I alternate therefore I generalize: how the intrinsic resistance risk of fungicides counterbalances their durability

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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 evolution of resistance, relative to continuous fungicide use, but results in higher levels of generalism in evolved lines. We demonstrate that the relative risk of resistance intrinsic to fungicide alternation probably underlies a trade-off between the number of fungicides and the frequency 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: experimental evolution; fungicide resistance; selection drivers, generalism, ecological specialization, environmental variation, temporal heterogeneity

Introduction

Human societies face a number of crucial challenges, including obtaining sufficient feed, food, fuel, and fiber, and treating infectious diseases. The protection of crops, and of animal and human health still relies heavily on the use of pesticides and drugs to control diverse microbes and pests. However, the efficacy of these agents has been compromised by the generalization of their use, leading to the rapid and widespread evolution of resistance [1, 2, 3]. Resistance can be seen as phenotype optimization in response to the selection imposed by pesticides and drugs, and has provided many examples of contemporary adaptive evolution[4]. The costs engendered by the evolution of resistance in pathogens, arthropods and weeds amount to billions of US dollars each year[5, 6], and the development of resistance may lead to the loss of millions of lives[7, 8], and entail hidden costs, due to greater pesticide use[9] and impacts on biodiversity[10, 11]. The use of smart strategies for preventing and delaying the evolution of resistance is, thus, crucial.

Diverse agents, with different modes of action, are often available for controlling pests and pathogens. Strategies can therefore be developed in which these agents are skilfully deployed over space and time so as to limit the evolution of resistance[12, 13]. More precisely, the optimization of sequences, mixtures, alternation, mosaic or dose strategies for pesticides (and, indeed, for drugs in the medical context) involves maximizing the heterogeneity of the selection pressure exerted on pathogen and pest populations[14, 15, 16, 17, 18]. The alternation (also known as cycling, rotation, or periodic application) of unrelated active ingredients (AIs) was first proposed by Coyne in 1951[19] and provides multiple means of intergenerational killing, maximizing the probability of killing the offspring of resistant individuals[13]. The efficacy of alternation for preventing resistance remains a matter of debate, as discrepancies between modeling and empirical data have been noted, for control strategies for insects, weeds and pathogens[13, 20, 21, 22]. The biological traits of the organisms concerned may partly account for this divergence, as these traits were not systematically made explicit in the mathematical models, and different numbers of generations were considered in the 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 identity 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 or patients, time to reach a certain amount of resistance, effective life of the pesticide or drug). 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[23, 24]. Nevertheless, the diversity of resistance mechanisms observed in microbes, animals and plants is considerable. The evolutionary origins of these mechanisms may also differ[25]. Target site resistance (TSR) affects the structure and/or expression of the target of the pesticide or drug, resulting in cross-resistance to AIs with the same mode of action. This type of resistance is considered to be a specialist adaptation. 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 modes of action and is usually considered a generalist trait[26, 27, 28, 29]. Theory, with support from experimental evolution experiments, suggests that specialists evolve in homogeneous environments, whereas generalists are more likely to evolve in heterogeneous habitats[30]. Thus, in terms of adaptation to pesticides and drugs, we would expect generalist resistance to be more readily selected with increasing heterogeneity of selection pressure, for any given strategy. This hypothesis has been validated for herbicide mixtures, for which increasing use 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[31]. In the clinical

environment, generalist resistance has also been shown to be more frequently selected in situations in which antibiotics are alternated[32]. Overall, these studies highlight the importance of treatment frequency relative to the pathogen life cycle[33, 34]. 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[35, 36] — to determine how the components of fungicide alternation drive the quantitative and qualitative performance of this strategy. *Z. tritici* has evolved resistance in the field to four different unisite modes of action in Western Europe, resulting in a decrease in field efficacy depending on the strategy deployed, with contrasting resistance dynamics in France[37, 38, 39, 40, 41, 42]. The resistance of *Z. tritici* is determined by various specific target-site mutations, but also by overexpression [43, 44, 45, 46] or enhanced efflux, a generalist mechanism causing weak multidrug resistance (MDR) when not associated to TSR and affecting at least three modes of action[44, 47]. We made use of the haploid yeast-like easily-culturable form of this pathogen[48], in an experimental evolution approach[49] 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 interplay between alternation components clearly guided the evolutionary trade-off between the qualitative and quantitative 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 evolution of resistance

We observed the dynamics of *Z. tritici* resistance to benzovindiflupyr (B), carbendazim (C) and prothioconazole-desthio (P), here representing 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 global resistance evolution rate ρ) was used as a proxy for increasing resistance. Resistance evolved in all populations continuously exposed to B, C or P, and was generalized after three, six and six cycles, respectively. Resistance also evolved in lines treated with these three fungicides in alternation, regardless of the alternation rhythm and the number of fungicides alternated, although resistance never generalized, even after 12 cycles, in some situations (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 reflecting the hierarchy of the relative intrinsic risks of resistance associated with benzimidazoles (high; C), SDHIs (medium; B) and DMIs (low; P) (SI 3). The global 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 carbendazim to a level similar to that observed for medium- and low-risk fungicides in conditions of continuous exposure. The benefits of alternation 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 decrease the evolution of resistance relative to single fungicides in continuous use.

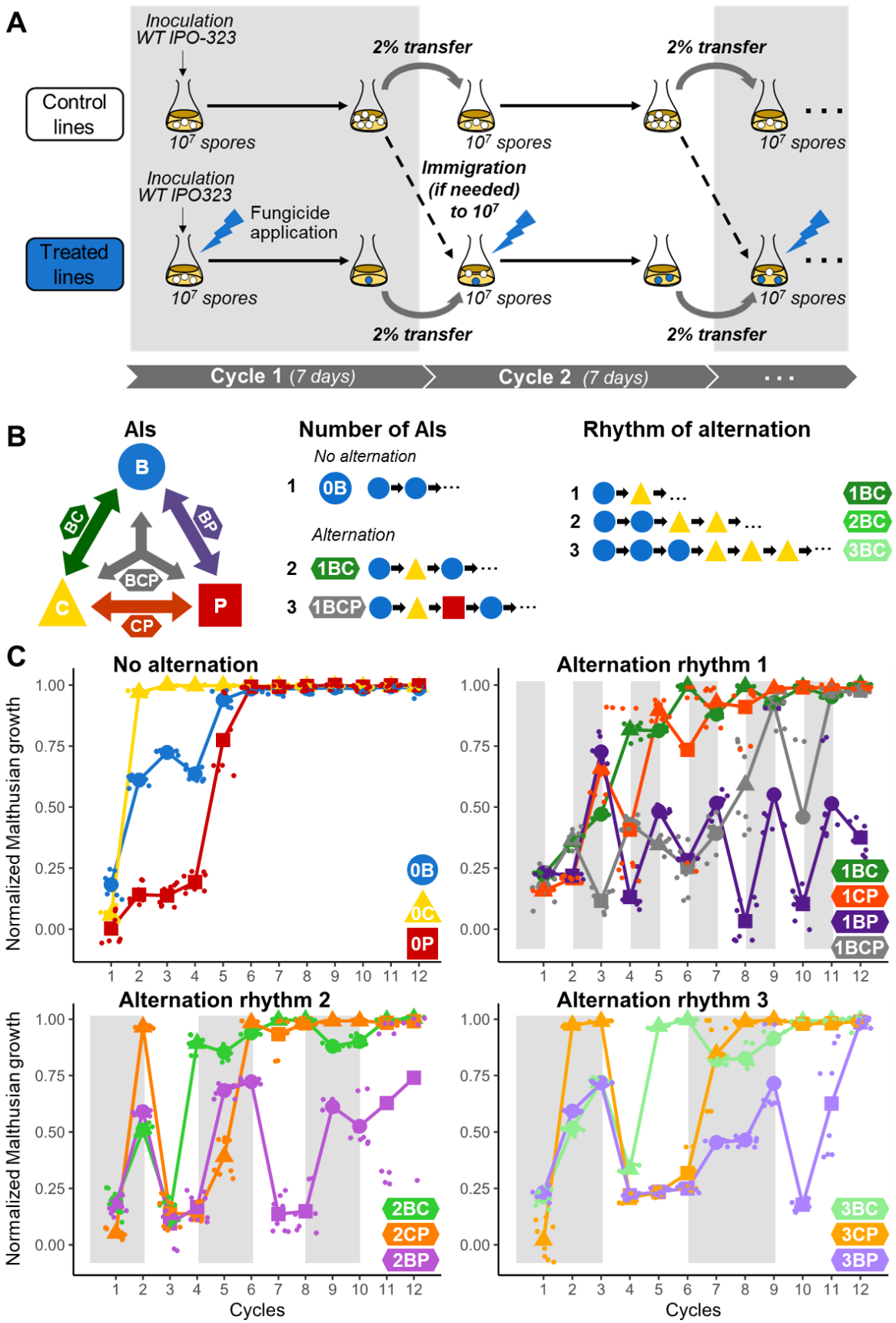

Figure 1. Experimental evolution of fungicide resistance under treatments with contrasting temporal heterogeneities. (**A**) The sensitive strain IPO-323 of Z. *tritici* was used to found 56 lines, with each Erlenmeyer flask inoculated with 10^7 spores. Fungicides were added to the treated lines to mimic 14 patterns of selection.

(Fig. 1 continued) 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 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 generalized within six cycles in lines with continuous exposure, whereas generalization in lines subjected to alternating exposure was either delayed or had not yet been reached after 12 cycles (1BP, 2BP).

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 global 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), and by alternation component (Fig. 2A). The impact of alternation on the rate of selection of resistance to a given fungicide 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 rate of evolution of resistance to a particular fungicide 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 dynamics, 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%) and a generalization phase (here, over 90%)[16]. 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 Malthusian growth (Fig. 2B). Alternation decreased the resistance evolution rate of the high-risk fungicide mostly towards the end of the selection phase and during the generalization phase, rather than during the early phases (delaying the generalization 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 generalization phase was observed only in the

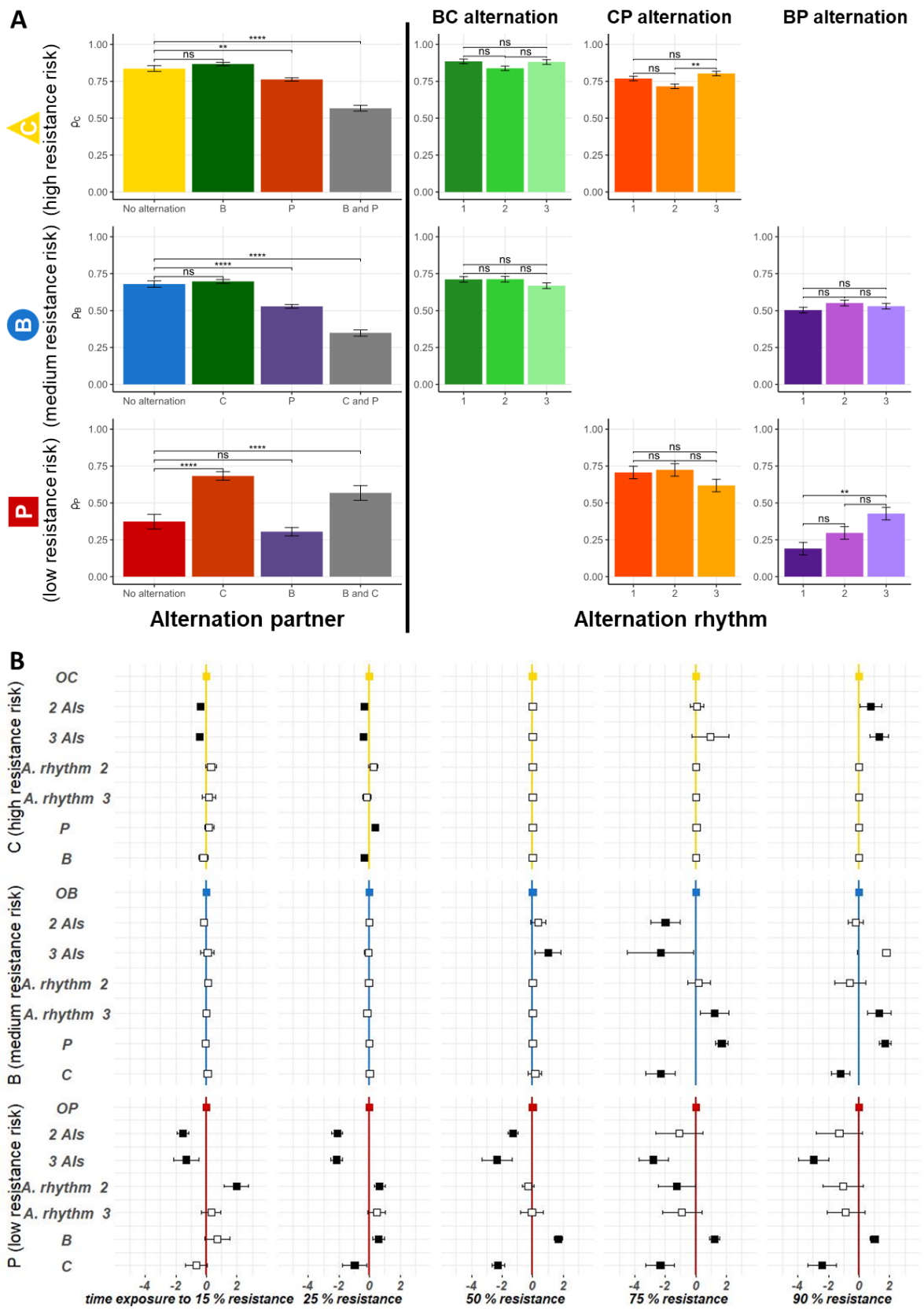

Figure 2. Effects of alternation components on the evolution of resistance. (A) ρ_f, the mean rate of resistance evolution for time of exposure to fungicide *f,* is shown as a function of alternating AIs on the left, and for each alternation pair, as a function of alternation rhythm on the right. The error bars represent the standard error. *P*-values for pairwise comparisons were calculated with linear models (Tukey's *post-hoc* correction). "ns"

(Fig. 2 continued) 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 generalization 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.

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 generalization 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[50], *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 structure 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[44].

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. The profiles of strains evolving under direct selection regimes were generally narrower and less diverse than those of strains evolving under alternation regimes (Fig. 4A and 4B). 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 long alternation rhythm.

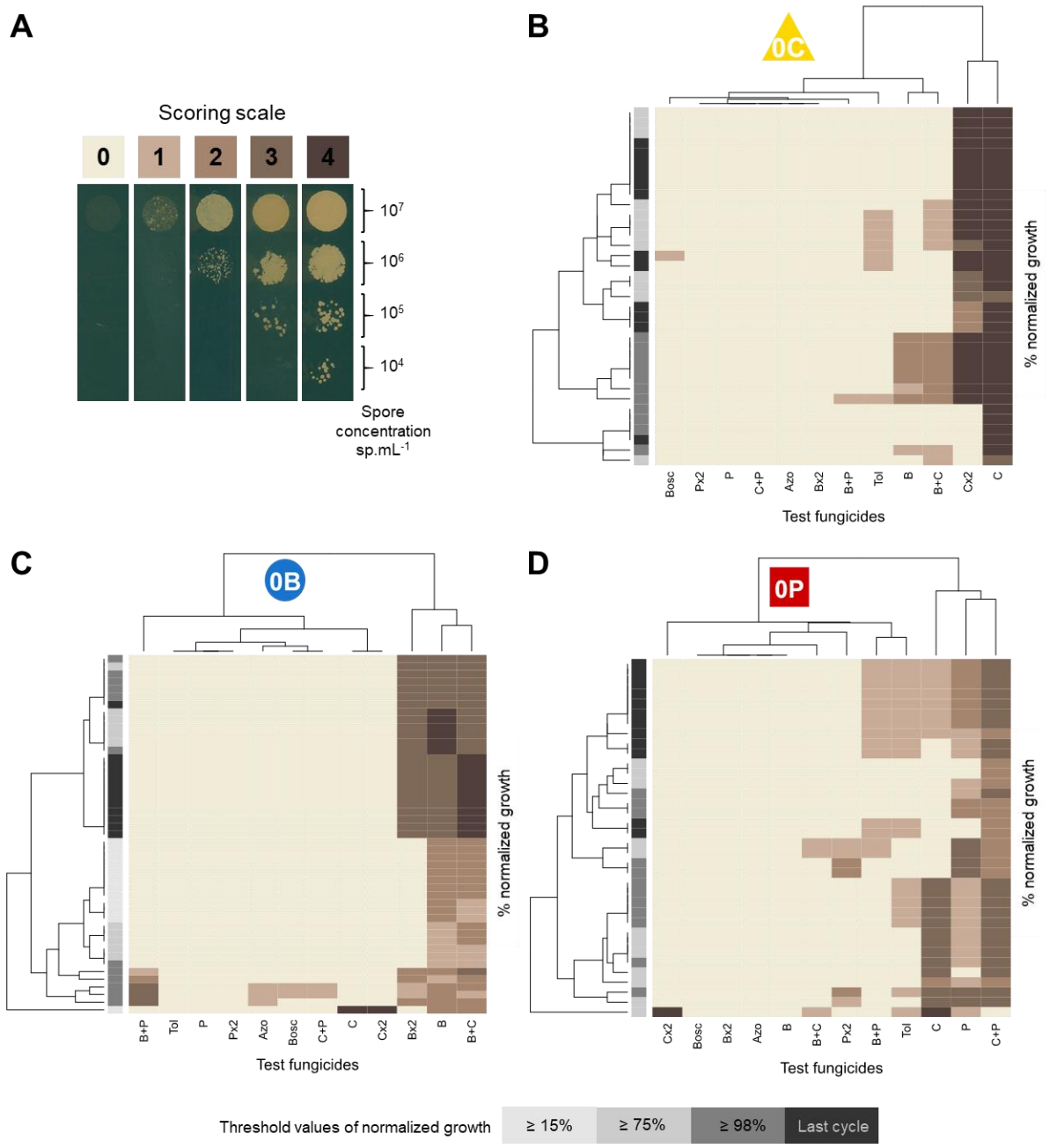

Figure 3. Evolution of phenotype resistance profiles during experimental evolution. (**A**) Scoring scale used to assess the growth of isolates on solid medium supplemented with discriminatory doses of fungicides (droplet tests). For each isolate, four spore concentrations were tested on the same plate. 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; $0.273<F_{st}<0.453$; $P<0.001$).

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[51, 52]. No other change was recorded relative to the 330 full-gene sequences established for *tub2, cyp51, sdhB, sdhC* or *sdhD.* 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*[44]. In field strains, it is determined by insertions in the promoter region of the MFS1 transporter gene[53]. Systematic genotyping of the 249 isolates collected in this experiment revealed no changes in the length of the *mfs1* promoter, particularly in strains displaying tolnaftate resistance. Overall, these findings suggest that alternation components favor generalist phenotypes, with enhanced efflux due to unknown transporters and/or mutations more 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[49, 54, 55]. Here, we used an experimental evolution approach and the economically important wheat pathogen *Z. tritici* 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 [13, 20] and mediate generalism in evolved individuals[30, 56], as previously demonstrated for weeds, insects and bacteria[15, 32, 57, 58, 59, 60].

Specifically, using three different fungicides, we found that alternation was either neutral or beneficial in terms of delaying global resistance, 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, and fitness cost may help to decrease the frequency of resistance during the time window in which the fungicide is not used[24]. However, this would suggest that resistance evolution rate due to exposure to a given fungicide (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 fungicide resistance risk, 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 resistance evolution rate of a fungicide, depending on the intrinsic risk of resistance for the added AI relative to those already present in the alternation. Finally, the temporal heterogeneity of environments may

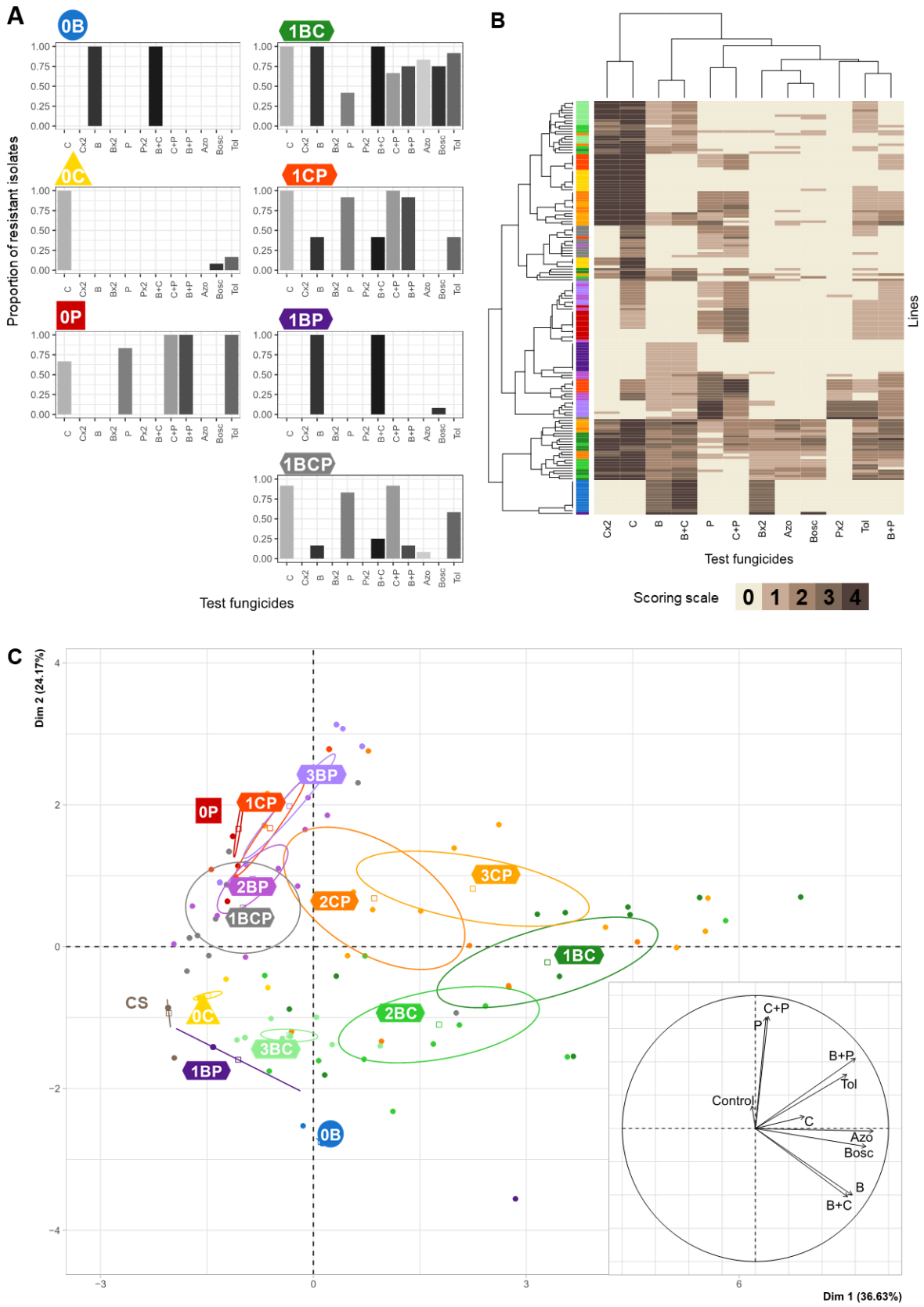

Figure 4. Phenotypic resistance profiles selected at the end of experimental evolution. (**A**) The average phenotypic resistance profile obtained with the different selection regimes (*i.e.* after continuous or alternating exposure; alternation rhythm = 1 cycle) at the end of the experiment. Diagrams show the proportion of isolates

(Fig. 4 continued) 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.

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[61, 62]. 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.

We confirm here, for the first time 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 consistent with the continual change in population structure, 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[63]. 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[64]. Combining the respective paths associated with each AI, 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[32, 65]. 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, with possibly additivity or synergy, 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. 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, which may also operate *in natura*, without our suspecting it and which may, therefore, have an overlooked impact on resistant traits[66, 67, 68]. 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[69]. 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 stabilization phase of the dynamics of resistance to the high-risk fungicide, suggesting a higher relative fitness cost in the final stages of adaptation than for the other fungicides. 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, possibly because the high-risk fungicide was more efficient at controlling mutations with a low resistance intensity.

Finally, experimental evolution has proposed a simplified model for evolution *in natura* [55]. 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[70]. Gene flow is also expected to occur between evolving metapopulations. 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. 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. We would also argue that resistance management *via* temporal heterogeneity is possible provided that the environmental grain is appropriate[62]. 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[24], to control emerging mutations as efficiently as possibly, 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. 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[71].

Methods

Ancestral population, culture conditions and assessment

We used IPO-323, as an ancestral isolate, to found all lines. 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 defrosted 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. 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% RH 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[72].

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 x 10^5 sp.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 sp.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. 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_{450} 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 active ingredients (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[73]. 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 sp.mL^{-1} and each Erlenmeyer flask was inoculated with 10^7 spores (500 µL). The resulting starting concentration of 4 x 10^5spores.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 lines were not treated with

fungicides. Control solvent lines received 0.5% solvent, whereas treated lines were treated as shown in Fig. 1B, giving rise to 56 independent lines.

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 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 ("global 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 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 SI 1.

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% (generalization of 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*[44]. 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[74]. AMOVA is generally used for molecular data. Here, we assumed that different scores for a particular test corresponded to different 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 SI 1.

Genotyping of evolved isolates

One strain for each resistance phenotype profile was selected at random and kept for MFS1 genotyping. Insertions into the promoter of this gene have been shown to cause MDR in field isolates of *Z. tritici.* DNA was isolated and amplicon size after PCR for this gene was systematically determined for these 249 isolates, to check for any MFS1 promoter alteration, with previously described protocols and reference strains[53].

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[37, 39, 42, 43]. 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 SI 2, 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.

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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

CD and PD have no competing interests to declare. AB contributed as part of a PhD studentship partly funded by Syngenta, supervised by ASW, FC and AD.

Supplementary Information 1:

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).

Supplementary Information 2:

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	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	-
mfs1			
MFS1_consensus_2	GCAAGGATTCGGACTTGACG (F)	66.9	Omrane *et al* (2017)
MFS1_consensus_4	CTGCCGGTATCGTCGATGAC (R)	67.8	-

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

2. Regular PCR Mix

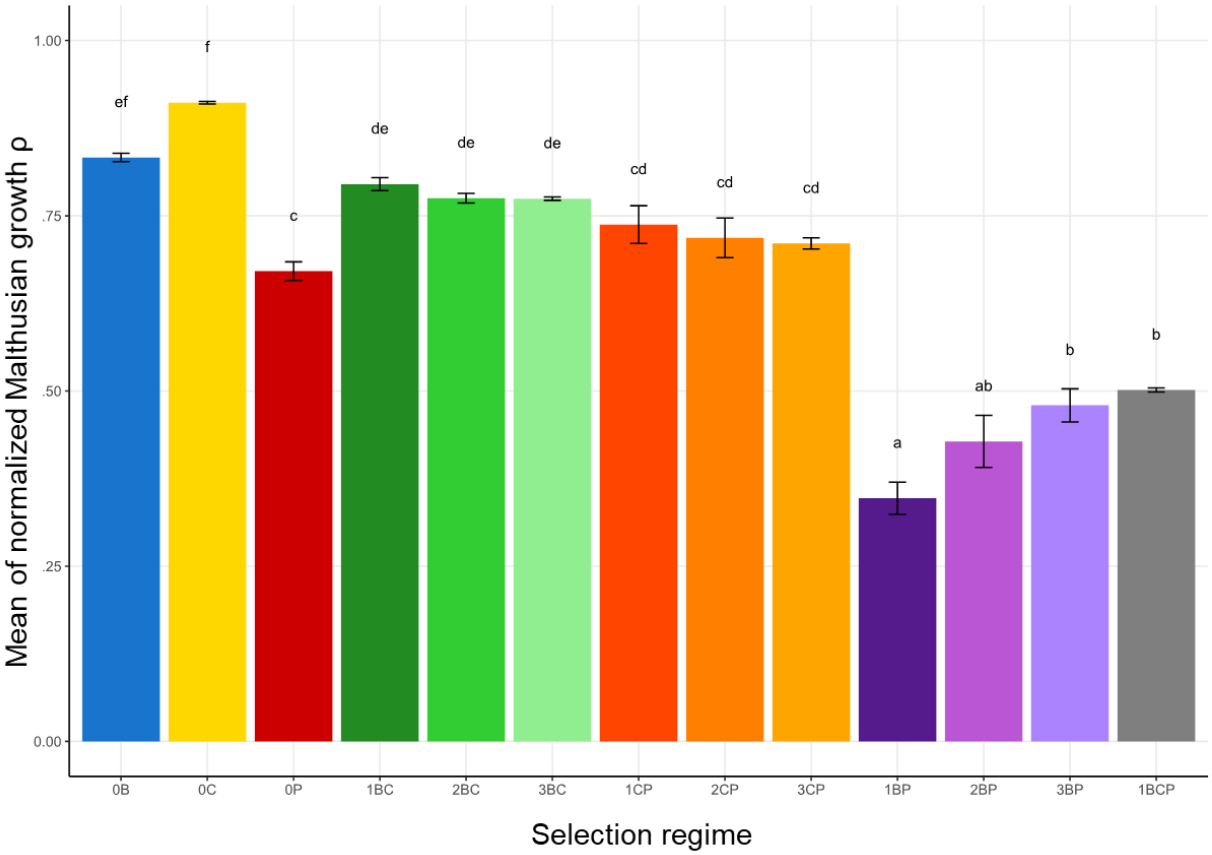
Mix	1 tube (µL)
DNA	1
dNTP (25mM)	0.5
FW (10 µM)	1
RV (10 µM)	1
Titanium buffer (10X)	5
Titanium *Taq* polymerase (50X)	0.5
Deionized H_2O	41
Total volume	50

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	

Supplementary Information 3:

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.