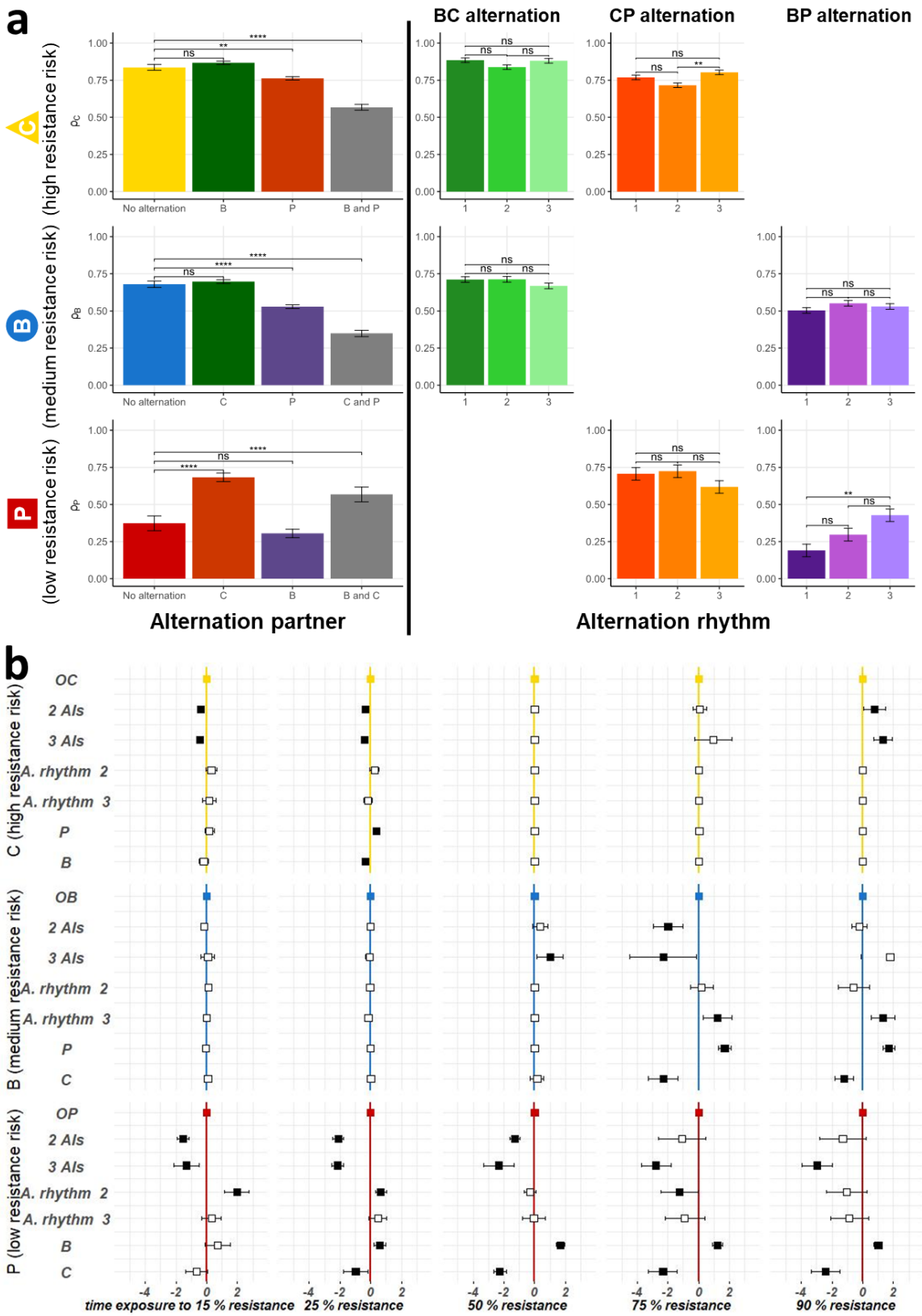


Figure 2. Effects of alternation components on the evolution of resistance. (a) ρ_f, the mean rate of resistance evolution for time of exposure to fungicide *f,* is shown as a function of alternating AIs on the

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

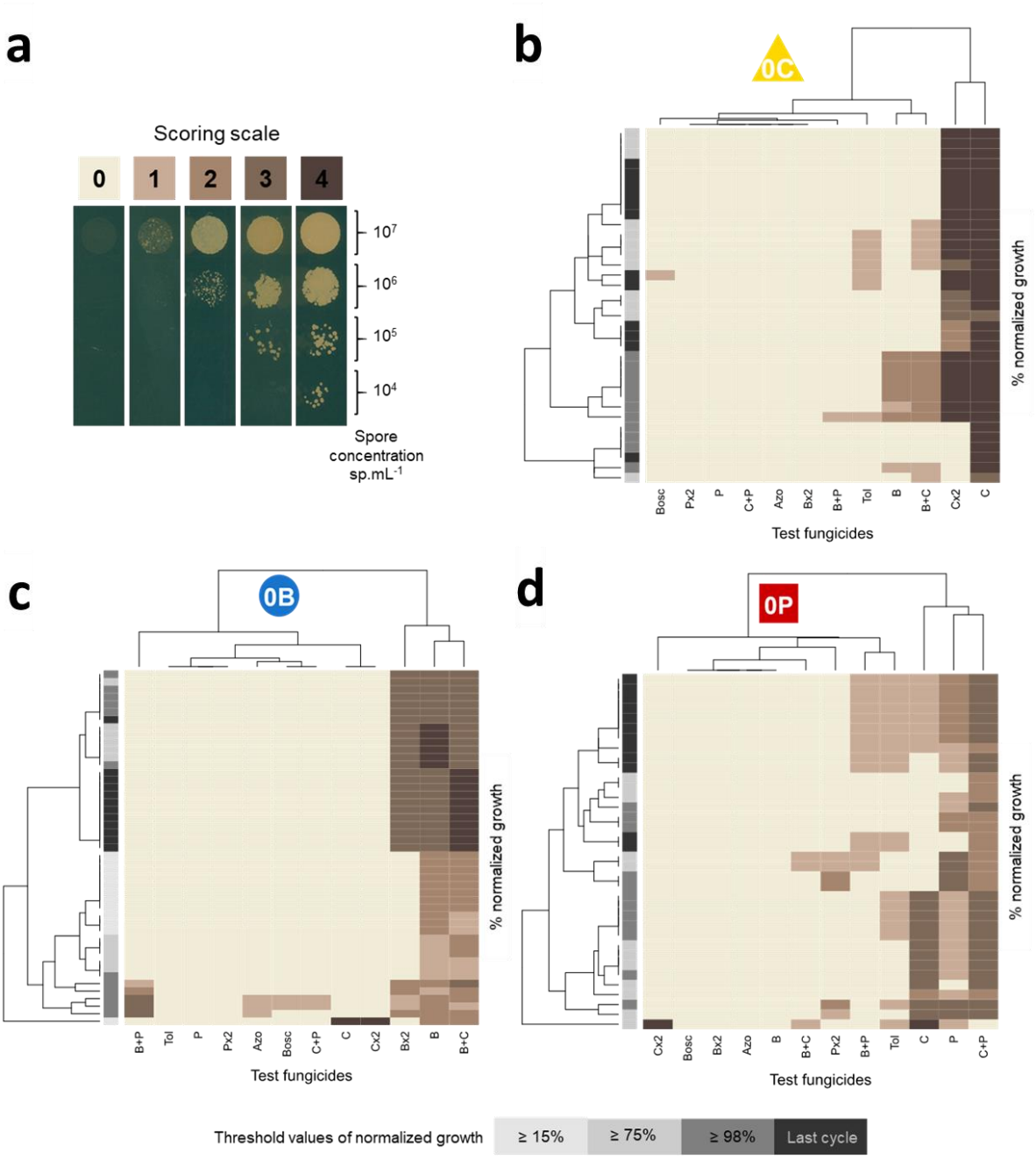


Figure 3. Evolution of phenotype resistance profiles during experimental evolution. (**a**) Scoring scale used to assess the growth of isolates on solid medium supplemented with discriminatory doses of fungicides (droplet tests). For each isolate, four spore concentrations were tested on the same plate.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

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

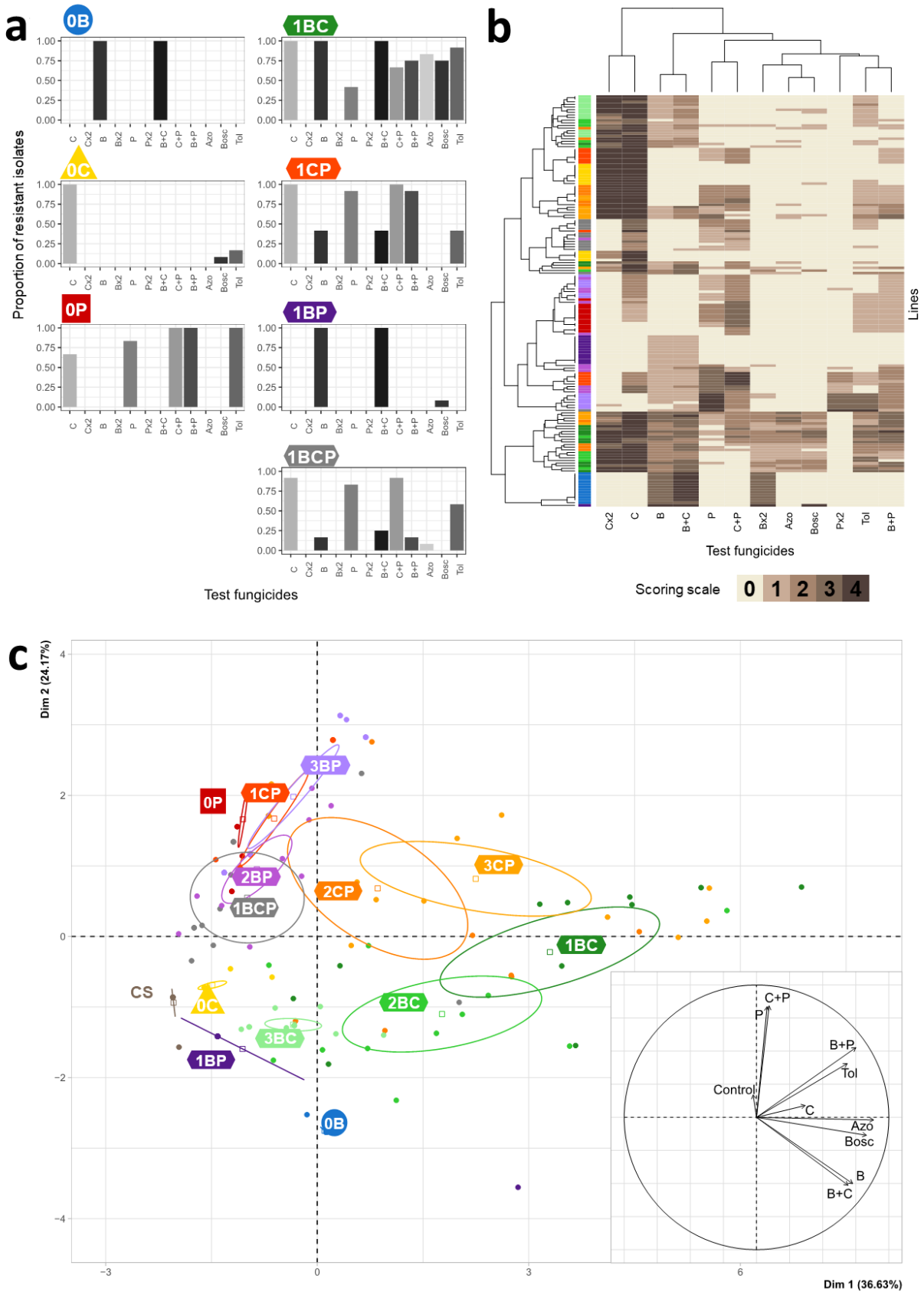
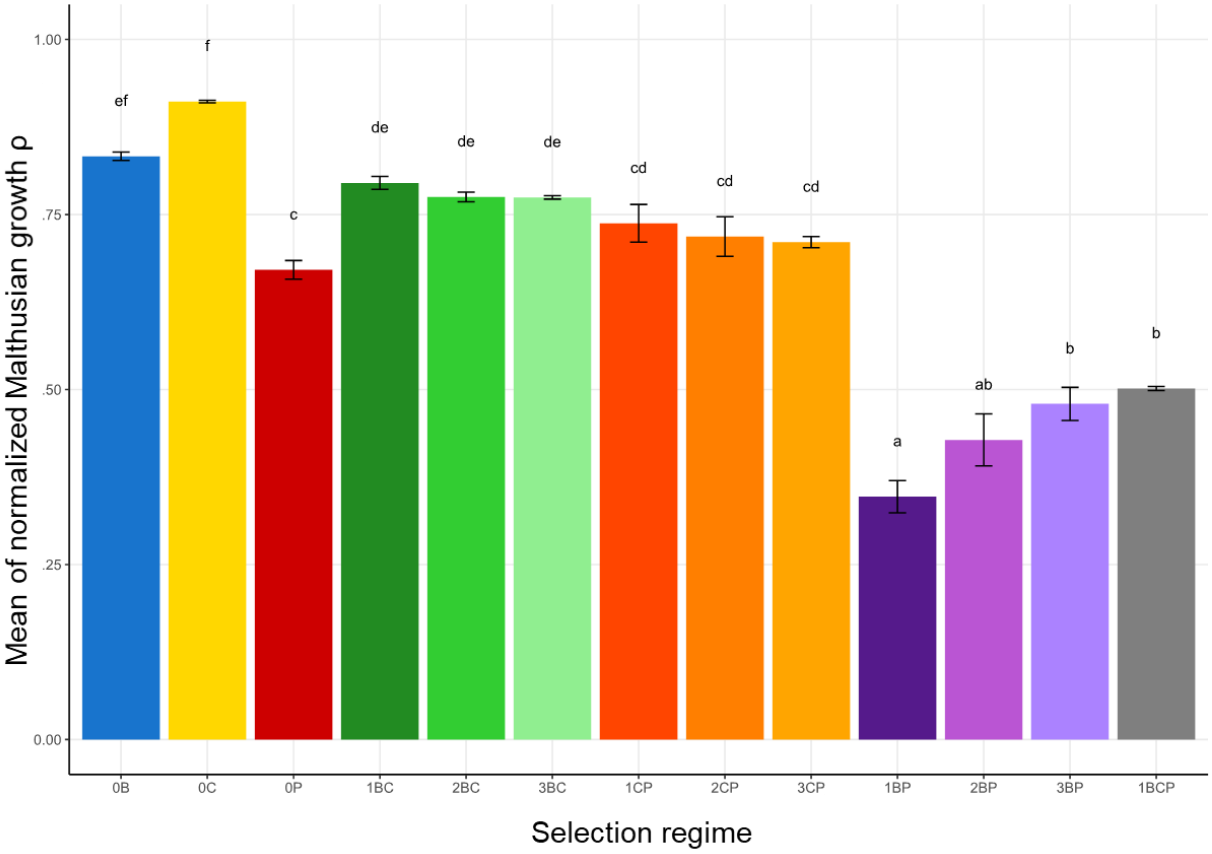


Figure 4. Phenotypic resistance profiles selected at the end of experimental evolution (**a**) The average phenotypic resistance profile obtained with the different selection regimes (*i.e.* after

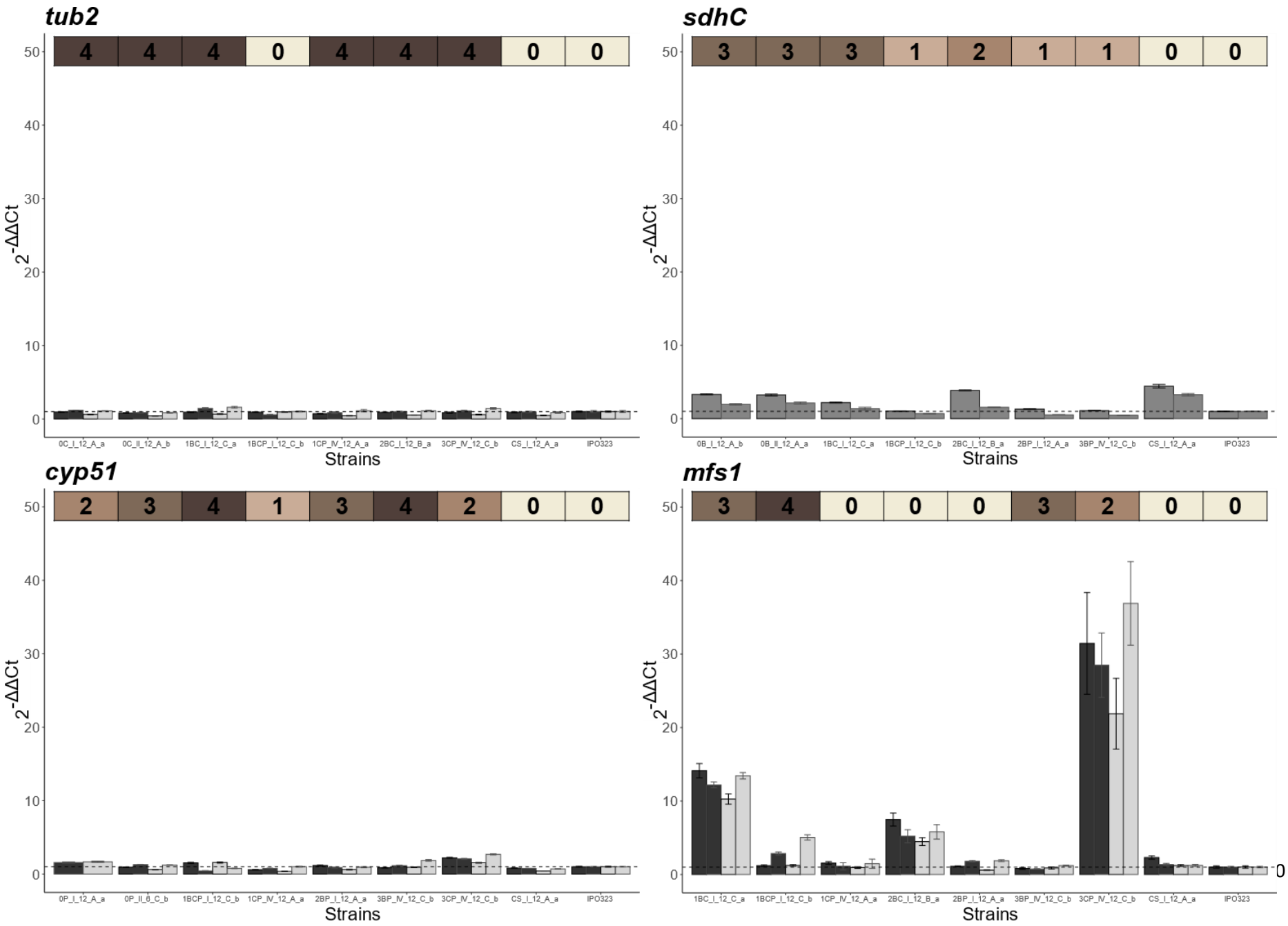
continuous or alternating exposure; alternation rhythm = 1 cycle) at the end of the experiment. Diagrams show the proportion of isolates resistant (rating score >0) to the test fungicides among the 12 collected from the four replicate lines of each selection regime. The test and scores are described in the legend to Fig **3**. (**b**) Heatmap of the phenotypic resistance profiles at the end of experimental evolution. The resistance rating scores (0-4; represented by the brown scale) are shown for each of the 12 isolates collected for each of the 13 selection regimes (4 replicate lines per regime; represented by the rainbow scale, as described in Fig **1**) and for each fungicide tested. The structure of the lines differed significantly between selection regimes (AMOVA; F_{st}=0.455, P<0.001). Alternation favored generalist phenotypes: the number of AIs to which a line is resistant (excluding tolnaftate) increased with the number of AIs used in the selection regime (χ^2=36.5; df=2; P=10^{-8}) and was also dependent, to a lesser extent, on alternation partner. Similarly, resistance to tolnaftate, an indicator of multidrug resistance in several pathogens, increased with the number of AIs used in the selection regime (χ^2=4.91; df=2; P=8 x 10^{-3}) and alternation rhythm (χ^2=3.85; df=2; P=0.022). The intensity of resistance, assessed with rating scores, was significantly affected by all components of alternation. In particular, alternations including the medium- and low-risk fungicides decreased the intensity of resistance (F=70.17; df=1,225; P=6 x 10^{-15} and F=57.06; df=1; P=10^{-12}, respectively). By contrast, alternation rhythm and the number of AIs used in the selection regime slightly increased the intensity of resistance (F=4.91; df=2; P=0.008 and F=3.85; df=2; P=0.02, respectively). (**c**) PCA on the phenotypic resistance profiles for all lines at the end of the experiment showed structuring according to independent AIs (not used for selection during the evolution experiment; Azo and Bosc) and according to the low-risk fungicide (P). CS: control solvent lines. **Supporting Information Figure S1: Variation of resistance evolution in selection regimes**

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


Mean of normalized Malthusian growth ρ
1.00
.75
.50
.25
0.00
ef
f
c
de
de
de
cd
cd
cd
a
ab
b
b
0B
0C
0P
1BC
2BC
3BC
1CP
2CP
3CP
1BP
2BP
3BP
1BCP
Selection regime

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

Relative gene expression (expressed as $2^{-\Delta\Delta Ct}$) of *tub2*, *sdhC*, *cyp51*, encoding the target proteins of carbendazim, benzovindiflupyr and prothioconazole-desthio, respectively, shown for eight isolates collected from various selection regimes. The expression of the drug transporter MFS1 was also quantified, as enhanced efflux is recognized as a generalist resistance mechanism in field isolates of *Z. tritici.* Isolates were chosen to represent the diversity of sensitivity the various fungicides, as shown from their scores in droplet tests (shades of brown, on the top of each graph). The ancestral strain IPO-323 was added as negative control. Two biological replicates were tested independently. The expression of *tub2*, *cyp51* and *mfs1* was normalized to that of the genes encoding EF1α (black bars) and ubiquitin (light grey bars). The expression of *sdhC* was normalized to that of the gene encoding β-tubulin (grey bars).
The overexpression of the three target genes was not observed among isolates resistant to B, C or P. The overexpression of *msf1* is noticed for few isolates exhibiting moderate to high resistance to tolnaftate, suggesting that other unknown additional mechanisms must be involved.


tub2
4 4 4 0 4 4 4 0 0
sdhC
3 3 3 1 2 1 1 0 0
cyp51
2 3 4 1 3 4 2 0 0
mfs1
3 4 0 0 0 3 2 0 0
2-ΔΔCt
0
10
20
30
40
50
0C_I_12_A_a
0C_II_12_A_b
1BC_I_12_C_a
1BCP_I_12_C_b
1CP_IV_12_A_a
2BC_I_12_B_a
3CP_IV_12_C_b
CS_I_12_A_a
IPO323
0B_I_12_A_b
0B_II_12_A_a
2BP_I_12_A_a
3BP_IV_12_C_b
0P_I_12_A_a
0P_II_8_C_b
2BP_I_12_A_a
Strains

Supporting Information Methods S1: Statistical programs used for data analysis

All analyses were performed and all figures were generated with R 4.0.4 and R Studio 1.4.1106.
R Core Team. *R: A Language and Environment for Statistical Computing*. (R Foundation for Statistical Computing, 2021).
RStudio Team. *RStudio: Integrated Development Environment for R*. (RStudio, PBC, 2021).

The additional packages used for this work are listed below.

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

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

1. **Primers used to sequence fungicide target genes and to detect alterations of the *MFS1* promoter in *Z. tritici***

Primer name	**Primer sequence**	**Tm** (°C)	**Reference**
Tub2			
Tub5	CAGTCACTTGCAAACGGGTA (F)	63.7	This work
Tub14	GAAGCCTCCTGGTACTGCTG (R)	63.9	-
Tub15	GTCGACCAGGTTCTCGATGT (F)	64.1	-
Tub13	AACCGACCATGAAGAAGTGG (R)	63.8	-
cyp51			
CypFra2r	TCCTTCTCCTCCCTCCTCTC (R)	64.0	Brunner *et al* (2008)
EBI112	TTCAGCACGCTCGCCATCCTCC (F)	76.5	Brunner *et al* (2008)
Cyp9	TCTATACTGCCAGCCGATCA (R)	63.3	This work
Cyp12	CACTAACAGGCCAACTGCAA (F)	63.8	-
NB1	CACTCTTCATCTGCGACCGAGTC (R)	69.4	Leroux *et al* (2007)
sdhB			
Mg SDHB	TTCGGCACGTCGAGTTTCTGGCCATGCTGGCGG (F)	89.2	This work
Mg SDHB Rv	CCACAATGCCGCAAAATCGGCACAACTGCTCCC (R)	86.1	-
Mg SeqSDHB Fw	ACATCTACCGATGGAACCC (F)	61.2	-
Mg SeqSDHB Rv	TGTGGCATCGGTACAAGC (R)	63.5	-
sdhC			
Mg SDHC Fw	TCTCTTCGTCGACATCACCA (F)	64.7	This work
Mg SDHC Rv	ACGCACTCGCAACACTCA (R)	64.7	-
sdhD			
Mg SDHD Fw	ACAGCTTCCGAGGTTTCGCG (F)	70.8	This work
Mg SDHD Rv	GCCATCTGTATATACACGCCC (R)	63.0	-

Brunner, P. C., et al. (2008). "Evolution of the CYP51 gene in *Mycosphaerella graminicola*: evidence for intragenic recombination and selective replacement." Molecular Plant Pathology **9**(3): 305-316.
Leroux, P., et al. (2007). "Mutations in the CYP51 gene correlated with changes in sensitivity to sterol 14 alpha-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*." Pest Management Science **63**(7): 688-698.

2. **Regular PCR Mix**

Mix	1 tube (µL)
DNA	1
dNTP (25mM)	0.5
FW (10 µM)	1
RV (10 µM)	1
Titanium buffer (10X)	5

Titanium *Taq* polymerase (50X)	0.5
Deionized H_2O	41
Total volume	50

DNA polymerase: Titanium *Taq* (Takara Bio)

3. Regular PCR program

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

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

1. **Primers used to detect length polymorphism in the promotor sequence of fungicide target genes in *Z. tritici***

Primer name	**Primer sequence**	**Tm** (°C)	**Reference**
tub2			
Zt_tub2_prom_F1 Zt_tub2_prom_R1	ATTGCTGTGCTTGTGTTCGA (F) CGACATACCCGTTTGCAAGT (R)	55.2 57.3	This work -
cyp51			
Mg51proF MgCyp51-5'-1r	GTGGCGAGGGCTTGACTAC (F) CAAGTTTCCAGAGGCTGGTC (R)	61.0 59.4	Chassot *et al,* 2008 -
sdhC			
Zt_sdhC_prom_F1 Zt_sdhC_prom_R1	GACCGGTCGAATGCTTGC (F) GGTGAGCTTCTGTGCCAAC (R)	58.2 58.8	This work -
mfs1			
MFS1_consensus_2 MFS1_consensus_4	GCAAGGATTCGGACTTGACG (F) CTGCCGGTATCGTCGATGAC (R)	66.9 67.8	Omrane *et al,* 2017 -

Chassot, C., et al. (2008). Sensitivity of *Cyp51* genotypes to DMI fungicides in *Mycosphaerella graminicola*. Modern Fungicides and Antifungals compounds V. G. U. Dehne DW, Kuck KH, Russell PE and Lyr H. Braunschweig, Germany, DPG Seebsterverlag**:** 129-136.
Omrane, S., et al. (2017). "Plasticity of the *MFS1* promoter leads to multidrug resistance in the wheat pathogen *Zymoseptoria tritici*." mSphere **2**(5).

2. **Regular PCR Mix**

Mix	1 tube (µL)
DNA	1
dNTP (25mM)	0.24
Primer FW (10 µM)	0.5
Primer RV (10 µM)	0.5
Titanium buffer (10X)	2.5
Titanium *Taq* polymerase (50X)	0.3
Deionized H_2O	19.96
Total volume	25

DNA polymerase: Titanium *Taq* (Takara Bio)

3. Regular PCR program

For *cyp51, sdhC* and *mfs1*

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

For *tub2*

Initiation	95°C	3 min	
Denaturation	95°C	30 s	15cycles
	68°C	1mn	
Denaturation	95°C	30 s	35 cycles
Annealing	Tm°C	30 s	
Elongation	72°C	1min	
Final	72°C	5 min	

Supporting Information Methods S4: Quantifying the expression of *tub2*, *cyp51*, *sdhC*, *mfs1* in *Z. tritici* by qRT-PCR

1. **Origin of tested isolates**

Isolate	Selection regime	Tested genes
IPO323	None (ancestral strain)	*tub2*, *cyp51*, *sdhC* and *mfs1*
CS_I_12_A_a	Control with 0.5% ethanol as solvent	*tub2*, *cyp51*, *sdhC* and *mfs1*
0C_I_12_A_a	0C	*tub2*
0C_II_12_A_b	0C	*tub2*
0B_I_12_A_b	0B	*sdhC*
0B_II_12_A_a	0B	*sdhC*
0P_I_12_A_a	0P	*cyp51*
0P_II_6_C_b	0P	*cyp51*
1CP_IV_12_A_a	1CP	*tub2, cyp51* and *mfs1*
3CP_IV_12_C_b	3CP	*tub2, cyp51* and *mfs1*
1BC_I_12_C_a	1BC	*tub2, sdhC* and *mfs1*
2BC_I_12_B_a	2BC	*tub2, sdhC* and *mfs1*
2BP_I_12_A_a	2BP	*cyp51, sdhC* and *mfs1*
3BP_IV_12_C_b	3BP	*cyp51, sdhC* and *mfs1*
1BCP_I_12_C_b	1BCP	*tub2, cyp51, sdhC* and *mfs1*

2. **Primers used in qPCR**

Primer name	Primer sequence (5'-3')	Reference	Use
β-tubulin_Fw β-tubulin_Rv	AACGAGGCTCTCTACGACATCTG GGCGGAGACGAGGTGGTTG	Omrane *et al*, 2017	Expression of *tub2*, encoding β-tubulin
CYP51_Fw CYP51_Rv	TTCTCTTCCGTGGCAAGTTGTC GCCGTATGTGATGGTGCTTCC	S. Patry-Leclaire, unpublished	Expression of *cyp51*, encoding sterol 14α-demethylase
MFS1_F MFS1_R	CAATGGGGCGGCAGCAAATAC TTGGATGGTGGCGAAGATGAGG	Omrane *et al*, 2015	Expression of *mfs1,* encoding a MFS drug transporter
UBC_Fw UBC_RV	GTCTGCGGACCACAATACC CGACCTTTCCTTGCCTCTG	Omrane *et al*, 2017	Ubiquitine housekeeping gene

EF1α_Fw	AAGATTGGTGGTATCGGAACAG	Omrane *et al*, 2017	Elongation factor EF1α housekeeping gene
EF1α_Rv	GACTTGACTTCGGTGGTGAC		
qPCR_SDHC1_fwd	GGCAACAACTCCTTCCAACAG	Steinhauer *et al,* 2019	Expression of *sdhC*, encoding the C subunit of the succinate dehydrogenase
qPCR_SDHC1_rev	ACCAGGTTATTTGCGGTTTGT		
qPCR_SDHC1_probe (CY5/BHQ2)	CCAAGTAACAGCAGCCGCCGTCT CCGAATC		
qPCR_TUB1_fwd	AGCGCATGAATGTCTACTTCAA	Steinhauer *et al,* 2019	β-tubulin housekeeping gene (for *sdhC* only)
qPCR_TUB1_rev	CCTTGGCCCAGTTGTTTCC		
qPCR_TUB1_probe (HEX / BHQ1)	TCGGTCAGCTCTTCCGCCCAGA		

EF1α and *UBC* were used as reference genes to quantify the expression of *tub2, cyp51* and *mfs1. tub2* was used as a reference gene to quantify the expression of *sdhc.*

Steinhauer, D., et al. (2019). "A dispensable paralog of succinate dehydrogenase subunit C mediates standing resistance towards a subclass of SDHI fungicides in *Zymoseptoria tritici*." PLOS Pathogens **15**(12): e1007780.Omrane, S., et al. (2015). "Fungicide efflux and the MgMFS1 transporter contribute to the multidrug resistance phenotype in *Zymoseptoria tritici* field isolates." Environmental Microbiology **17**(8): 2805-2823.
Omrane, S., et al. (2017). "Plasticity of the *MFS1* promoter leads to multidrug resistance in the wheat pathogen *Zymoseptoria tritici*." mSphere **2**(5).

3. Protocols

Isolates were grown in the dark at 18°C, with a RH of 70% and shaking at 150 rpm for four days, in 50 mL borosilicate Erlenmeyer flasks containing 25 mL YPD medium and no fungicide. Two independent biological repeats, grown successively, were produced for each isolate. Fungal cells were freeze-dried and RNAs were extracted using a commercial kit (Monarch total RNA Miniprep; New England Biolabs) and the manufacturer's recommendations. Retrotranscription was achieved using the protoScript II First Strand cDNA Synthesis kit (New England BioLabs) according to the manufacturer's protocol. qPCRs were achieved as follows, with three technical repeats for each sample:

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

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

Regular qPCR program for *tub2, cyp51, mfs1, EF1α* and *UBC*

95°C	5 min	
95°C	15 s	45 cycles
60°C	1min	

Regular qPCR program for *sdhC*

95°C	3 min	
95°C	10 s	45 cycles
60°C	20 s	

4. Determination of the relative gene expression, $2^{-\Delta\Delta Ct}$

First, ΔCt values were calculated as:

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

with Ct the value of cycle threshold and i each isolate tested.

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

$$\Delta\Delta \mathrm{Ct} = \Delta \mathrm{Ct_i} - \Delta \mathrm{Ct_{IPO323}}$$