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# Évaluation des stratégies de gestion des résistances aux fongicides par une approche d'évolution expérimentale : le cas de *Zymoseptoria tritici*, agent causal de la septoriose

Agathe Ballu

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**Evaluating fungicide resistance management  
strategies by means of experimental evolution:  
the case of *Zymoseptoria tritici*, the causal agent  
of Septoria leaf blotch**

Évaluation des stratégies de gestion des résistances aux  
fongicides par une approche d'évolution expérimentale :  
le cas de *Zymoseptoria tritici*, agent causal de la septoriose

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à Paris-Saclay, le 17 septembre 2021, par**

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A Manon, pour m'avoir insufflé une partie de ta curiosité, ta naïveté et ton émerveillement  
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*« La curiosité mène à tout : parfois à écouter aux portes, parfois à découvrir l'Amérique », et ...  
parfois à faire une thèse.*

José Maria Eça de Queiros





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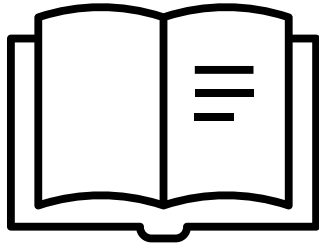
## ABBREVIATIONS

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<b>Acronym</b>	<b>Description</b>
<b>AI</b>	Active Ingredient
<b>AOX</b>	Alternative Oxidase
<b>B</b>	benzovindiflupyr
<b>C</b>	carbendazim
<b>DMI</b>	Sterol 14 $\alpha$ -DeMethylation Inhibitor
<b>Dpi</b>	Day post Inoculation
<b>DRC</b>	Dose-Response Curve
<b>DST</b>	Decision Support Tool
<b>DTH</b>	Degree of Treatment Heterogeneity
<b>EC<sub>x</sub></b>	Effective Concentration that induces x% of the maximal response
<b>EE</b>	Experimental Evolution
<b>EPPO</b>	European and Mediterranean Plant Protection Organization
<b>EU</b>	European Union
<b>FRAC</b>	Fungicide Resistance Action Committee
<b>HGT</b>	Horizontal Gene Transfer
<b>INRAE</b>	Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement
<b>IPM</b>	Integrated Pest Management
<b>MBC</b>	Methyl Benzimidazoles Carbamates
<b>MDR</b>	MultiDrug Resistance
<b>MIC</b>	Minimum Inhibitory Concentration
<b>MoA</b>	Mode of Action
<b>NTSR</b>	Non-Target Site Resistance
<b>OD</b>	Optical Density
<b>PPP</b>	Plant Protection Products
<b>P</b>	prothioconazole-desthio
<b>QiI</b>	Quinone Inside Inhibitor of cytochrome b
<b>QoI</b>	Quinone Outside Inhibitor of cytochrome b
<b>R4P</b>	Research and Reflection Ring on Pesticide Resistance
<b>RRA</b>	Resistance Risk Assessment
<b>RF = RL</b>	Resistance Factor = Resistance Level
<b>Rpm</b>	Revolution Per Minute
<b>SBI</b>	Sterol Biosynthesis Inhibitor
<b>SDH</b>	Succinate Dehydrogenase
<b>SDHI</b>	Succinate DeHydrogenase Inhibitor
<b>SGV</b>	Standing Genetic Variation
<b>SHA-SDHIs</b>	Stretched Heterocycle Amide SDHIs
<b>STB</b>	Septoria tritici blotch
<b>TSR</b>	Target Site Resistance
<b>WHO</b>	World Health Organization

# **Part I.**

## **Introduction**



# Introduction

## 1 Resistance as a global concern

### 1.1 Resistance provides pathogens the capacity to escape pesticides or drugs

Pesticides and drugs are active ingredients (AIs) used in agriculture and human or veterinary medicine that kill or prevent the growth or the reproduction of target organisms (either plants, animals, bacteria, fungi, or virus) (European Commission, 2016). Pesticides and drugs often disturb (inhibit or mimic) a protein active in an essential biological pathway (Gould et al., 2018). Those AIs can be of mineral, chemical (natural extract or synthetic compound), or of biological origin (generally, whole microbial organisms).

#### 1.1.1 Definitions

*Biological resistance* is the capacity of an individual to survive a pesticide or drug treatment correctly applied that would normally kill it, and to transfer this ability to a viable offspring. Biological resistance is heritable and results from intraspecific polymorphism whereby one or several genetic changes (mutations, insertions, deletions, TE mobilization; see § 1.1.2.3) confer reduced susceptibility. This acquired trait is advantageous in the specific environment where the fungicide is applied (Chapman, 1998; Cloete, 2003; European Commission. Directorate-General for Health and Consumers, 2010; Poole, 2002; R4P Network; Russell, 2003; Tabashnik et al., 2014). Resistance is defined as *monogenic* if the genomic change(s) affect(s) only one gene and *polygenic* if several genes are involved. From an evolutionary point of view, *acquired biological resistance* is the adaptive response to the introduction of pesticides and drugs in the environment (Jansen et al., 2013). It is then the phenotypic optimization of an individual in response to the selection pressure exerted by pesticides or drugs (Orr, 2005). Some biological characteristics of the organisms may favour resistance and then increase the adaptive potential of a species. In particular, pathogen adaptation is favoured by specific biological traits such as a high mutation capacity due to short generation time and large effective



population size, high gene flow and multiple-scale migration (Croll and McDonald, 2012; Jackson et al., 2011; McDonald and Linde, 2002; McDonald and Stukenbrock, 2016). Pathogens, and fungi in particular, usually display several modes of reproduction (sexual, asexual or parasexuality) which may favour the combination of advantageous alleles and their amplification in populations. Their genome is often highly plastic due to the intense dynamics of transposable elements, integrons or accessory chromosomes. Such genome architecture may allow them to adapt quickly, for example to pesticide or drug (Álvarez et al., 2020; Bennett, 2004; Bertazzoni et al., 2018; Capy, 1998; Croll and McDonald, 2012; Tsushima et al., 2019).

*Resistance in practice* or *field resistance* occurs when a decrease in pesticide or drug treatment efficiency is observed in field or clinical situations. It requires that the frequency of individuals carrying biological resistance reaches a certain threshold (Figure 1). For plant pathogens in particular, biological resistance may not systematically imply field resistance, depending on resistance intensity (resistance factors; developed later) and on the frequency of resistant variants in populations.

Acquired biological resistance must not be assimilated to *natural resistance* (also referred to intrinsic resistance or natural insensibility), which is the innate ability of a pathogen to escape from a drug or pesticide action due to its inherent biological characteristics (structural or functional) (Fajardo et al., 2008; Lucas et al., 2015). For example, penicillin is an antibiotic targeting the peptidoglycans. Gram-positive bacteria possess high levels of this polymer in their membrane while gram-negative bacteria have lower levels that are surrounded by lipopolysaccharides (LPS). Consequently, without any adaptation, most of the gram-negative bacteria are intrinsically less susceptible to penicillin than gram-positive bacteria (Breijyeh et al., 2020; Salton and Kim, 1996; Wright, 1999). Among plant pathogens, *Tapesia acuformis*, causing eyespot in cereals is naturally resistant to azole fungicides, whereas its sister species *T. yallundae* is fully susceptible (Albertini et al., 2003). Another example is *Botrytis pseudocinerea* that is naturally resistant to the sterol biosynthesis inhibitor fenhexamid, whereas *B. cinerea* acquired resistance to this fungicide after its introduction (Walker et al., 2011). This natural resistance, pre-existing the introduction of fenhexamid in the field, is associated with the metabolization of fenhexamid in *B. pseudocinerea* only (Billard et al., 2011). Natural resistance may define the range of efficacy

of pesticide and drugs prior to their use. Where species complexes are frequent, natural resistance may also explain why pesticide or drug treatments do not control a fraction of the heterogeneous population (Coleman, 2016; Paudel et al., 2020; Turner and Butler, 2014).

### 1.1.2 Resistance as an adaptive process to the selection pressure of pesticides and drugs

Resistance evolution is a dynamic process, reflecting the evolution of the frequency of advantageous variants in populations, ultimately leading to resistance in practice. The dynamics of resistant variants is generally considered to take place in three phases. When a resistant allele arises, it may spread in the population to the point it is unlikely to die out by chance (van den Bosch et al., 2011). This is the emergence phase. Most of the resistant mutations that appear will not reach this threshold, for example because of genetic drift or a too high fitness cost. The selection phase refers to the further increase in frequency of this variant, which generally follows a sigmoid curve. The stabilization phase is characterized by a low rate of resistance increase and a high frequency of resistant variant. Resistance can ultimately be generalized (Figure 1).

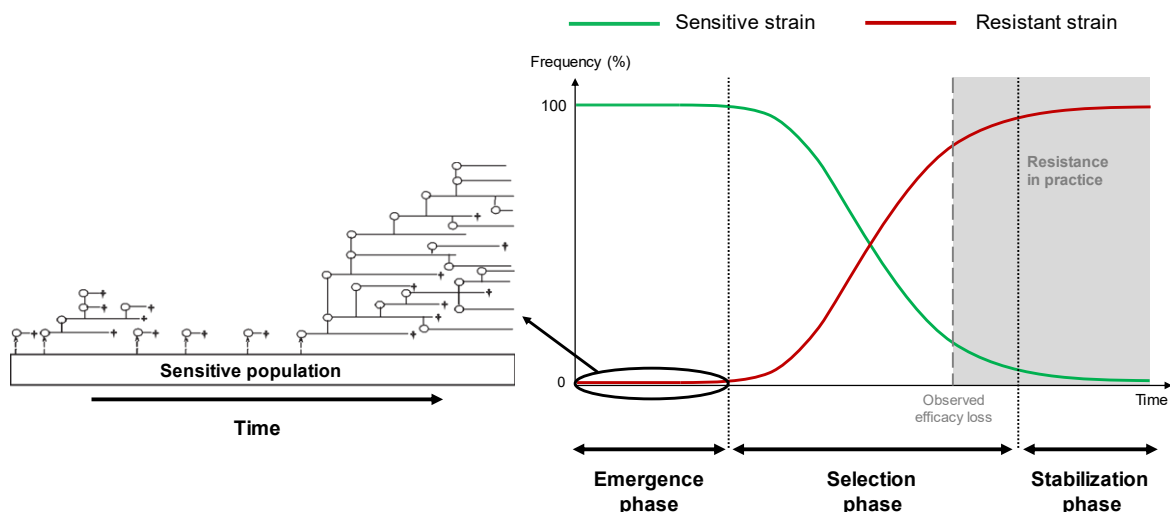


Figure 1: Phases of resistance dynamics in a population undergoing fungicide selection pressure and focus on the emergence phase allowing the rise of resistant strains in an initially susceptible population. Adapted from (van den Bosch et al. 2011).

### 1.1.2.1 Origins of resistance and the emergence phase

Adaptation of a population to a new environment exposed to pesticides or drugs can have several origins. First, a new mutation (or *de novo* mutation) can occur after an environmental change and then be selected in the population. This *de novo* mutation can be the result of a natural mutation, or more scarcely it can be induced by a mutagenic effect of the pesticide or drug. As an example, the *in vitro* exposure to sublethal doses of various fungicides highlighted multiple mutations throughout the genome of several strains of *Sclerotinia sclerotiorum* (Gambhir et al., 2021). The global mutation rate per nucleotide per generation is estimated around  $10^{-9}$  -  $10^{-10}$  for unicellular organisms, and  $10^{-8}$ - $10^{-9}$  for multicellular ones (Bezmenova et al., 2019; Lynch et al., 2016b). As many pathogens like fungi and bacteria have a short generation time and a large population size, their mutation capacity is higher than that of weeds and insects, and it is likely that all possible mutations already exist waiting to be selected (Hawkins et al., 2018). When arising in a population, the initial frequency of resistant mutations is very low. Only mutations with sufficient beneficial effects (with no or limited fitness cost and high resistance levels; § 1.1.2.2 and § 1.1.3 ) may emerge and fix into populations (Hawkins et al., 2018). The time needed for such an adaptive mutation to emerge is called the waiting time (

Figure 2) and will depend on the mutation rate, the population size and the selection pressures in the environment (Chevalier et al., 2019; Hawkins et al., 2018; Karasov et al., 2010; Smith et al., 2010). This waiting time can be even more important for a recessive resistant allele in a diploid or polyploid organism as its presence in each chromosome is needed (Chevalier et al., 2019; Hawkins et al., 2018).

Resistance polymorphism can also pre-exist prior to drug or pesticide exposition as the standing genetic variation (SGV). Neutral or (slightly) deleterious alleles are preserved in the original population because of multiple neutral forces such as recurrent mutations, drift or gene flow (Barrett, Schluter 2008 ; Hawkins et al. 2018 ; O'Donnell et al. 2014). As reviewed in Hawkins et al. (2018), this pre-existing resistance polymorphism can be issued from previous adaptations of the pathogen population to natural compounds similar to the pesticide/drug. Indeed, some pesticides or drugs are extracted or are synthetic analogues of natural compounds. For example, plant pathogenic fungi and pests are exposed *in natura* to a large range of natural inhibitors (as host defence compounds or toxins) which could explain the selection of efflux

transporters or detoxification enzymes in populations (Hawkins et al. 2018). The introduction of a new drug/pesticide then “reveals” this pre-pesticide adaptation by gene amplification, overexpression or gain-of-function mutations. Finally, pre-pesticide resistance can also be a pleiotropic effect of a non-related adaptation (Hawkins et al., 2018). In the case of SGV origin, as resistance is already present in the population, the waiting time is hardly reduced (Figure 2). Resistance is then more likely to emerge quicker than with a *de novo* origin (Barrett and Schluter, 2008; Chevalier et al., 2019; O’Donnell et al., 2014). Allele frequency from SGV origin may be sufficiently high in populations so that some diploid or polyploid organisms are homozygous in the population. An adaptation *via* a recessive allele selection is then more likely to come from SGV for which its frequency is higher than *de novo* mutation or hybridisation (Chevalier et al., 2019; Hawkins et al., 2018). In the case of SGV origin, even partial resistance with weak effect or resistance with some fitness cost that may have lower selective advantage may emerge and be fixed as such alleles are more likely to escape from stochastic loss (Barrett and Schluter, 2008; Hawkins et al., 2018; Hermisson and Pennings, 2005). This does not imply that large effect mutations are necessarily coming only from *de novo* origin. Indeed, resistance with high resistance factors can also emerge from SGV through the selection of compensatory mutations with smaller effects or epistatic interactions (Andersson and Hughes, 2010; Hawkins et al., 2018).

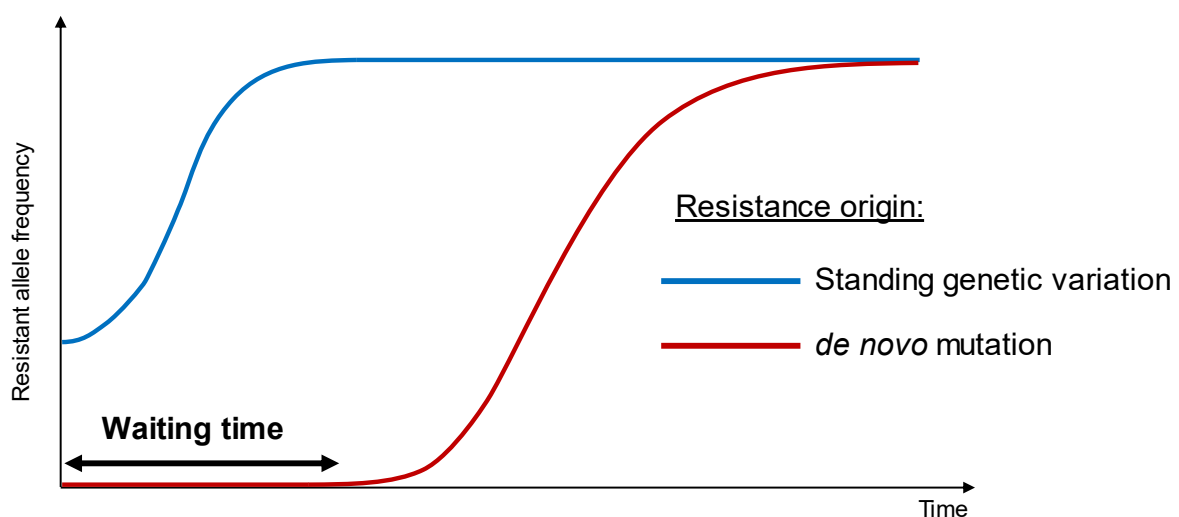


Figure 2: Influence of the origin of resistance on the rate of adaptation. Adapted from (Chevalier et al., 2019)

Three methods allow to determine whether an adaptation was acquired from *de novo* mutation or arose from SGV (Barrett and Schluter, 2008; Hawkins et al., 2018). The first one consists in the detection of “selective signatures”, using genomic polymorphism to assess hard or soft selective sweeps, corresponding respectively to *de novo* mutation or SGV (or recurrent *de novo* mutations). The second method consists in identifying the presence of the fixed allele(s) in an ancestral population, suggesting SGV. If it is not found, *de novo* mutation is more likely to be the source of adaptation. However, this method is only possible if a non-exposed ancestral population is available. Finally, the last method is the one of “phylogenetic dating”. This method analyses DNA sequences to generate a gene’s phylogenetic tree for multiple species. Their comparison allows identifying gene transfers or ancient presence of standing variation. Using these approaches, it was demonstrated that herbicide resistance was often due to the selection of polygenic metabolic resistance from standing variation. Fungicide resistance was most commonly due to *de novo* target-site mutations. Insecticide resistance displayed cases of both *de novo* mutations and selection from standing variation, in target-site and major metabolic enzyme-encoding genes (Hawkins et al., 2018).

At last, and more scarcely, resistance can be transmitted by another resistant species *via* interbreeding or horizontal gene transfer (HGT) (Hawkins et al., 2018). For example, *Mus musculus domesticus* adapted to rodenticides thanks to the interbreeding with the resistant *Mus spretus* (Song et al., 2011). HGT is quite common in bacteria and its association with the acquisition of extrinsic resistome (set of genes involved in antibiotic resistance) has been highlighted for *Aeromonas spp* (Bello-López et al., 2019). HGT has also been described in fungi (*e.g.* in *Pyrenophora tritici-repentis*) but not yet for fungicide adaptation (Hawkins et al., 2018). However, even if HGT exists in some fungi, it is most common in bacteria.

### 1.1.2.2 Resistance evolution in the selection phase

If mutation is the evolutionary force driving resistance emergence, resistance invasion in populations relies mostly on selection and genetic drift (Serre, 2006; Zhan and McDonald, 2004). Natural selection allows the transmission of advantageous alleles to the next generation. *Absolute fitness*,  $W$ , is the ability of organisms to survive and reproduce in a given environment (Orr, 2009). The *relative fitness*,  $w$ , which quantifies the average contribution to the gene pool of the next generation, is the absolute fitness of each genotype divided by the absolute fitness

of the genotype with the highest absolute fitness (or the “referent” genotype). Thus, for the referent genotype,  $w$  is equal to 1. Adaptation to a given environment refers to the relative fitness (or selective value) of individuals and is driven by their difference of fitness. The selective value of an individual varies depending on its environment: individuals with higher absolute fitness in a given environment will better compete, survive and reproduce, compared to individuals with lower fitness in the same environment (Orr, 2009). Consequently, their alleles will be more frequently transmitted over the generations. Then, the selection coefficient  $s$  can be defined as the measure of one allele relative fitness respectively to another one:

$$s = w_1 - w_2,$$

with  $w_1$  the relative fitness of the first allele and  $w_2$  the relative fitness of the second (Hartl and Clark, 1997; Solignac, 1995). If we consider the first allele as a pesticide or drug resistant one and the second a susceptible, then

$$s = w_R - w_S.^1$$

If  $s > 0$ , the resistant allele is relatively fitter than the susceptible one and therefore its frequency will increase over generations by contrast to the frequency of the susceptible, and conversely if  $s < 0$  (Figure 3).

Pesticide or drug applications favour resistant individuals, which exhibit greater selective value in treated environments. Under selection pressure, the relative fitness of susceptible individuals is lower than the one of resistant individuals ( $w_R > w_S$ ). The selection coefficient,  $s$ , is positive and resistance frequency increases. In untreated environment, if the absolute fitness of the resistant genotype is lower than the absolute fitness of the susceptible genotype, this fitness difference is qualified as “*cost of resistance*” or “*fitness cost of resistance*” or “*fitness penalty*”. Biological resistance is not always associated to a cost of resistance (R4P Network, 2020; Vogwill and MacLean, 2015). However, when such a fitness cost entails the selective value of the resistant individuals, the difference between the fitnesses of resistant and susceptible individuals decreases in the treated environment, but the selection coefficient can still be positive, allowing resistance to increase, but at a lower rate. Conversely, in untreated environments, the

---

<sup>1</sup> This definition of the selection coefficient is derived from population genetics equations. In theoretical models (*i.e.* epidemiological models) describing the evolution of resistance, the selection coefficient is presented as  $s = r_R - r_S$  (van den Bosch et al., 2014a; Milgroom and Fry, 1988). From a mathematical point of view, assuming an exponential growth of populations, these two coefficients are equal after exponential transformation, and therefore, both of them can be used equivalently (Garnault et al., 2019).

selection coefficient becomes negative ( $w_R < w_S$ ), and resistance frequency decreases naturally in populations (Figure 3).

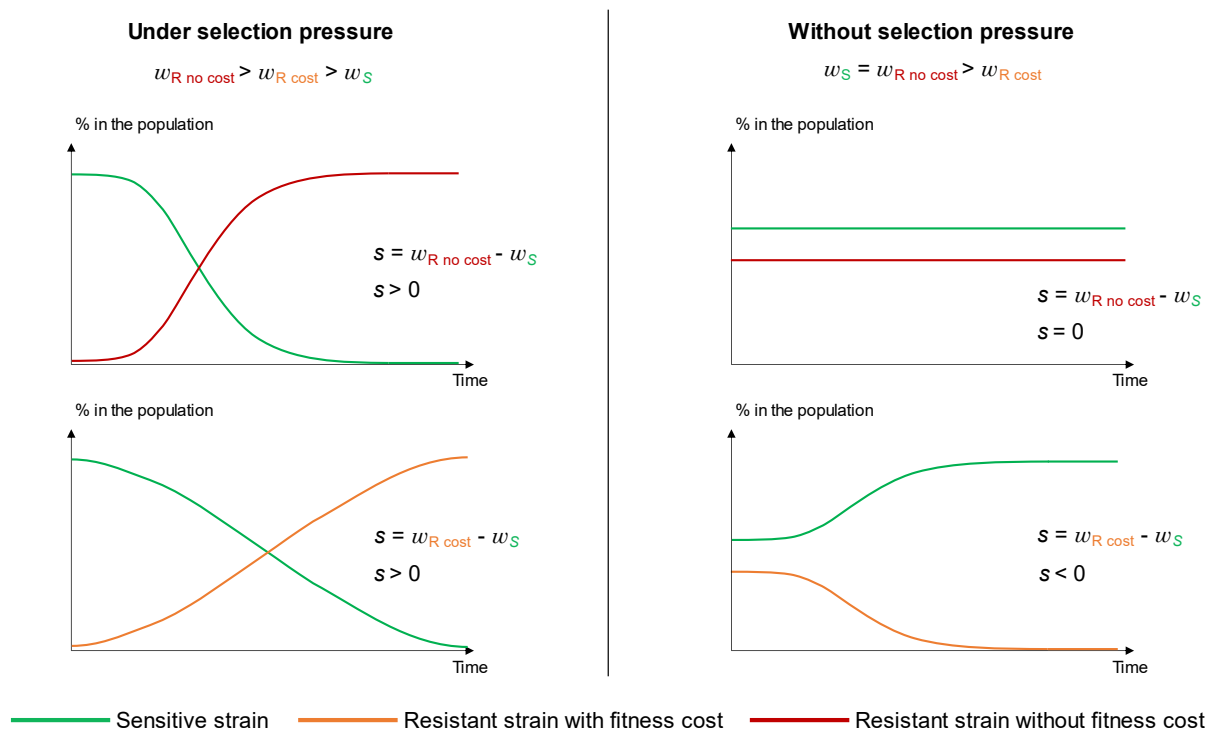


Figure 3: Evolution of allele frequency in a population undergoing or not the selection pressure exerted by fungicides. With  $w$  the relative fitness, the susceptible allele (not adapted to the fungicide) in green, the resistant allele associated with a fitness cost is in orange and the resistant allele without fitness cost is in red.

The rate of the selection then relies on the variation between the respective selective values of the categories of individuals. Each environment is characterized by a mutational landscape (*i.e.* fitness landscape) which possesses either a global phenotypic or fitness optimum (smooth landscape) or sometimes several possible local optima (rugged landscape) (Orr, 2005)(Figure 4).

The closest a strain is from one of these optima, the higher its fitness. Adaptation will drive population phenotypes to the nearest peak. However, the adaptive walk and the fixation time depends on the environment (Ogbunugafor et al., 2016). Indeed, different selection pressures (*e.g.* drug or pesticide applications) shape different selective landscapes (Mira et al., 2015; Palumbi, 2001). The selective value of an individual then not only depends on the environment but also on its potential fitness penalty. In particular, resistance mechanisms may be associated to distinct direct fitness costs (allocation costs, functional trade-offs). Indirect fitness cost

(linked genes or clonal replacement) can also result from the origin of resistance (Hawkins and Fraaije, 2018).

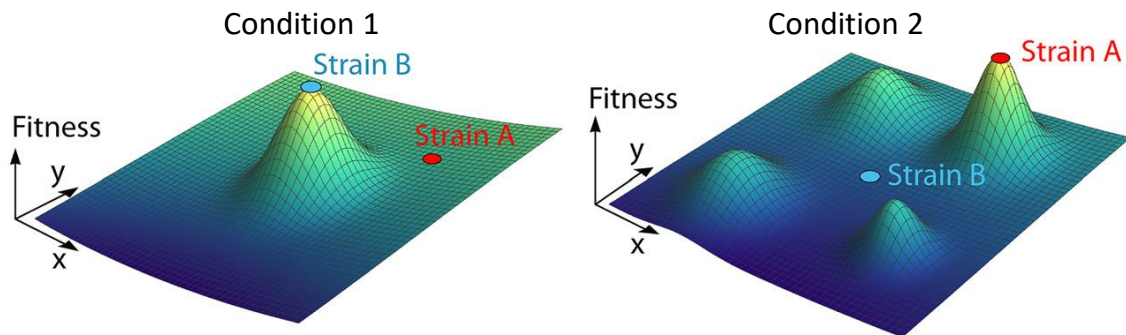


Figure 4: The exploration of fitness landscapes during resistance selection. The z axis represents fitness and the xy plane the different possible combinations of genotypes possible. Only one fitness peak is present in condition 1 (smooth landscape) while several fitness peaks are present in condition 2 (rugged landscape). In condition 1, strain B is fitter than strain A and conversely in condition 2. Adapted from (van den Bergh et al., 2018).

In addition to selection, genetic drift may also alter alleles trajectories just by chance (Caballero, 1995; Lanfear et al., 2014; Mallard et al., 2019; Serre, 2006). Genetic drift is more efficient in small size populations, for example resulting from a bottleneck. Then in absence of selection pressure, and depending on their initial frequency and population demography, resistant alleles are eliminated or fixed by genetic drift. As an example, genetic drift was modelled as the main evolutionary force decreasing the number of alleles in *Zymoseptoria tritici* (Zhan and McDonald, 2004). Migration or gene flow may either increase the frequency of resistance while bringing resistant individuals in a population or dilute resistance with individuals from susceptible reservoirs. For example, immigration accelerated the evolution of resistance in bacterial populations of *Pseudomonas aeruginosa*, and high rate of immigration associated to a rapid increase of antibiotic concentration contributed to increase levels of resistance to antibiotics (Perron et al., 2008). Mutation can still occur in populations during the course of adaptation, as similar mutational events but selected in different genetic backgrounds. Additional resistance mutations can contribute to increase resistance factors or enlarge resistance spectra, in combination or not with the one that first emerged. Compensatory mutations may also help restoring the selective value of resistant individual by reducing fitness cost of resistance either in absence or presence of selection pressure (Levin et al., 2000; zur Wiesch et al., 2011).



### 1.1.2.3 Patterns of resistance dynamics and underlying genetic mechanisms

#### Box 1: Main patterns of selection dynamics

Selection modifies population structure, and especially the frequency of adapted phenotypes. However, several patterns of selection are recognized.

- **Stabilising selection** is characterized by an increase of intermediate phenotypes frequency and the removal of extreme individuals. It is associated with a decrease of phenotypic variation. This selection pattern might be favoured by a fluctuating environment (Hallsson and Björklund, 2012; Kopp and Matuszewski, 2014).
- **Directional selection** favors one extreme phenotype, either selecting advantageous ones ( $s > 0$ ) (*i.e.* positive selection) or counter-selecting deleterious ones ( $s < 0$ ) (*i.e.* negative selection or purifying selection).
- **Disruptive selection** (sometimes also referred as diversifying selection) favours simultaneously several extreme phenotypes at the expense of intermediate ones. Fitness is higher for the individuals with extreme phenotypes than for the individuals with intermediate trait values.

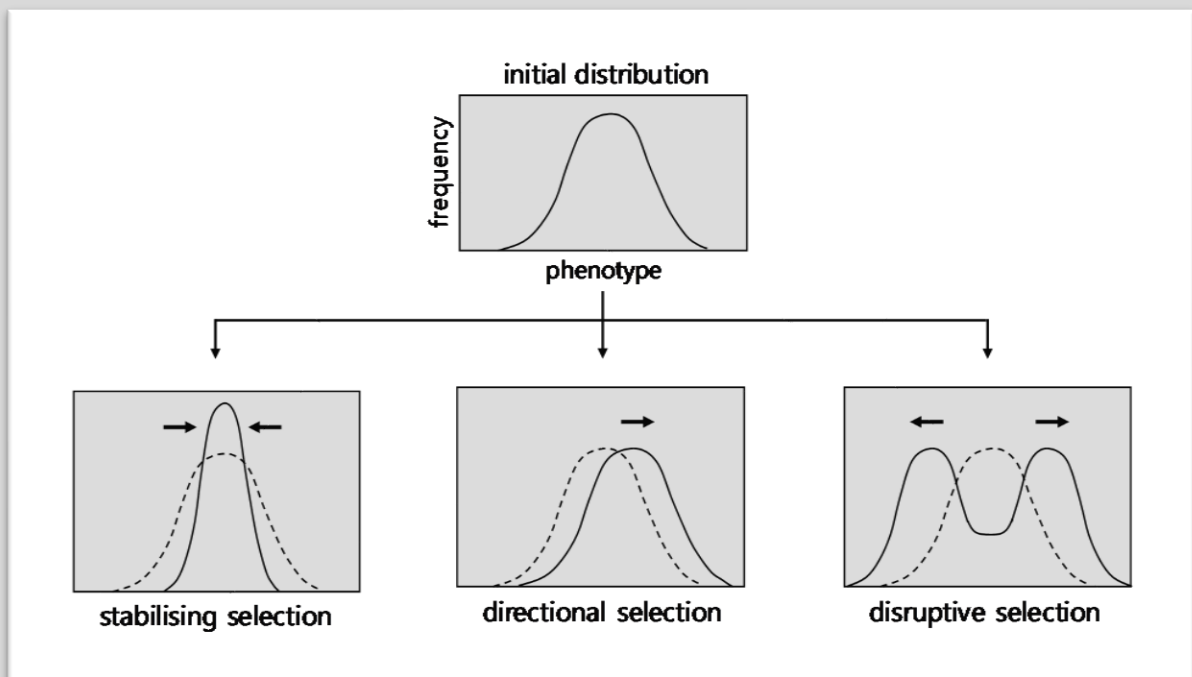


Figure 5: Population phenotypic distribution before (dashed curve) and after (full curve) the action of the main types of selection. Arrows are illustrating the shift of population distribution towards positively selected traits. Adapted from (Boixel 2020).

Theory predicts distinct patterns of selection, depending on how they structure population (Bürger, 1999; Kingsolver and Pfennig, 2007; Mitchell-Olds et al., 2007; Nielsen, 2005; Pélabon et al., 2010) (Box 1).

In the case of pesticide or drug treatments, selection is often described as directional and may result in the sharp increase in frequency of a single resistant allele of high selective value, and eventually in its fixation (Mitchell-Olds et al., 2007; Murray et al., 2018; Petersen et al., 2007; Seoighe et al., 2007; Stanton et al., 2020). However, the global increase of resistance frequency over time, attributed to the directional selection of resistant phenotypes, may follow different dynamics (*i.e.* qualitative or quantitative), in relation with the genetics of resistance (see Figure 6) (Brent and Hollomon, 2007a; Sang et al., 2016).

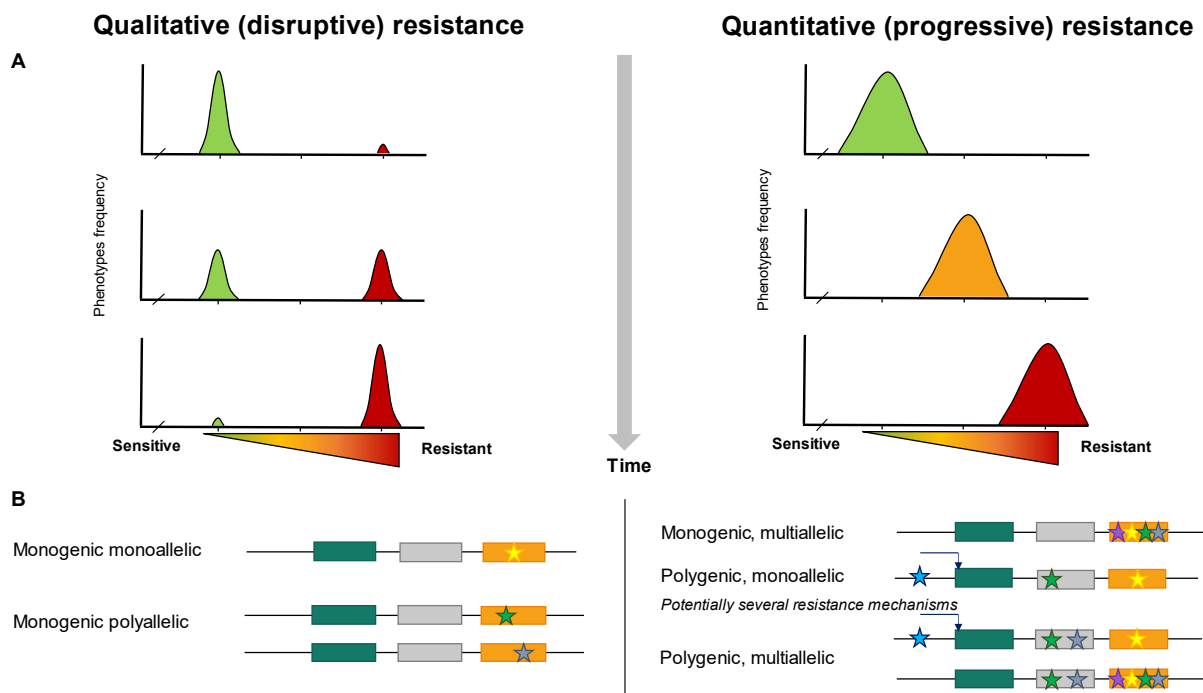


Figure 6: Dynamics of resistance and underlying resistance genetics for qualitative and quantitative resistance dynamics. **A.** Phenotype distribution: the evolution of resistant phenotypes is presented over time. **B.** Stars represents mutations in resistance genes; each colour is a different mutation. Mutations can be located either in the resistance gene itself or in its promotor (blue star).

*Qualitative resistance* (also called disruptive or discrete resistance) refers to the direct selection of one, or a few, resistant phenotypes, usually of high resistance factor and controlled by a unique mutation (monogenic resistance) affecting the target site (Figure 6). Conversely, *quantitative resistance* (also named multistep or continuous or progressive) displays a continuous variation of phenotypes, from susceptible to resistant, succeeding in populations. Resistance

factors gradually increase over time. Resistance is associated to the accumulation of different resistant genes (polygenic resistance) and/or of several resistant alleles (multiallelic resistance) (Figure 6). Understanding resistance dynamics and the underlying genetic mechanisms is a prerequisite to implement sound management, aiming at delaying the emergence of resistance when it is possible and slowing down selection. Resistance management aims at stabilising selection and preventing the move towards resistant optima. These studies are also very important to adapt prediction models to the reality of a resistance case.

### 1.1.3 Biological characterization of resistant phenotypes in the laboratory

Resistance characterization is important to understand its potential impact and future evolution in populations, as well as to feed models, and then estimate the practical consequences for resistance management (§ 2). Several traits can be characterized in the laboratory, either *in vitro* or *in planta*, such as resistance intensity, spectrum of cross-resistance, resistance stability and heritability and fitness.

*The intensity of resistance* measures the amount of a given active ingredient necessary to inhibit by 50% the survival of the tested individual ( $EC_{50}$ ; Box 2). The *resistance factor* (RF) or *resistance level* (RL) is the ratio of the respective pesticide or drug concentrations required to obtain the same effect on resistant individuals and on reference susceptible ones of the same species. It is usually expressed as

$$RF = EC_{50} \text{ resistant} / EC_{50} \text{ susceptible.}$$

In natural populations the genetic backgrounds are often heterogeneous, then RF is often described as followed  $RF = \text{mean of } EC_{50} \text{ of the resistant genotypes} / \text{mean } EC_{50} \text{ of the susceptible genotypes}$ . The higher the RF is, the more resistant are the tested individuals.  $EC_{50}$ s and RFs are practical tools to compare intrinsic activity (potency) and resistance intensity determined by various mechanisms or associated to different AIs (§ 1.1.4) or to distinct multiples alleles of the same gene.

The comparison of  $EC_{50}$ s for several active ingredients, sharing or not the same mode of action, is useful to describe the *profile of resistance* or *resistance spectrum* (R4P Network, 2016, 2020) which represents the range of pesticides/drugs the individual is resistant to. Establishing the resistance spectrum is essential to avoid any ineffective or inappropriate treatment and to assess resistance mechanisms (§ 1.1.4).

## Box 2: Characterizing the susceptibility to a pesticide or a drug

The  $EC_{50}$  is the pesticide or drug concentration that produces a half-maximum response (Auerbach, 2016). In particular, for pathogens, this concentration inhibits by 50% the value of phenotypic trait (*e.g.* its growth, survival, reproductive success, sporulation, spore germination, germ-tube length...). The  $EC_{50}$  is determined from a dose-response curve establishing the relation between the value of the chosen trait and a range of concentrations (Figure 7). Data are then modelled (with a logistic regression) to calculate  $EC_{50}$ , but also the  $EC_{90}$  or the  $EC_{100}$  (or MIC for minimum inhibitory concentration). Tests on pathogens are generally achieved *in vitro* on solid or liquid medium (Petri dishes or microtiter plates; Figure 8), or *in planta*, but their design largely depends on the biology of the tested organism (R4P Network, 2016).

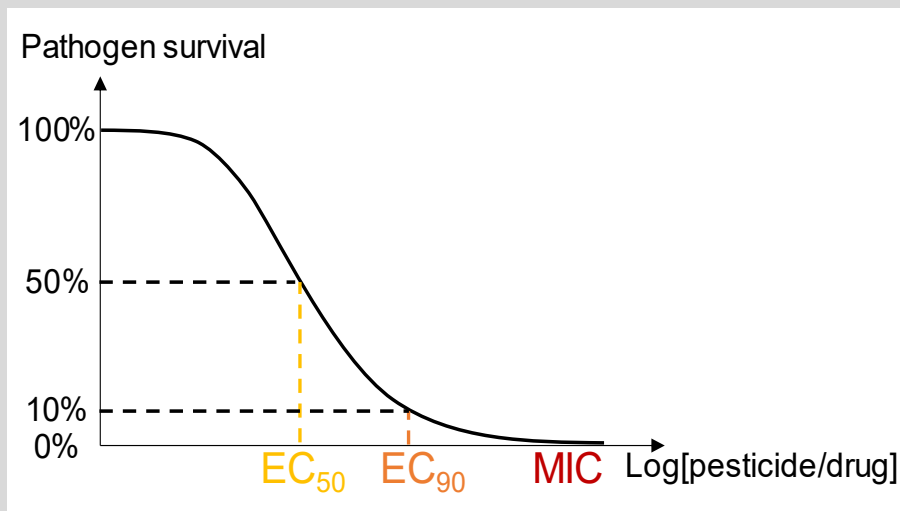


Figure 7: Determination of  $EC_{50}$ ,  $EC_{90}$  and MIC from a dose-response curve.

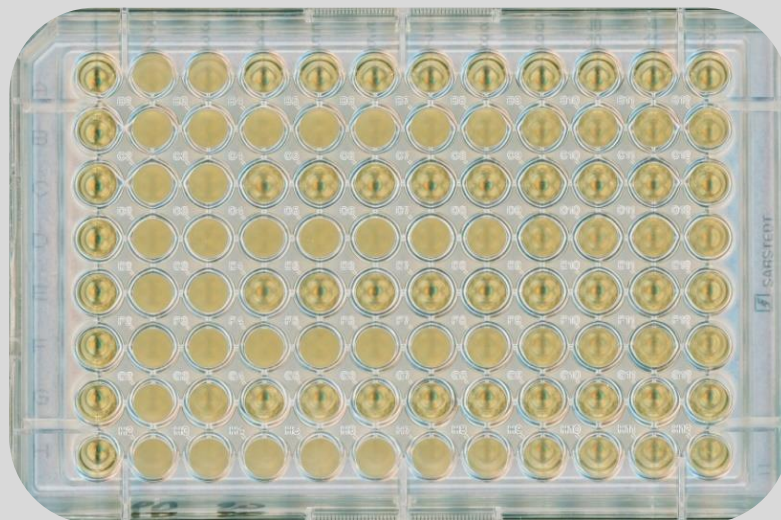


Figure 8: Microtiter plate test used to determine  $EC_{50}$  in *Zymoseptoria tritici*. Fungicide dose increases from left (2nd column) to right. Two strains are alternated every line.

Resistance spectra can be explained by cross-resistance or multiple resistance (Figure 9). *Cross-resistance* occurs when one mutation (or by extension one mechanism) confers resistance to several AIs. Then cross-resistance is qualified as positive. Multidrug resistance (MDR) is a particular case of positive cross-resistance where a mutation affecting membrane transporters enhances the activity of efflux pumps and allows resistance to unrelated modes of action (Nikaido, 2009). Note that cross-resistance can also be qualified as negative when resistance to an AI leads to the hypersusceptibility towards others (R4P Network, 2016). As for *multiple resistance*, resistance to several AIs is due to the co-selection of several different mutations. Each mutation is responsible for one resistance, making the accumulation of those independent mutations the genetic mechanism behind multiple resistance. This definition of multiple resistance can also imply different resistance mechanisms conferring resistance to several different AIs.

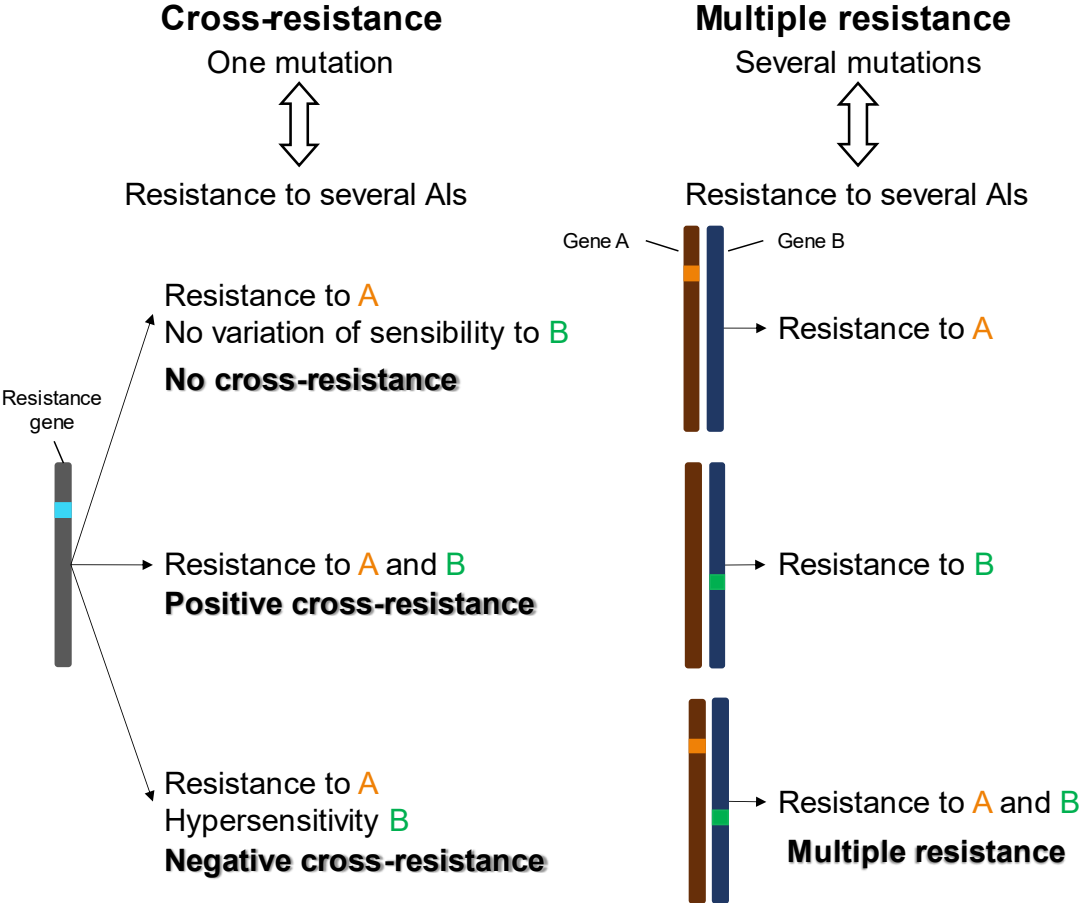


Figure 9: Genetic mechanisms underlying cross- and multiple resistance.

Resistance acquisition can be associated with pleiotropic effects on the target site function or enzyme's activity, *i.e.* with fitness cost. Those can directly trigger the fitness and spectrum of

resistance of the resistant individuals. The cost of resistance may also mitigate the selection of resistance which justifies its characterization. Ideally, as many traits as possible are measured and may include the number of viable offspring or the growth rate (or Malthusian fitness for population with continuous growth). This last trait is often considered a good proxy for absolute fitness (Orr, 2009; R4P Network, 2020; Vogwill and MacLean, 2015).

#### 1.1.4 Mechanisms of resistance towards pesticides and drugs

Generally, a given drug or pesticide binds to its specific receptor, *i.e.* target site, and then constitutes an alternative to the natural ligand. AIs can also bind to ion channels, enzymes or membranes carriers (Lambert, 2004; Salahudeen and Nishtala, 2017). Ligands exhibiting only one target site are referred as unisites, and multisites otherwise. Drug and pesticide ligands compete with natural ones by mimicking the interaction with their receptor. Several interactions are possible. First, drugs or pesticides that induce the expected biological response are qualified as agonist. If the produced response is not complete, the drug or pesticide is labelled as partial agonist. Conversely, if the induced response is opposite to the response with the natural ligand, the drug is called inverse agonist (negative efficacy). Finally, antagonists are ligands that do not generate biological response once bound to their receptor. Antagonists usually obstruct the action of the natural ligand on the receptor. It can be a competitive antagonist meaning that the interaction is reversible and can possibly be surpassed or non-competitive when the binding is irreversible. Most of the AIs used in agriculture fall into this last category (R4P Network, 2018). Before drugs and pesticides interact directly with their biochemical target site, an AI has to enter into the cell and reach its target, which might involve transport systems. Its concentration should also be sufficient to modify the biological response, despite metabolization or export processes. Thus, mechanisms at work in resistant strains may either alter the ligand with the natural receptor or limit or prevent the journey of the ligand to the localisation of the receptor (Cloete, 2003; Gaines et al., 2020; Kennedy and Tierney, 2012; Saves and Masson, 1998). This allows the organization of resistance mechanisms into two categories: (1) toxicodynamically derived mechanisms, in which the interaction with the target sites is modified and (2) toxicokinetically derived mechanisms, which alter the way in which organisms absorb, biotransform, and excrete chemicals (Kennedy and Tierney, 2012). Those categories are

most often referred as target site resistance (TSR) and non-target site resistance (NTSR) mechanisms, respectively (R4P Network, 2016).

TSR includes the 3D modification of the target receptor (Figure 10) (Khan et al., 2020; Lambert, 2005; Reygaert, 2018). The change of its structure affects the affinity of the ligand or modify its function. This change is generally associated to SNPs (mutation, insertion, deletion) in the gene encoding the target protein. TSR also includes target overexpression due to an up-regulation of the target gene. This upregulation is usually correlated with inserts in the promoter sequence or in the transcription factors regulating the target gene. TSR usually implies cross-resistance between AIs sharing the same MoA but exceptions rely on the residual affinity between the AI and its mutated target (Casida, 2017; Devine and Shukla, 2000). By contrast, NTSR covers a variety of mechanisms reducing AI absorption (*e.g.* cell wall impermeability, altered influx or import), increase of AI elimination (increased efflux), transformation of AIs into less toxic metabolites (metabolization/detoxification) or limitation of the activation of the pro-drug/propesticide into its active form. AIs can also be sequestered in cellular organelles (*e.g.* vacuoles of weed species) or be “inactivated” by the binding of a protein. Resistance can also be caused by the up-regulation of alternative pathways, circumventing or compensating the decrease in activity of the inhibited target. NTSR mechanisms do not rely on highly specific interactions with the AI’s target. Therefore, they are considered generalist mechanisms and may imply hardly predictable cross-resistance between AIs, regardless of their MoA. Resistance mechanisms are reviewed in Figure 10.

The occurrence of these mechanisms depends on the considered taxons. TSR is described for a large diversity of resistant taxa (including bacteria, fungi, weeds, insects, or mammals) but occurs at various degrees. Target alteration, and to a lesser extent, target overexpression, is highly described for phytopathogenic fungi, as they are associated to high resistance factors and therefore of practical relevance, which may also reveal that other mechanisms are poorly explored in this kingdom (Costa-de-Oliveira and Rodrigues, 2020; Lucas et al., 2015). TSR is concerning almost all unisite fungicide MoAs. Examples of NTSR to fungicides include:

- 1) enhanced efflux leading to multidrug resistance (MDR) (principal NTSR mechanism described in fungi) (*e.g.* *Sclerotinia homoeocarpa* resistant variant display overexpression of ABC transporters and also cytochrome P450 enzymes with detoxification capacity (Sang et al., 2018)),

2) the reduction of the penetration of the fungicide (by a modification of the cell wall composition in some *Candida* and *Aspergillus* species (Lima et al., 2019)),

3) the activation of the alternative oxidase (AOX) pathway (*e.g.*, in *Venturia inaequalis*, AOX is a source of resistance to respiration inhibitors like QoIs or QiIs (Wood and Hollomon, 2003)),

4) the metabolization into inactive forms (*e.g.* fenhexamid detoxification in *Botrytis pseudocinerea* (Billard et al., 2011)), and

5) the default in activation of profungicide into their active forms (*e.g.* cymoxanil in *Botrytis cinerea* (Tellier et al., 2009)).

TSR is also common in weeds (*e.g.* inhibition of PSII due to one mutation in the chloroplastic *psbA* gene, responsible for triazine resistance) but tends to be downplayed face to the importance of NTSR (especially metabolization due to cytochrome P450 enzymes affecting many MoAs and Glutathione S-Transferases (GST), or the translocation of glyphosate for example) which prevents herbicide from reaching its target site (Délye et al., 2013; Powles and Yu, 2010). Resistance to triallate can also result from a default ins activation due to a reduced sulfoxidation (Das and Mukherjee, 2018).

Insecticides are also frequently facing metabolization (due to esterases, oxidases and GSTs enzymes, for example, acting on several chemical classes like pyrethroids, organophosphates and carbamates) and sequestration issues, in addition to target site modification (TSR) (not very common in insects of agronomic relevance) (Bass et al., 2014; Mitchell et al., 2014; Panini et al., 2016).

Most common resistance mechanisms in bacteria prevent the interaction of the antibiotic and its target (NTSR) by inactivating or destructing the antibiotic (*e.g.* aminoglycosides inactivated by enzymatic phosphorylation) or even decreasing the uptake across the outer membrane barrier in Gram-negative bacteria. The overexpression of multidrug efflux pumps is also particularly worrying in Gram-negative bacteria as they are major determinants of multidrug resistance with broad antimicrobial profile to existing antibiotics and also potential future ones (Cesur and Demiröz, 2013; Dever and Dermody, 1991; Munita and Arias, 2016; Nikaido, 2009; Poole, 2005). It is also very frequent that the target site is affected (structurally or functionally) (*e.g.* fluoroquinolone).



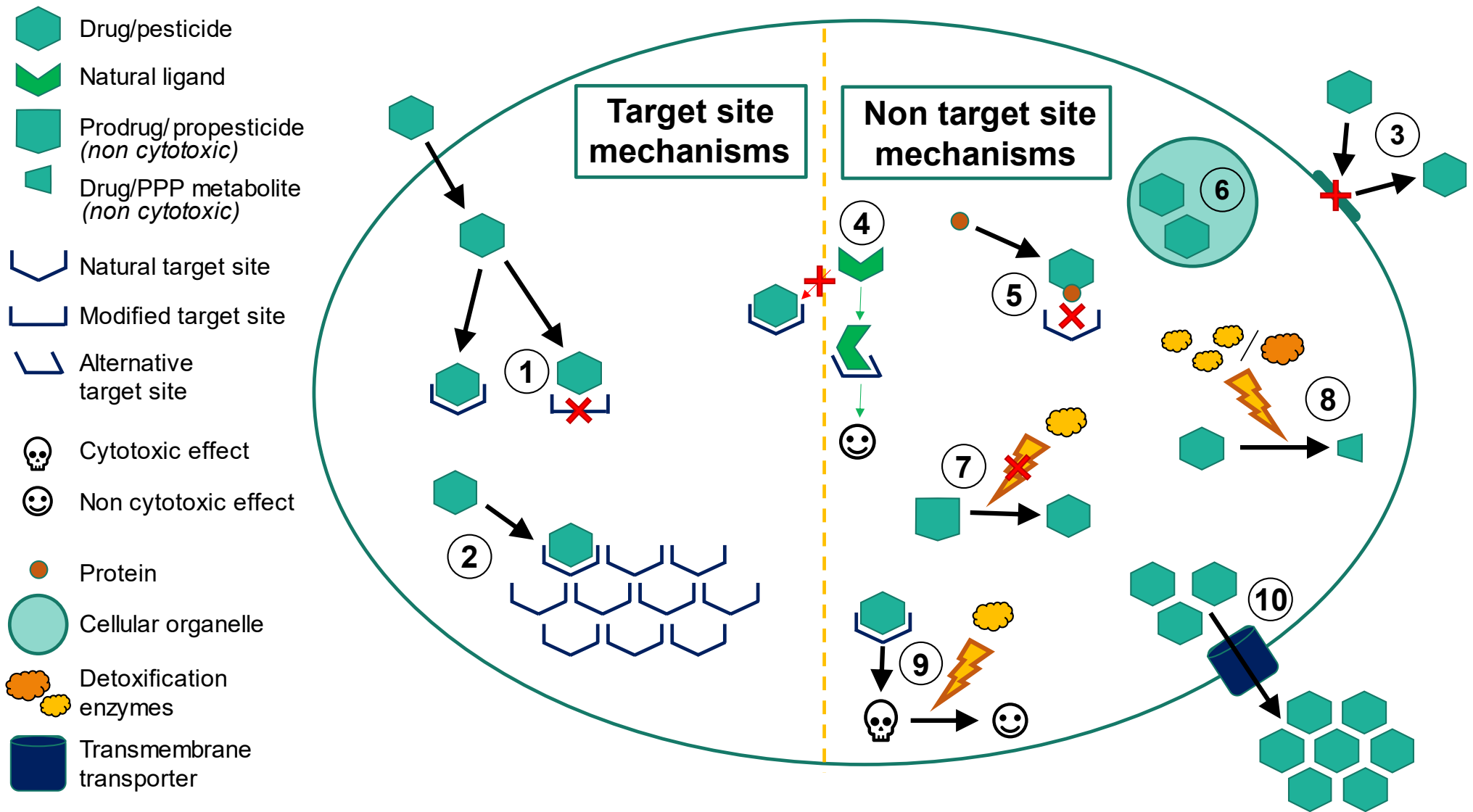


Figure 10: Mechanisms of resistance towards drugs or pesticides. Adapted from (R4P Network 2016).

Target Site Resistance (TSR): 1) Target site structural alteration decreasing the affinity for the drug or pesticide, 2) Target site overexpression

Non-Target Site Resistance (NTSR): 3) Reduced absorption of the drug/pesticide by the binding of a protein, 4) Bypass of the target site function by an alternative pathway, 5) Inactivation of the drug or pesticide by the binding of a protein, 6) Sequestration of the drug/pesticide in cellular organelles, 7) Default in activation of the prodrug or propesticide, 8) Detoxification in less toxic forms by more neutralizing enzymes or more active enzymes, 9) Neutralization (detoxification) of the interaction product of drug or pesticide and its target site, 10) Enhanced efflux of the drug or pesticide outside the cell

Those resistance mechanisms are independent, but can be combined in the same individual, leading to multiple resistance and potentially increasing RFs and/or enlarging the spectrum of cross-resistance, depending on combinations (Li et al., 2014; Mitchell et al., 2014; Panini et al., 2013; Powles and Yu, 2010; Vázquez-García et al., 2020). For example, Cowen et al. (2015) precise that not all described fungicide resistance mechanisms (*e.g.* target site alteration, overexpression, drug uptake, efflux and MDR regulation) are present in any clinical *Candida albicans* strains yet, but this pathogen may have the potential for such a prospect. Isolates of the phytopathogenic *Botrytis cinerea*, were found resistant to five and more fungicide MoAs of fungicides, due to the accumulation of multiple mutations in several target genes but also due to enhanced efflux (MDR) (Chen et al., 2016). Similarly, some bacterial species exhibit simultaneously three different mechanisms of resistance to fluoroquinolone: target site modification, over-expression of efflux pumps and target protection (homologues competing for the binding site) (Munita and Arias, 2016). The combination of these advantageous resistant alleles can be facilitated in different ways. MoAs may have been used sequentially, the innovative ones replacing those concerned by resistance. This process leads to the selection of the new resistances in already resistant backgrounds, and then to multiple resistance. In bacteria, horizontal gene transfer may also contribute to successful combinations of alleles. At last, sexual reproduction may also recombine independent mechanisms of resistance initially selected in different individuals. Such phenomenon increases the genotype diversity of recombinant individuals and may discard deleterious mutations, both of which accelerating adaptation (Bürger, 1999; Heitman, 2006; McDonald and Linde, 2002). Depending on their respective frequency in populations comparatively to susceptible alleles, sexual reproduction may then link advantageous alleles but also unlink them, leading to the loss of advantageous combinations. By contrast, asexual reproduction may not create innovative allele combinations but may amplify them largely in populations. Therefore, organisms with dual mode of reproduction are often considered highly adaptive and at high risk regarding resistance evolution.

## 1.2 The challenges of resistance for pest and disease management

### 1.2.1 Pests and diseases: a burden for agriculture and health care from the start

Human diseases and pests directly impact our life quality, while those of plants or animals may alter it indirectly through our food and environment. Indeed, all around the world, infectious diseases cause high morbidity and mortality. Before the 20<sup>th</sup> century, they were responsible for about one third of the recorded deaths (Adedeji, 2016; Armstrong, 1999). The antibiotic revolution contributed to progress in medicine and life expectancy doubled in every continent in about one century (Roser et al., 2013). Nonetheless, three of the ten main causes of death are communicable (infectious and parasitic diseases and maternal, perinatal and nutritional conditions). Then, pests and diseases are still a major concern (WHO, 2020). The mortality rate due to fungal diseases in human is now comparable to the one of tuberculosis and HIV (Fisher et al., 2018). Additionally, a sufficient and good quality food production and conservation has been looked for in every society. However, diseases and pests are responsible for high variability in crops and cattle yield and quality (Regnault et al., 2012). For example, phytopathogenic fungi may be globally responsible for up to 20% of crop yield losses (30% when considering post-harvest losses) but may reach much higher yield reduction depending on the crop, pathogen and epidemic intensity (Fisher et al., 2018). In addition, some species, (*e.g.* *Fusarium* species) produce mycotoxins which can be highly toxic and/or carcinogenic for humans, animals and plants (Jimenez-Garcia et al., 2018). However, variability between geographical regions is important and the regions most impacted by pathogens and pests are located where there is a food-deficit with fast-growing populations, and frequent emerging or re-emerging pests and diseases (Savary et al., 2019). Considering all pathogens and pests, the estimated yield loss reaches more than 40% in some regions (*e.g.* rice (40.9%), maize (41.1%) in the Indo-Gangetic Plain) (Savary et al., 2019).

Therefore, crop protection and medicine have been at the heart of human concerns since the first civilisations. For example, sulphur was yet used around 2500 BC for its properties to control insects and arthropods (Abubakar et al., 2020). The first records of “drugs” date back to 1500 BC in Egyptians civilizations where medicines were prepared from mixtures of plant, mineral or even animal products (Jones, 2011). In 1650, barberries (*Berberis vulgaris*) were removed from

farming areas to fight against *Puccinia graminis*, responsible for stem rust, and that even before knowing that it was an alternate host for this fungus (Regnault et al., 2012). But before the middle of the XIX<sup>th</sup> century, phytopathology was not considered as a proper discipline and decisions were mainly based on empirical observations. Tools to control pest and diseases developed along with science and technical progress. Bordeaux mixture (copper sulfate and quicklime) was invented and first used in 1885 in vineyards to cure downy mildew due to *Plasmopara viticola*. But it is really after World War II that organic chemistry really revolutionized crop protection and modern medicine with the introduction and the large diffusion of synthetic pesticides and drugs.

Those were easy to use, quite cheap, of regular efficacy and highly efficient: these advantages made them really popular and explain their massive use in many areas during the last decades. (R4P Network, 2016; Regnault et al., 2012; REX Consortium, 2010, 2013; Umetsu and Shirai, 2020). Nowadays crop protection highly relies on pesticides use to maximise yield despite the presence of weeds, pathogens, viruses and animal pests (Oerke, 2006) (Figure 11)

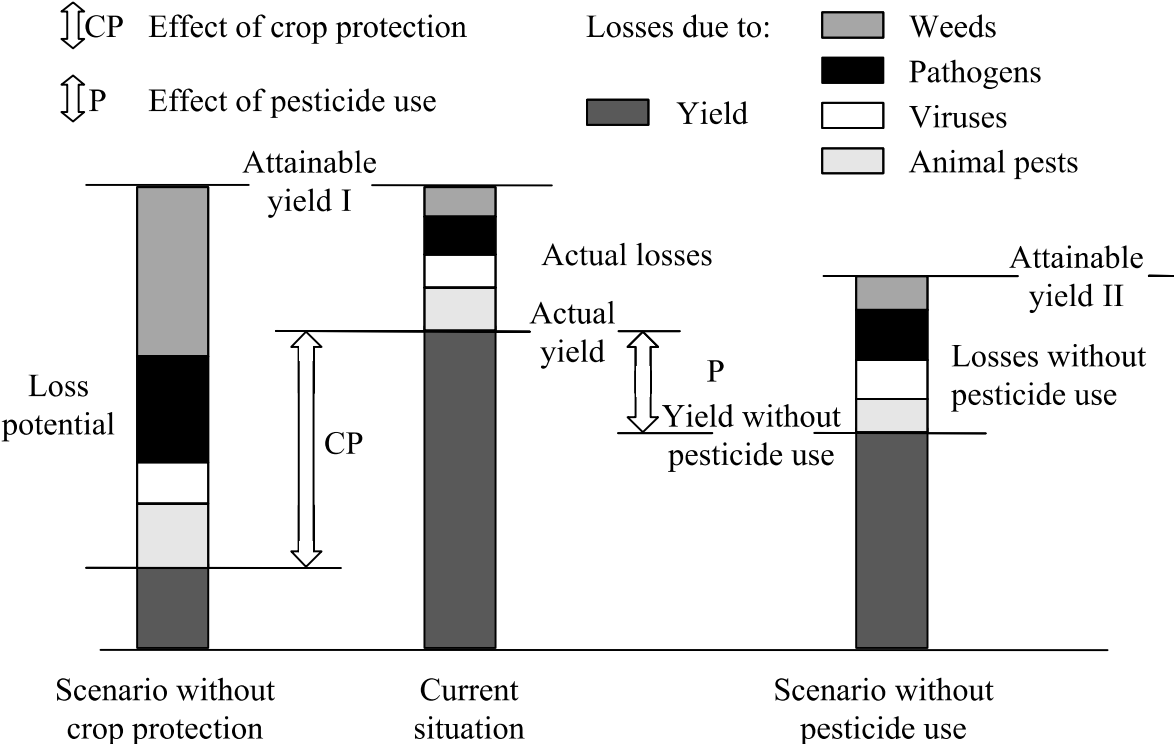


Figure 11: Estimation of crop losses and yield levels with or without crop protection and pesticides. (Oerke, 2006).

If pesticides and drugs are relevant for the society for the production of food and feed and preserve plant, human and animal health, their generalized use also have drawbacks. In addition to decried toxic effects on human and environmental health, one of those is the selection of resistance. First resistance cases were described in insect populations (efficacy decrease of sulphur-lime in San Jose scales, orchard aphids, or brown mites populations in 1914 (Melander, 1914)) but were rapidly followed by bacteria (*Streptococci* resistance to sulfonamides in the late 1930's (Sköld, 2001)), fungi (resistance of *Pyrenophora avenae* to organomercurials first reported in 1964 (Deising et al., 2008)) and weed cases (*Senecio vulgaris* to simazine in the 1960's (Holt, 1992)). Warnings about resistance management and the reasonable use of insecticides were already given by scientists describing the first resistance cases in 1914 (Gould et al., 2018). Indeed, ever-increasing numbers of resistance cases have now been described. More than 600 species of arthropods now exhibit resistance to at least one insecticide and more than 350 insecticides are subject to resistance (Mota-Sanchez and Wise, 2021). Fisher et al. (2018) emphasize the fact that the time before efficacy decrease in fungicides treatment is becoming shorter due to the cultural and clinical practices that supplement the inner traits of fungi to generates resistant variants (§ 1.2.2). The time before the first detection of resistance is also correlated to the cultural practices and the risk of resistance of the AI (Grimmer et al., 2014a). As listed in REX Consortium (2013), resistance to 30 fungicides has been observed in the field for 250 species of fungi. Just in France, in 2019, 32 pathogens were already described for fungicide resistance which was concerning 83 fungicides (R4P Network, 2021). Worldwide, more than 260 weed species also have resistance to herbicides and about 40% exhibit multiple resistance (Figure 12) (Haywood et al., 2021; Heap, 2021). Every actual herbicide class is concerned (Gould et al., 2018).

The situation for antibiotic resistance is also very worrying as some bacteria with high pathogenicity are resistant to all the current classes of antibiotic and many bacteria are resistant to a wide spectrum of antibiotics (Jansen et al., 2013; Munita and Arias, 2016; WHO). 250 antibiotics are concerned by resistance in 1700 species of bacteria (REX Consortium, 2013). Many genes (potentially 23 000) and mechanisms can be involved in antibacterial resistance (Cloete, 2003; Li and Webster, 2018).

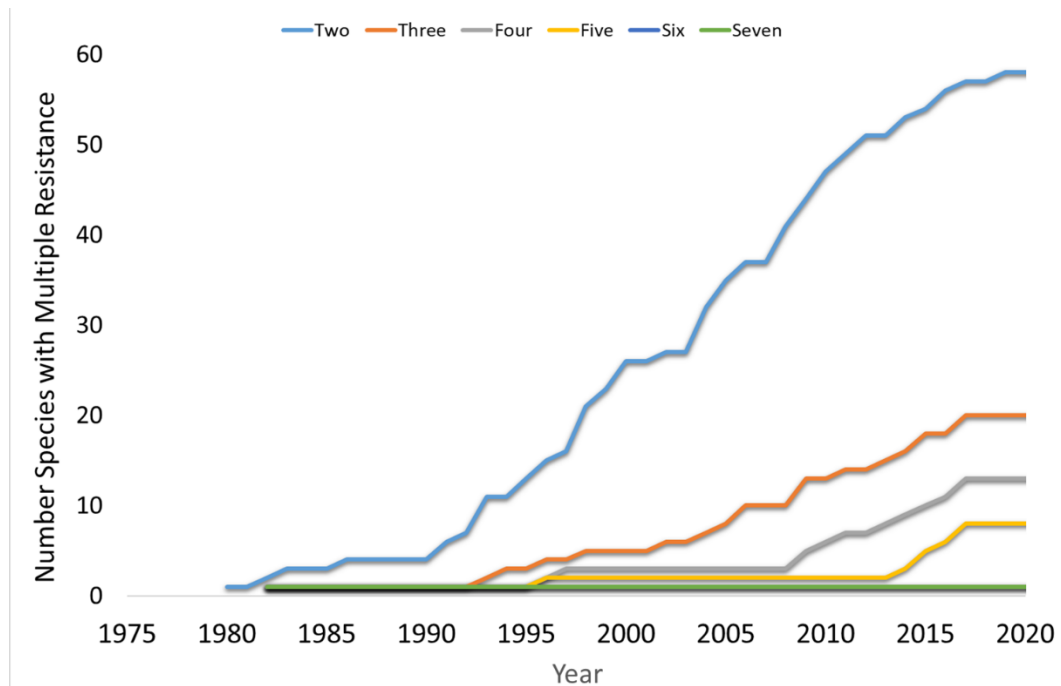


Figure 12: Evolution of the number of weed species displaying multiple resistance. The term multiple resistance does not necessarily refer to several sites of action in one individual plant but to sites of actions that can be found in several populations of a given species. From (Heap, 2021), weedscience.org.

### 1.2.2 Operational factors aggravating the selection of resistance to pesticides or drugs

The previous sections detailed the evolutionary processes underlying the emergence and the selection of resistance, as well as some biological and genetical pathogen/pest traits that favour this phenomenon. Such intrinsic factors of the pesticide or drug (as its MoA or chemical group, unisite vs. multisite, its complexity and molecular weight, ...) and of the pathogen or pest (e.g. reproduction mode, trophic type, number of hosts) can hardly be manipulated (EPPO, 2015; Gaines et al.,



Figure 13: Some operational factors invoked by the World Health Organization to explain the rise of antibiotic resistance.

2020; Grimmer et al., 2014b; SCENIHR, 2009). But besides those, operational factors may also contribute to aggravate the selection of resistance (i.e. increasing the selection coefficient; §1.1.2.2) (Figure 13). The intensive use of pesticides and drugs since the 1950s is surely the most important explanatory factor. As an example, the emergence and spreading of resistant bacteria have been highly correlated with antibiotic consumption (Ventola, 2015). This enhanced use may be justified by various reasons as detailed hereafter.

### 1.2.2.1 Production systems and societies promoting an intense use of pesticides and drugs

The “antibiotic revolution” and the “green revolution” are characterized by a change in clinical (both human and animal) and agricultural practices. Indeed, after World War II the chemical industry started to massively produce drugs and pesticides which become cheaper and easily available. This shift of practices also occurred in developing countries (Olsen, 2013; Ventola, 2015; Woolhouse and Farrar, 2014).

#### *Intensification of the use of pesticides and drugs*

The intensification of agriculture worldwide and the increase of monoculture from the 1960s (Regnault et al., 2012), which can be driven by economic advantages of a given crop for example (Gould et al., 2018), displayed favourable conditions for pathogen development (McDonald and Stukenbrock, 2016). Indeed, pathogen transmission and development are made easier in uniform and dense host populations of little genetic variation. Consequently, pesticides were massively sprayed over large areas, as a progress to control pathogens and pests, but exerting strong and rather homogeneous selection pressure.

Medical uses of drugs increased from their introduction around the 1930's because of the growing and ageing worldwide population (additionally to injured soldiers from the World war). Because of ageing and previous diseases (*e.g.* HIV infection, cancer chemotherapy, transplantation, diabetes, ...), more immunocompromised patients are now susceptible to bacterial, fungal and viral infections (Palumbi, 2001; Revie et al., 2018; Robbins et al., 2017; Ventola, 2015; Woolhouse and Farrar, 2014). In developed countries, overcleaning and hygienic focus might

also have limited the development of acquired immunities to some pathogens, as well as increasing the use of bactericides and virucides outside clinical context (Ventola, 2015). The use of antibiotics for animal infections or wastewater are other examples of the spread of selection on antibiotics in many areas which results, even indirectly, in a global increase of selection pressure (Gaviria-Figueroa et al., 2019).

### *Large number of pathogens and pests targeted with the same modes of action*

In addition to the broad use of pesticides and drugs, selection of resistance is also enhanced by the fact that similar modes of action can be used in many economic areas, as many of them are generalist modes of action, exhibiting an activity over a large spectrum of taxa. For example, azole fungicides are used in human, animal, and crop protection and in the industry, as general biocides. Similarly, antibiotics production is also strongly addressed to animal husbandry, to promote growth and prevent infections. As an example, about 80% of the antibiotics sold in the USA were used for veterinary purposes (Aminov, 2010; Palumbi, 2001; Prestinaci et al., 2015; Ventola, 2015). The use of antibiotics as animal growth promoter is now banned in the EU (2006) and in the USA (2017) (Center for Disease Control and Prevention, 2021) but their worldwide use for animal health is still increasing (Dall, 2020; Woolhouse and Farrar, 2014). This global use of pesticides and drugs, focused on a limited number of modes of action may have promoted resistance development. They might as well have affected interactions between environmental microbiomes (Davies and Davies, 2010; Ventola, 2015). A striking example is the discovery of clinical isolates of *Candida albicans* and *Aspergillus fumigatus* bearing azole-resistant mutations usually selected in agricultural environments, which strongly suggests a transfer between *a priori* separate worlds (Fisher et al., 2018; Parker et al., 2014). Similarly, antimicrobial resistance in human pathogens may have been influenced by the agricultural use of antibiotics (Laxminarayan et al., 2006). Non-primarily targeted organisms can then undergo indirect selection pressure and develop resistance after drugs or pesticides applications. The likelihood to acquired resistance by HGT also increases with co-infection.



### 1.2.2.2 Overuse and misuse of AIs

Resistance selection may also result from inappropriate use of pesticides and drugs, aggravated by the lack of knowledge on resistance management and a large and easy access to AIs (lack of regulation, particularly on sales, in some parts of the world) (Fair and Tor, 2014; Palumbi, 2001; Ventola, 2015). There are several examples of pesticides misuses with lasting consequences. For example, in the 1980's, the resistance risk of *Oculimacula spp* wasn't considered very important, therefore the fungicides benzimidazoles have been massively used (alone, several times and at full dose) to control this fungus but this latter failed within a few years (Lucas et al., 2015). Despite the withdrawn of those substances for eyespot control (in 1991 in France), the populations remain until today largely resistant. Additional treatments and/or a dose increase can be implemented as a response to resistance evolution. However, it may also enhance selection of certain resistant phenotypes even more, which results to be counterproductive (Fones et al., 2017). A lack of knowledge of the factors and the dynamics of resistance evolution often appears at the source of misuse. Social pressure may also influence the overuse, or the misuse of pesticides and drugs. With the decline of mortality, medical inefficacy is less accepted by modern societies and drugs prescriptions are favoured. In agriculture, pesticides are also an opportunity to satisfy consumers requesting high quality products without visual defaults and at low price. Prophylactic treatments in both medicine and agriculture generate an intense use on large spatio-temporal scales that impacts resistance evolution. By providing a preventive action rather than a curative, prophylactic treatments are often judicious for resistance management but may sometimes reveal unnecessary particularly in medicine, by being applied when disease pressure is still very low. In 2001, Palumbi was assessing that one third of paediatricians in the USA were overprescribing antibiotics and sometimes instead of an appropriate treatment. The choice of a wrong treatment, AI, duration or dose may represent 30% to 50% of the prescription cases (Luyt et al., 2014; Ventola, 2015). Consequently, antimicrobial resistance due to long periods of prophylactic use, insufficient doses, irregular or inappropriate treatments are still worldwide commonplace despite warnings (Fisher et al., 2018; Palumbi, 2001; Parker et al., 2014; Ventola, 2015).

### 1.2.2.3 Facilitated migration of resistant organisms

Globalization by increasing the movement of people and agricultural products has also been a vector of new pests in some territories and facilitated the dispersal of resistant individuals worldwide (e.g. Figure 14) (McDonald and Stukenbrock, 2016). Several examples of importation of multidrug-resistant *Plasmodium falciparum* (causing malaria) from immigrants, tourists or expatriates returns have been noticed (MacPherson et al., 2009). Similarly, insecticide-resistant corn rootworm *Diabrotica virgifera virgifera* has invaded Europe from northern America through five independent introduction events (Ciosi et al., 2008). Global climate change, by modifying ecosystems and intensifying pathogen dispersion, can then be a vector of resistance spread across the world (Fones et al., 2017).

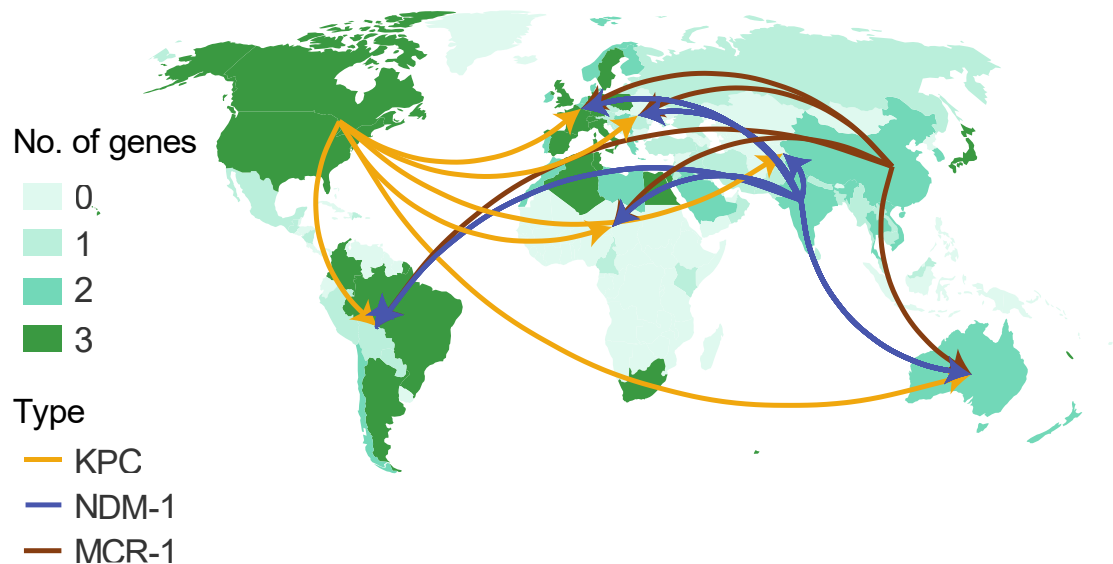


Figure 14: Global distribution and spread of three plasmid-borne genes associated with multiple antibiotic resistance and their likely origins. (Jørgensen et al., 2018b).

### 1.2.2.4 Low diversity of available modes of action

Managing resistance supposes the access to a variety of modes of action (§2.2) (Fair and Tor, 2014; Jørgensen et al., 2018b; R4P Network, 2016; REX Consortium, 2013). However, such possibilities are not always wide, or even existent. Only a limited number of AIs and MoAs has been discovered and registered during the last decade, shifting the selection to the MoAs already approved and potentially concerned by resistance (Figure 15) (Haywood et al., 2021).

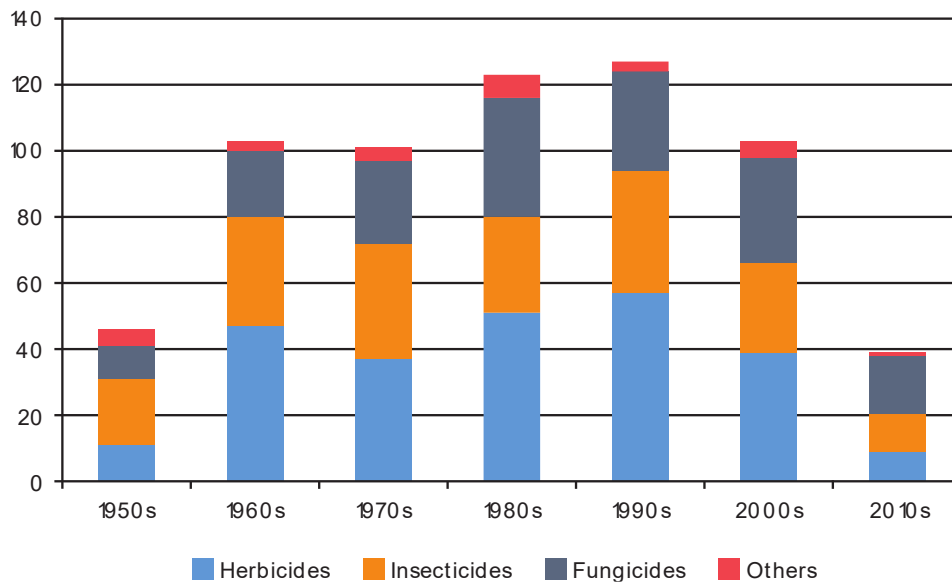


Figure 15: Number of new pesticide active ingredients introduced per decade: 1950s to present day. (McDougall 2018)

Since the early 90s, the pesticide market has extremely evolved. On one side, standards for certification are now considering seriously health and environmental effects of pesticide, hopefully increasing safety for users and biodiversity. New regulations (*e.g.* Regulation (CE) No 1107/2009 for pesticides) are in place, with further studies on toxicological and ecotoxicological risks and environmental impacts. Those additional studies were representing about 25% of the cost of a new pesticide (2010-2014) (McDougall, 2016). In addition to the tougher regulation, the obstacle to the development of new drug or pesticide can also be economical. Indeed, the cost necessary to research, develop and register a new AI to be used in agriculture or in medicine has increased of about 88% between 1995 and 2014 (McDougall, 2016). In its study McDougall (2016) estimated that, between 2010 and 2014, only one molecule on about 160,000 synthesized was registered and that the investment costed in average US\$ 285 million and 11.3 years (between the first synthesis and the first sales). Pharmaceutical industries are more actively looking for selective pesticides and drugs to avoid the undesirable effects on non-target organisms and humans (Umetsu and Shirai, 2020). However, this specificity can also imply an easier acquisition of resistance and therefore short benefits. Consequently, some drug companies now consider that the potential profits of a new antibiotic do not justify such investment. Indeed, antibiotics are sold at a low price (particularly off-patents molecules into their generic forms) which shaped the vision of buyers that are not expecting to change this

habit even for a more complex new AI (Ventola, 2015; Woolhouse and Farrar, 2014). To counteract this disinterest for antibiotics research and development, new programs have recently been launched to promote such investment, by instances like the World Health Organization, the European Center for Disease Prevention or the Control or the Infectious Diseases Society of America (Fair and Tor, 2014).

On the other side, raising safety standards for pesticides and drugs registration also induced the withdrawn of many AIs over the past 20 years. From the original 900-1000 active ingredients that were available in the pesticide market, only about 350 remain nowadays (Leadbeater, 2011). A striking example is the gradual removal of multisite fungicides (such as chlorothalonil, largely used in cereal markets) and of azole fungicides, because of their endocrine disrupting properties. For all these reasons, the balance between the introduction of new AIs and their removal is largely in deficit but this hopefully contributed to preserve human health and biodiversity. Besides, the reduction of the number of registered MoAs escalates the prevention and management of resistance, since it relies on a diversity of mode of action and the use of low resistance risk AIs.

#### 1.2.2.5 Low availability or use of alternative control measures

Besides pesticides and drugs, other measures (*e.g.* prophylaxis; host immunity, detailed in § 2.2.2.1) are available to control pests and diseases. Alternative solutions are already at work in the field or are in development. Umetsu and Shirai (2020) noticed that there is a shift from R&D on synthetical chemical pesticides in favour to biological ones and predict that this trend will expand. However, the challenge remains to implement them in the field or clinics at accessible cost (even for developing countries) and with comparable and regular efficacy as the “conventional” solutions (Ghosh et al., 2019). As an alternative to antibiotics, several technologies are already clinically tested (antibodies, probiotics, bacteriophages, antimicrobial peptides) while others are still at the research stage (silencing of resistance genes with oligonucleotides for example) (Ghosh et al., 2019; Woolhouse and Farrar, 2014). One of the alternative solutions with the highest efficacy against pathogens in agriculture is their genetic control with the use of resistant or tolerant cultivars (see the example of *Zymoseptoria tritici* in § 3.2.1.2). Even if this trend is recently evolving, for years, varietal selection has been mainly based on yield criteria rather than on tolerance to pest and diseases (Bailey-Serres et al., 2019). This has

led to more susceptible crop populations needing even more treatments. Yet, pathogens may also adapt to plant resistance genes and resistant cultivars are not available for all crops, especially minor ones, which again defers control through pesticides. For example, cultivars of wheat resistant to yellow rust have been introduced from 1975 in Northern Europe. Once virulence has been first detected in 1994, it only needed four years for the generalisation of *Puccinia striiformis* f.sp.*tritici* variants able to bypass this host resistance (Bayles et al., 2000). The introduction of new control methods (*e.g.* biocontrol, varietal resistance) will slow down the selection of resistance to pesticides or drugs, but will defer, by diversification, selection pressure onto other genes.

### 1.2.3 Social, economic and environmental consequences of resistance

#### 1.2.3.1 Direct impact of resistances: decrease of treatment efficacy

As detailed previously, resistance selection is the gradual increase of resistance frequency in populations, which may lead to practical resistance in some situations. This default in efficacy has direct consequences on human mortality and available food quantity and quality. Public health is severely affected by resistance to drugs and has major social and economic impacts. Resistance of insect vectors of malaria (*as Anopheles* mosquitoes) costs more than \$260 million each year in sub-Saharan Africa and many lives (Gould et al., 2018). For Fullybright (2019), about 25,000 people will die yearly in Europe due to antibiotic resistance. In 2019, the Centers for Diseases Control and Prevention (CDC) estimated that, in the USA, more than 2.8 million antibiotic-resistant infections occur each year, and more than 35,000 people die as a result. However, Aslam et al. (2018) considered that this number is higher and that there are 99,000 deaths each year in the USA due to antibiotic-resistant pathogen-associated hospital-acquired infections (HAIs). In any case, the yearly economic burden for USA of these antibiotic resistant infections has been estimated to about \$20 billion in health care costs and \$35 billion in productivity loss (Golkar et al., 2014). Those costs are associated to longer hospital stays and disabilities, increased medical attention, and more complicated treatments (higher doses, alternative drugs more expensive or toxic) (Adedeji, 2016; Ventola, 2015). In agriculture, the lack of pesticide efficacy impacts yield but also quality (for example, because of the presence of mycotoxins including during post-harvest conservation) of the agricultural production. The

economic impact of resistance on agriculture is hardly assessed but just in the United States, it was estimated around US\$10 billion per year (Palumbi, 2001). Just the glyphosate resistance in corn, cotton and soybean fields in the USA may cost annually US\$1 billion (Frisvold et al., 2017). If severe epidemics were occurring simultaneously on five major crops (hopefully not likely), food security would be jeopardized as only 39% of world population would be correctly fed (Fisher et al., 2012).

In addition to this direct economic impact, resistance also arises the social and political issue of food sovereignty and more globally questions the need to feed a growing worldwide population with qualitative products.

### 1.2.3.2 Indirect consequences of resistance: an increasing of doses and number of treatments

The erosion of pesticides and drugs' efficacy has also various indirect consequences. This includes, when authorized, the increase in applied doses or/and additional treatments, possibly with other MoAs not concerned by resistance but of greater toxicity (Leroux et al. 2007). These catch-up practices are not without consequences for the global pesticide use and its negative effect on human health and biodiversity.

#### *Economic impact*

Additional treatments may decrease the potential benefit (for farmers or health care system) as a second-line treatment is often more expensive than the first pesticide or drug application (Adedeji, 2016; Laxminarayan et al., 2006; Ventola, 2015). In the medical field, malaria management would suffer from an additional cost of about US\$200 million yearly due to resistance and supplementary treatments. Such economic impact will also be indirectly experienced by consumers.

#### *Impact on human health*

Increasing the number of treatments or substituting them with more toxic alternatives globally increases communities exposure to health damages (Adedeji, 2016; Ventola, 2015).

This issue principally concerns workers that would have not been protected (because of ignorance of safety guidelines or lack of protective equipment) or would have been involved in an accident. Non-target population can also be exposed due to spray drift or by food and water contaminations (which stresses the importance of regulation on pesticide and drug residues) (Damalas and Eleftherohorinos, 2011).

Direct or indirect exposure to pesticides have consequences on human health either by a toxic/poisoning effect or chronic exposure. For example, some molecules can be toxic (with fatal or non-fatal outcomes) or carcinogenic and may result in reproductive and neurodevelopmental disorders and endocrine disruption (Bourguet and Guillemaud, 2016). The effect of chronic exposure is more complicated to assess and quantify economically. They are suspected to cause severe diseases such as cancers, diabetes, blindness, etc.

### *Impact on biodiversity*

Intensified exposure due to resistance does not only affect humans. Many studies described the negative effects of pesticides or drugs on their environment. Early after their introduction, toxic effects of pesticides on natural fauna have been reported (*e.g.* the book "Silent Spring" from Rachel Carson published in 1962). Abiotic compartments (air, soil and water) can also be highly contaminated with variable quantities of pesticides or residuals of pesticides on short or long periods, depending on their persistence. This contamination is mainly local. But dissemination due to spray drift or seep in soil and water may also take place on a wider scale. Living organisms, as vertebrates and invertebrates animals, plants, algae and microorganisms, of these abiotic compartments can be affected. Therefore, pesticides may mitigate ecosystemic services, change natural communities, modify ecosystems and even select resistance in wild species (Jørgensen et al., 2018b). Pesticides can be toxic, harmful or affect the survival or reproduction of non-target organisms, *via* direct and indirect effects (Bernardes et al., 2015; Bourguet and Guillemaud, 2016; Regnault et al., 2012; REX Consortium, 2013).

#### 1.2.3.3 A decrease of pesticides uses hard to reach

Due to the consequences on health and the environment, societies, and politics (particularly in Europe) are willing to decrease this impact by a global reduction of pesticides use.

In France, the “Plan Ecophyto II+” traduces this wish to reduce and improve the use of phyto-pharmaceuticals products (Ministère de l’agriculture et de l’alimentation, 2021). The first version of this plan was proposed in 2008 but has been reviewed several times, facing the difficulties of reaching the original objectives. Indeed, resistance omnipresence in pathogens does not facilitate this plan, as treatment failures lead to a global increase of pesticide use.

Today, this project is based on a global implication of all actors and a support to farmers. It aimed at reducing, if possible, the use of pesticides by 25% for 2020, and by 50% for 2025, at better handling pesticides risks and impacts and finally enhancing the use of pesticides alternatives. We can note that in some very particular cases, research of new AIs or alternatives is accelerated “thanks” to the presence of resistance (for example when there is a generalized resistance to every drug or pesticide available for a pathogen of major importance). For example, the WHO established a list of pathogens for which research and development must be a priority (e.g. *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae* are classified as critical priorities) (Tacconelli, 2017).

#### 1.2.4 At the crossroads: the urge for resistance prevention and management

In the face of these enormous consequences in human, environmental and financial aspects, solutions to prevent and manage resistance are highly expected. The arrival on the market of new products efficient on resistant organisms is probably the most expected solution but should be of short-term efficacy without management, as resistance to those new pesticides will quickly occur as well (Gould et al., 2018; Jansen et al., 2013). That is why, in parallel to new compounds discovery, management is needed to delay resistance selection, while maintaining a good control of the diseases and pests. The next section of this introduction will detail the ins and outs of resistance management.

Recent dramatic cases of resistance contributed to change mindsets. Susceptibility to drugs or pesticides should be considered as a common good. By preferring practices with short term benefits but selecting resistances on the long term, users are spoiling this resource at the expense of the whole community and are heading towards a “Tragedy of the Commons” (Hardin,



1968). Resistance phenomenon and its consequences are now considered by politicians regulating pesticide use and by companies through the work of Resistance Action Committees (RACs) from CropLife International. In order to delay resistance as much as possible, resistance risk assessment (RRA) before the introduction of a new AI in the market is now recommended by the Food and Agriculture Organization of the United Nations and is even required for pesticides authorisation in several areas (R4P Network, 2021). This is for example the case in the EU and Australia. In the EU, there are two key principles in Directive n° 2009/128/CE about resistance management. First, suitable anti-resistance strategies must be applied when an important resistance risk has been established. Secondly, the strict minimal volume of pesticides necessary to reach an acceptable disease control must be used (attached with the promotion of non-chemical alternatives for example), so that resistance development does not increase in populations. Recently, even China increased its requirements around RRA while in the USA, New-Zealand and Japan there it is still not compulsory. Besides RRA, actions may be implemented for a better management of resistance and its consequences. For example, Jepson et al. (2020) provided guidelines and a list of pesticides with a minimum risk for human and environmental health.

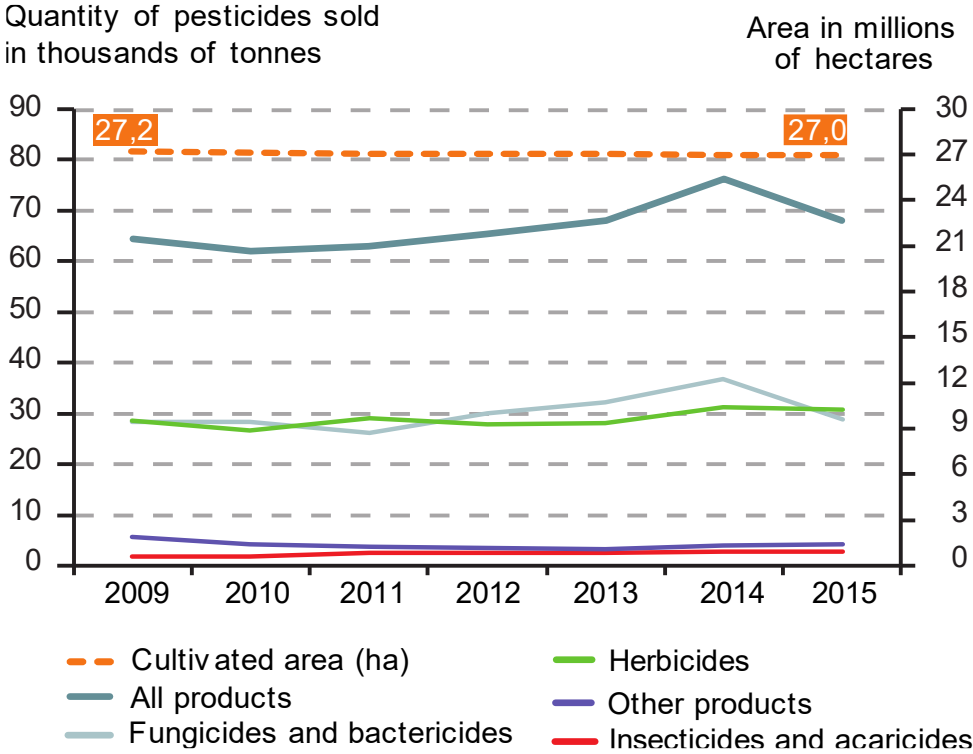


Figure 16 : Evolution of pesticides sales in France between 2009 and 2015. Adapted from (Ministère de la transition écologique, 2017).

Similarly, Adedeji (2016) reminds the need for education on drugs resistance. For example, campaigns like “antibiotics are not automatic” have been launched for more than ten years by public authorities and the World Health Organization organises each year the World Antimicrobial Awareness Week.

Unfortunately, despite the awareness around resistance issues, pesticides and drugs use hasn't changed sufficiently (Figure 16).

Socio-politics are recently investigating this field to propose new approaches for the example of France. Associated with alternative methods, scientific research on resistance management is therefore still needed to achieve an efficient transition towards systems less reliant on drugs and pesticides.

## 2 The prediction and management of resistance to pesticides and drugs

As detailed in the previous section, resistance is the adaptive response of pathogens to a massive and often homogeneous use of drugs or pesticides. Depending on the pathogen, the considered AIs and the selective forces at play, this adaptation process may differ for its dynamics and underlying mechanisms. Nevertheless, the emergence and selection of resistance may ultimately lead to resistant phenotypes invading the population with major consequences for the agricultural or medical systems. Prediction studies might be useful to estimate which resistant alleles might arise and to describe the associated phenotypes. Once resistance has emerged, prediction of resistance dynamics may allow the implementation of adapted strategies (Figure 17). In addition, according to zur Wiesch et al. (2011), prevention measures should be considered when resistance has not emerged yet (or do not pre-exist), whereas management measures should be applied once resistance is present in the population.

This section presents the principles underlying the prediction and management of resistance in a general manner, but with a specific regard to fungi, as they constitute the biological model for this thesis.

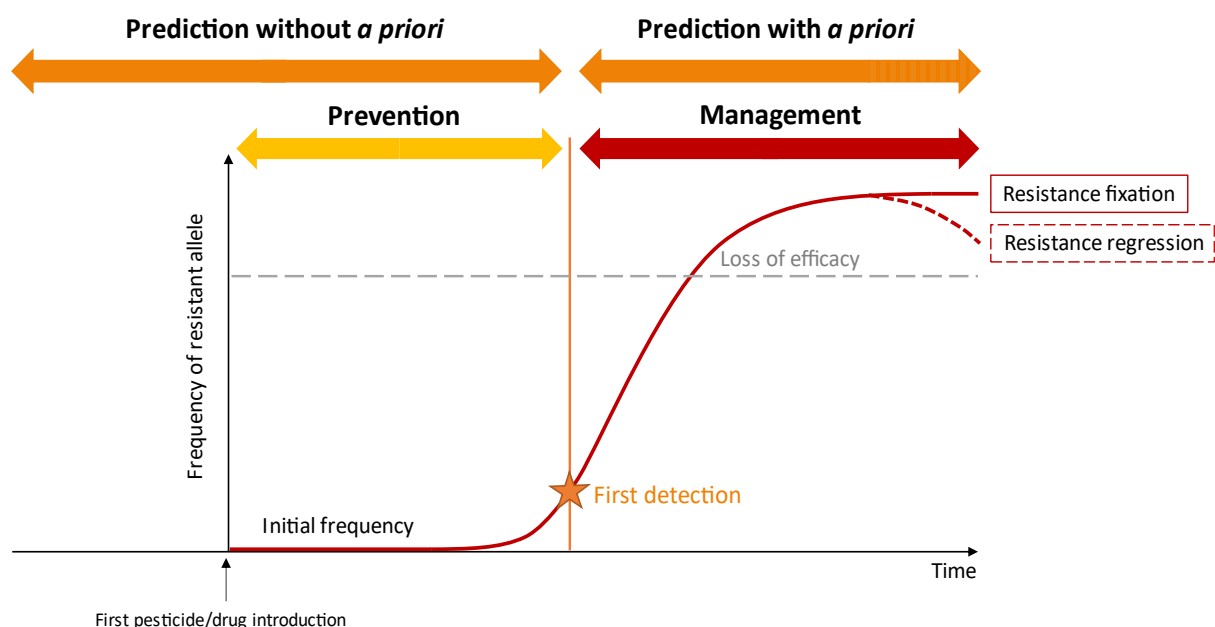


Figure 17: Scientific and operational questions and the evolution of resistance. The initial frequency of resistance will influence the duration of the prevention phase.

## 2.1 Predicting resistance to pesticides and drugs

Predicting adaptation is a key issue for evolutionary biology and requires answering several questions. In the context of adaptation to pesticides and drugs, one of them is to predict which variant (*i.e.* which mutation, and then which resistance mechanism) will be selected after exposure to a pesticide or drug. This is crucial to adapt management strategies since their efficacy depends upon the fitness of this variant and its spectrum of cross-resistance. This is especially important for new modes of action, for which resistance is still unknown, and for pathogens or pests not yet concerned by resistance. A second question to be answered is how this resistance will evolve (*i.e.* what will be the dynamics of the resistance). In particular, it is useful to predict the time for emergence of resistance and the rate of resistance progression at various spatio-temporal scales. Different strategies and tools aim at answering these two sets of questions that contribute to RRA (National Research Council, 1986).

### 2.1.1 Predicting resistant variants most likely to emerge in a population

Even before the detection and characterisation of the first resistant mutant, and thus without having a precise preconception of the most likely mutant to emerge, it is possible to make predictions based on the study of other organisms already facing resistance to this MoA. Indeed, some mutations and/or resistance mechanisms to a given MoA might be shared by several species. This recurrence can result from interbreeding, HGT or from independent parallel evolution meaning that the same genetic changes arose independently in multiple species (Hawkins et al., 2018). Being selected in many different organisms gives mutants an increased probability of also being selected in the target organism. Laboratory mutants carrying those genetic changes can also be produced, in order to verify their susceptibility to the MoA (*via* functional validation if possible) and to assess their fitness. The docking between the mutated target protein and the AI can be modelled *in silico* to speculate on their loss of affinity and compare the potential resistant alleles that could emerge. For example, it has been predicted that the presence of a type I intron after the codon 143 of *cytb* in plant pathogens would likely prevent the emergence of the substitution G143A responsible for QoI resistance in several fungi (Grasso et al., 2006).

If the parallel with other organisms cannot be made, as resistance has not been described in other species (particularly for a new MoA), the resistant mutants can be generated experimentally by directed evolution. The same analyses (resistance level, fitness, docking) and validation as described before can then be carried out on those mutants obtained by mutagenesis or experimental evolution. Such directed evolution is possible either on solid or liquid medium and with or without mutagenic agents (chemicals, UV) (Gutiérrez-Alonso et al., 2017; Hawkins and Fraaije, 2016; Lalève et al., 2014). For example, chemical mutagenesis production of mutants of the *Leishmania* parasite, followed by drug selection of mutants and combined with genome-wide screens revealed the biological recurrence of mutations associated with the antimonial resistance phenotype and the corresponding resistance mechanism (Bhattacharya et al., 2019). By contrast, Fouché et al. (2020) selected resistance to a new fungicide MoA with an experimental evolution protocol (see Box 3 for further information on this approach) including several applications of the AI on *Z. tritici*, without any additional mutagenic agent. The experiment was conducted over several cycles in order to select the fittest mutants among those that appeared without direct intervention and thus establish the mutants the most likely to arise. Such investigations may influence preventive actions of resistance management and may guide research and development.

### 2.1.2 Predicting resistance dynamics

Predicting resistance dynamics involves forecasting the evolution of the frequency of resistance mutations in populations over time and space, while identifying the factors that most affect resistance selection and including them in prediction models. The risk of resistance evolution then depends on hazards, inherent to the pest or pathogen, inherent to the drug or pesticide, and resulting from their interaction, *i.e.*, from resistance genetics. It also depends on population exposure to pesticides or drugs. Indeed, a strong resistant variant will be hardly selected in a population poorly exposed to the relevant compound. RRA then relies on the detailed knowledge accumulated on hazardous traits from pests or pathogens and drugs or pesticides and on a precise view of the selection pressure applied on populations. RRA is preferably achieved with preconception about resistance past evolution in populations, resistant variants and the past and current use of pesticides and drugs.

### 2.1.2.1 Hazard characterization

#### *Hazard inherent to the pest or pathogen*

Depending on its evolutionary potential, a pest or a pathogen will be more or less susceptible to develop resistance rapidly (Holt et al., 2013), and this hazard depends on its biological and genetic characteristics (Grimmer et al., 2014b; Moss et al., 2019). Indeed, as introduced in § 1.1.1, the mode of reproduction, generation time, number of host species impact the size of a population, whereas its structure is influenced by the mutation rate, the possibility of gene recombination or accumulation, the genome size and plasticity, among other traits. The larger the population, the greater the mutation load and then the possible emergence of resistance, especially from SGV (Barrett and Schluter, 2008; Hawkins et al., 2018; O'Donnell et al., 2014; Orr, 2005). The dispersal capacity of a pathogen or pest is also important to consider (by the air, rain or transport by humans or animals). Indeed, resistance can be acquired through the immigration of resistant individuals.

The occurrence of resistance mechanisms (*ie.* TSR *vs.* NTSR) also differs among taxa, some of them being less susceptible to display NTSR mechanisms. For example, weeds commonly metabolise herbicide, reduce its uptake, translocate or/and sequester it in vacuoles (Gaines et al., 2020; Jugulam and Shyam, 2019) while NTSR seems rather rare in pathogenic fungi, where only a few examples of enhanced efflux and metabolization have been described yet (Lucas et al., 2015).

#### *Hazard intrinsic to drugs and pesticides*

Certain MoAs have been easily overcome by the emergence of resistance mechanisms, while for others resistance has never been described. This underlines a variety of intrinsic resistance risks between MoAs, which mainly depend on the structural conformation of the AI and its target site (Délye et al., 2013), and the number of target sites concerned by this MoA (unisite *vs.* multisite) (Gaines et al., 2020; Grimmer et al., 2014b). Indeed, in a highly preserved target, a single mutation can induce major conformational changes. Hence, resistance to an AI may occur but this structural modification might be responsible for the loss of the initial function of the target. On the contrary, some other target sites may endure several mutations without losing their primary function, leaving more opportunities for resistance to emerge.

### *Interaction between pests or pathogens and pesticides or drugs*

The resistance mechanism and its genetic complexity (polygeny, polyallelism, degree of dominance) must also be considered when assessing the risk of resistance. A resistance of low intensity (low RFs), usually associated with quantitative resistance and/or NTSR is generally considered at lower risk of practical resistance, at least in the first stages, than high-resistance mutations associated with qualitative resistance. For example, a single substitution at codon 119 (close to a catalytic serine) of the *Ace-1* gene (coding for acetylcholinesterase) in *Anopheles* and *Culex* mosquitoes confers high resistance to carbamates and organophosphates and has been associated to rapid increase in mosquitoes survival to treatments (Alout and Weill, 2008; Weetman et al., 2015). By contrast, a single mutation in *grlA* in *Staphylococcus aureus* is responsible for low-level resistance to the antibiotic ciprofloxacin. A high level resistance may be achieved but through the accumulation of mutations in *gyrA*, *gyrB*, *grlA*, *griB* or *norA* promoter region, which therefore is either less likely or takes more time to establish (Jones et al., 2000). By influencing both the level and the spectrum of resistance, the mechanisms responsible for the resistance shape the hazard associated to a given AI. Depending on the AI, the resistance mechanisms preferentially selected may differ. For example, resistance to herbicides from groups G and D is generally NTSR due to altered translocation, whereas herbicides from groups A and B generally select for active metabolization in the same species (Délye et al., 2013). While increasing resistance spectra in an unpredictable manner, NTSR increases the hazard of practical resistance when associated to sufficient RFs in the considered species. At last, the degree of dominance, in interaction with the reproduction mode, may mitigate resistance evolution in non-haploid organism. As an example, the recessivity associated to the allele responsible for resistance to CAA inhibitors certainly explain the residual efficacy of these fungicides in population of the oomycete *Plasmopara viticola* (downy mildew of grapevine), as heterozygote individuals are still susceptible (Blum et al., 2010).

#### 2.1.2.2 Exposure assessment

Exposure assessment relies on the thorough description of the selection pressure applied on a population. This first includes the fraction of the population which is exposed to a

given pesticide or drug. This fraction is modulated by the maintenance of untreated reservoirs, and by agricultural or clinical practices (*e.g.* crop rotation, area or number of patients treated (*i.e.* untreated areas or untreated alternative hosts for pathogens) with a given AI, connection between the treated groups) (Ayala and Villela, 2020; Délye et al., 2013). Second, exposure also includes the amount of drugs or pesticides received in a given unit of time. This embraces the number and rhythm of treatments and the dose used. The dose of an AI may influence the mutation rate of a pathogen (Martinez and Baquero, 2000) or the type of mechanism selected (Jugulam and Shyam, 2019). This relation may be established by harm-dose response assessment, which reflects the quantitative relationship between the dose of an AI and the probability and type of resistance (Claycamp, 2015). At last, exposure assessment should also consider the respective used quantities and frequencies of each AI. Indeed, as mentioned previously, AIs might be associated to variable hazards, even within a single mode of action, and have different persistence and systemicity, which determines the duration of the exposure. Altogether, these traits mitigate the selection pressure quantitatively (*i.e.* govern the selection rate) and qualitatively (*i.e.* govern the associated resistance mechanism) (EPPO, 2015; Grimmer et al., 2014b; Moss et al., 2019; National Research Council, 1986).

In practice, the detailed information about the use of pesticide or drugs is recorded from sales or from user representative panels. Such information, strategic from a commercial point of view for concerned companies, is often private, and then hardly accessible, especially at a sufficient grain for fine analysis of the relation between resistance rate and selection pressure. Nevertheless, some national policies made then public, for example in Denmark (Kudsk et al., 2018), in UK<sup>2</sup> or very recently in France<sup>3</sup>.

*To conclude, the hazards inherent to the characteristics of pests and pathogens and of the drugs or pesticides can hardly be manipulated. By contrast, exposure of targeted populations can be limited or mitigated by smart resistance management (§2.2) actions which will ultimately decrease resistance risk (van den Bosch et al., 2011).*

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<sup>2</sup> <https://secure.fera.defra.gov.uk/pusstats/myindex.cfm>

<sup>3</sup> <https://www.data.gouv.fr/fr/datasets/ventes-de-pesticides-par-departement/>



### 2.1.2.3 Strategies and tools to predict resistance dynamics

#### *Resistance risk matrices and scoring systems*

It has been proposed to combine the individual components of RRA described above into simple tools such as resistance matrices. As an example, Figure 18 shows the matrix established for fungicides (Brent and Hollomon, 2007a). Briefly, hazards (hereafter mentioned as fungicide and pathogen risk by the authors) and exposure (hereafter mentioned as the agronomic risk) were subdivided into categories, ranked according to their specific risk level. Major plant pathogens and fungicides MoAs were assigned within these categories, based on published empirical studies and on expert knowledge. For example, FRAC published a risk table for fungicide resistance.(Figure 18).

<b>Fungicide Risk</b>	<b>Risk</b>	<b>Combined Risk</b>			<b>Agronomic Risk</b>
Benzimidazoles Dicarboximides Phenylamides QoI fungicides	High = 3	3 1.5 0.75	6 3 1.5	9 4.5 2.25	High = 1 Medium = 0.5 Low = 0.25
Carboxanilides SBI fungicides Anilinopyrimidines Phenylpyrroles SDHI*	Medium = 2	2 1 0.5	4 2 1	6 3 1.5	High = 1 Medium = 0.5 Low = 0.25
Multi-site fungicides MBI-R inhibitors SAR inducers	Low = 0.5	0.5 0.25 0.125	1 0.5 0.25	1.5 0.75 0.3	High = 1 Medium = 0.5 Low = 0.25
		Low = 1	Medium = 2	High = 3	<b>Pathogen Risk</b>
		Seed borne & soil borne pathogens Rust fungi	Eyespot <i>Rynchosporium</i> <i>Septoria</i>	<i>Blumeria</i> <i>Botrytis</i> <i>Plasmopara</i> <i>Magnaporthe</i> <i>Venturia</i>	

Figure 18: Fungicide resistance risk matrix. \*Medium to high according to FRAC. (Brent and Hollomon, 2007a).

The agronomic risk is assigned according to the agricultural practices implemented in the considered situation. Each category is associated to a score that enables to calculate the global resistance risk while multiplying the scores achieved for each of the three-risk components.

The higher the score, the more likely resistance is to evolve rapidly. A similar matrix has been published recently to predict the risk of resistance to herbicides (Moss et al., 2019).

This method is quite easy to use for well-known pathogens and pesticides and is of educational interest. However, comparing the time to resistance emergence predicted from the matrix and that observed in field conditions showed that the predictability of this method was unreliable for new MoA or emergent pathogens, and for unisite AIs in general (Grimmer et al., 2014a).

Without using a “matrix” strictly speaking, scoring systems are also used for antimicrobial resistance with some good quality prediction for some of them (Boyd et al., 2020). For example, the “GSDCS” scoring system identified risk factors (*e.g.* a stay of more than 5 days in an intensive care unit) and points were attributed for each of them, then summed up to establish a final score predicting infection by Resistant Gram negative bacilli (Vasudevan et al., 2014).

### *Statistical analysis of historical empirical data on resistance dynamics*

Empirical datasets on resistance evolution are usually generated through detection and monitoring plans. Those plans are designed to detect any new resistance emergence and quantify the evolution of the resistant known phenotypes over time and space (R4P Network, 2016). The regular and thorough description of resistance cases (their number, the associated mechanism, the environment in which they developed ...) contributes to RRA considered in resistance risk matrices previously detailed and give up-to-date resistance status to enlight decisions of stakeholders. Monitoring programs that collect such empirical data should therefore be implemented as soon as possible, long before resistance in practice is observed in order to provide (or complete) the information necessary to operational predictive tools (National Research Council, 1986; R4P Network, 2021).

These empirical data can also be used to infer the population dynamics of resistance *via* statistical modelling, *i.e.* models exploring the relation between the variation of resistance frequency or time to emergence and hazard or exposure traits. Equations of such models then do not describe the mechanisms occurring in populations and leading to resistance. These models are useful to identify and validate the key drivers of resistance evolution over time and space in field conditions for a given pathogen or pest. Using empirical data acquired along the way and information about the significant drivers for resistance evolution, such models can also be used in a predictive manner, integrating the history of selection in a local context, and

calculating projections for further years or new situations, enabling also to test future scenarios of pesticide use (Claycamp, 2015; Niewiadomska et al., 2019; Van Camp et al., 2020; Yu et al., 2018).

As an example, Grimmer et al. (2014b) established a linear model which quantifies the time (number of years) between the introduction of an AI and the first resistance detection depending on biological, fungicide and operational criteria. This model was calibrated using literature data and determined pathogen latent periods per year, fungicide molecular complexity, number of crop host species and the agronomic system as the best combined predictors of the emergence time. Traits most driving resistance have been analysed in *Zymoseptoria tritici*, taking advantage of a 20-year national-scale survey carried out in France (Garnault et al., 2021). The model underlying this analysis is now used routinely to predict resistance evolution at the regional scale, year after year, using historical data of resistance frequency and fungicide use (Garnault *et al.*, unpublished). Another national survey on herbicide resistance in the UK highlighted the number of herbicides applications as the main driver of resistance evolution (Hicks et al., 2018).

*RRA is necessary to anticipate the most worrying situations and particularly to guide detection and monitoring programs (Ngow et al., 2020). It is a prerequisite to implement early resistance management and reduce exposure to resistance hazards. Indeed, once resistance frequency is high in population, it is somewhat illusory to hope to reverse a population back to full susceptibility, except if a strong cost entails fitness of resistant individuals. Detection and monitoring are, thus, also essential (Raymond, 2019). That is why European Union through EPPO requires information on the likelihood of resistance development and imposes management strategies when potentially worrying resistance situations are assessed (EPPO, 2015).*

## 2.2 Resistance management relies on maximising the heterogeneity of the selection pressure exerted by pesticides and drugs

### 2.2.1 Theoretical principles to limit resistance evolution

Resistance management consists in the implementation of actions to delay the emergence of resistance and prevent, or at least limit, the increase in the frequency of resistant individuals in a population. In the best case, anti-resistance strategies may decrease the growth rate of resistance. Prevention measures apply when resistant mutants have not yet been detected in the population and therefore cannot be specifically adapted to the biological and genetic characteristics of the resistant variants. Once those have been detected and described, sound management measures can be implemented in full knowledge of resistance traits (Figure 17).

#### 2.2.1.1 Decreasing the occurrence of resistance mutations by controlling population size and gene flow and reducing exposure to pesticides and drugs

If resistance is not yet present in a population, resistance prevention lies in the reduction of population size, to reduce the probability that resistance emerges from *de novo* mutation (National Research Council, 1986). As detailed in § 1.1.1, the rate of emergence of resistant alleles from *de novo* mutation depends on its genomic architecture and on its fitness. The maintenance of low population size, for example by using other control measures, favours the loss of resistance alleles through genetic drift, especially when these alleles are still at low frequency and/or when the population is submitted to regular bottlenecks. An efficient reduction of population size has not only an impact on resistance emergence, but it also reduces resistance selection by diminishing the exposure of the population to the drug or pesticide selection pressure (“lighter” chemical treatment).

Gene flow may also contribute to increase resistance frequency in a population and to accelerate adaptation. Its limitation might then be desirable to decrease the possible introduction of resistance genotypes in the population or to increase the diversity of resistant populations. This may be achieved by preventing physical proximity between populations and interfering

with reproduction (Choudhuri, 2014). On the contrary, if gene flow is source of susceptible individuals' introduction from non-resistant populations, it shouldn't be prevented.

### 2.2.1.2 Limiting the selection of resistance

Once resistance has emerged in the population, the evolution of its frequency mainly depends on the selection pressure (quantified *via* the selection coefficient) and the duration of this exposure (van den Bosch et al., 2014a; National Research Council, 1986). Hence, to prevent the frequency of resistant allele from increasing, either the exposure time must be reduced, or the selection coefficient must be stabilized or decreased. As introduced in 1.1.2.2, the selection coefficient is defined as

$$s = w_R - w_S = W_R/W_S - 1,$$

where  $w_R$  is the relative fitness of the resistant strain, defined in relation to the susceptible strain ( $w_S = 1$ ), and  $W_R$  and  $W_S$  are the absolute fitnesses of the resistant and susceptible strains (Gillespie, 1998; Orr, 2009; Serre, 2006). Minimizing the selection coefficient  $s$  can be achieved either by decreasing  $W_R$  relatively to  $W_S$  or increasing  $W_S$ .

#### *Decreasing the exposure time to the selection pressures*

In a given environment, where the selection coefficient  $s > 0$ , the resistant alleles will be selected through time in the population. The probability that the frequency of this resistant alleles become high enough to be fixed in the population or that resistance in practice is observed will depend on the time of exposure to this environment. The less the population is exposed to these selection pressures favourable to the resistant allele, the less selection will operate, and the less its frequency will increase. Then time of exposure should be limited as much as possible to prevent resistance selection.

#### *Increasing the fitness of susceptible individuals by decreasing the dose of pesticides and drugs*

In an environment where the dose of a treatment is decreased, susceptible individuals survive more frequently than under the exposure to a full-dose treatment, in a manner correlated to the dose response curve established for the pesticide or drug and the resistance mechanism. Consequently, the  $W_R/W_S$  ratio may decrease with dose reduction. The reduction of the dose could be considered not only within a single application but also globally in a landscape or a timeframe.

Additionally, as some AIs might be slightly mutagenic, the dose reduction of pesticides and drugs may also decrease the emergence of *de novo* mutations.

### *Decreasing the fitness of resistant individuals with multidirectional selection*

An option for minimizing  $s$  would be to decrease the fitness of resistant individuals in treated environments, *e.g.* while increasing the dose of the pesticide or drug until it exceeds their MIC value. However, this option should be limited for genotypes exhibiting low to moderate resistance. Indeed, for highly resistant individuals, the MIC values might exceed the registered dose, *i.e.* the acceptable dose according to toxicological and ecotoxicological standards. This option may then not be operational and may be reserved for a limited number of situations. The selection coefficient,  $s$ , can also be minimized when a cost is associated with resistance as it reduces the fitness of resistant individuals relatively to that of susceptible strains. However,  $s$  would be negative only when populations are not exposed to pesticides and drugs ( $W_S > W_R$ ). Relying on this option might then be of limited interest, as fitness costs are not systematically observed when dealing with resistance to pesticides and drugs and compensatory mutations can occur.

Consequently, a better option to decrease the relative fitness of resistant individuals is to turn the treated environment into another one less favourable to resistant individual adapted to the given AI. Indeed, the per capita rate of increase (absolute fitness) of an individual is not dependent from a single trait. Then, alternative environmental conditions may favour some alleles and disadvantage some other ones, the absolute fitness resulting from a trade-off between these multiple alleles. By imposing multidirectional selection, the probability that a unique resistant phenotype is favoured in every environment is low. For example, Uecker and Hermisson (2011) mathematically analysed the probability of fixation of an allele from an haploid organism

in a periodically changing environment and concluded that the resistant one may be sometimes selected ( $W_R > W_S$ , selective advantage of resistant individuals) and sometimes counter selected ( $W_S > W_R$ , selective disadvantage of resistant individuals). In particular, the probability of fixing an allele depends upon its relative selective advantage in a given environment, and upon the duration of exposure to this latter. Thus, the more an environment is changing, the less adaptation may operate. Hendry et al. (2011), Jørgensen et al. (2018b) and REX Consortium (2013) translated this principle by recommending maximizing the heterogeneity (or dimensionality) of the selection pressures, measured as the degree of treatment heterogeneity (DTH), to slow down the evolution of resistance. The sources of heterogeneity are multiple and detailed in the next section. At last, it should be noticed the urge to implement strategies as early as possible, as stressed in modelling studies, since they are all the more efficient to delay resistance in practice than the initial frequency of resistance is low (Hobbelen et al., 2011; Milgroom and Fry, 1988). Therefore resistance management programs should be introduced as early as possible i.e. from the introduction of a new MoA (van den Bosch et al., 2014a). Today resistance management has become an integral part of the submission dossiers.

## 2.2.2 Translating theoretical principles into applied strategies to delay resistance selection

### 2.2.2.1 Decreasing population size, gene flow and exposure by the mean of non-chemical methods

Population size can be reduced in different ways. First, multiple non-chemical actions can be implemented to limit disease pressure. They differ according to the organism considered but mainly relies on the establishment of non-favourable conditions to the multiplication or transmission of the pest or pathogen. In agriculture, integrated pest management (IPM) promotes the use of all available methods to keep populations of pests and pathogens under thresholds of economic damage. Its principle is to reduce pesticides as much as possible to minimise their damage on human health, environment and agricultural sustainability (Ciancio and Mukerji, 2008; European Commission, 2017). In particular, crop rotation, prophylaxis, the use of resistant or tolerant varieties (non-compatible interaction with the host), plant defence stimulators, mechanical weeding or residue destruction or the release of beneficial insects (*e.g.* ladybirds as predators of aphids) have proven efficacy to control populations and are promoted

by current policies (Barzman et al., 2015; Bodin et al., 2020; Gilligan, 2008; Owen et al., 2014; Riddick, 2017). Unfortunately, non-chemical tools are sometimes not available to control some pests or pathogens, because of regulatory, technical or economic reasons (Dara, 2019). In health care, vaccination, general hygiene and non-physical interventions or community mitigation strategies highly participate in reducing the size of pathogenic microbial populations (Center for Disease Control and Prevention, 2020; Rainey et al., 2016; Wu et al., 2016).

When a possible introduction of resistance is identified, non-chemical measures can sometimes be taken to prevent a physical proximity with the “naïve” population or control the potential donor population (Raymond, 2019). To fight against antimicrobial resistance (as well as other diseases like Covid-19!), some physical distancing and quarantine measures can be implemented, even if they are mainly targeting microbial infections rather than the resistance problem itself (Nieuwlaat et al., 2020). Enhanced hygiene (*e.g.* hands washing, environment cleaning) and facemasks are promoted in prevention campaigns against the transmission of illnesses. Similarly, quarantine is a strategy of control used in agriculture to limit the spread of pests and diseases that could cause economic damage if introduced especially when carrying resistance alleles (*e.g.* *Diabrotica virgifera virgifera* in Europe (Ciosi et al., 2009)). This strategy also limits the exchange of genetic resources (Ciancio and Mukerji, 2008; National Research Council, 1993). Finally, methods as mating disruption with insects sex pheromones, or the removal of host reservoirs may also limit gene flow and population size (Damos et al., 2015).

Nevertheless, it is important to recall that even alternatives methods and biocontrol products are not necessarily environmentally friendly and safe for the health (Mahamoud Ahmed et al., 2018; Niedobová et al., 2019; Teyssier et al., 2020), and might also face adaptation from pests and pathogens (Bardin et al., 2015; McDonald and Linde, 2002). Besides, alternatives to pesticides or drugs may not always be as efficient as conventional chemical treatment to limit population size and gene flow. They sometimes need better adjustment or optimization (Verly et al., 2020). Even so, they are non-selective between susceptible and chemical-resistant individuals and then often reduce indistinctly both  $W_S$  and  $W_R$ , leading to unchanged population structure. It may happen that  $W_S$  and  $W_R$  are not equally affected by the non-chemical methods which may lead to a reduction of selection coefficient, especially in case of resistance cost. Therefore, while authorizing reduced exposure of populations to pesticides and drug, they



contribute to extend the effective life of pesticides and drugs. This reduction of exposure can be optimized by digital farming in agriculture or clinical decision support for health care.

**2.2.2.2 Delaying resistance while promoting multidirectional selection from pesticides and drugs**

Maximising the DTH in order to delay resistance evolution relies on the smart deployment, in space, time and dose, of the available AIs, i.e. on designing the strategies best adapted to a particular situation of resistance (Beckie and Reboud, 2009; Fisher et al., 2018; Jørgensen et al., 2018b; Laxminarayan et al., 2006; REX Consortium, 2013). Four anti-resistance strategies are usually considered, whose name differ according to the scientific community which promotes them (REX Consortium, 2013) (Table 1).

*Table 1 : Anti-resistance strategies combining pesticides or drugs available to delay resistance evolution and their names generally in use in the associated communities. In bold are the designations that will be used in this thesis. (REX Consortium, 2013)*

<b>Strategy</b>	<b>Antibiotics or antiviral drugs</b>	<b>Insecticides or Bt toxins</b>	<b>Fungicides</b>	<b>Herbicides</b>
<i>Responsive alternation</i>	Sequential use	Sequence, sequential use, and serial use	<b>Sequence</b>	Sequence and threshold strategy
<i>Periodic application</i>	Cycling, antibiotic rotation, Periodic application, and sequential use	Rotation, alternation, and sequential use	Rotation, <b>alternation</b> , and sequence	Rotation
<i>Mosaic</i>	Mixing, 50-50 treatment, antibiotic diversity, and multiple first-line therapy	Mosaic	<b>Mosaic</b>	Mosaic
<i>Combination</i>	Combination, antibiotic diversity, and simultaneous strategy	Mixture and pyramiding	<b>Mixture</b> and Combination	Mixture, Combination and double knockdown

These strategies are presented in Figure 19 as well as their effect on pest or pathogen generations. They are detailed in the following sections.

#### 2.2.2.2.1 Sequence

Sequence, or responsive alternation or sequential use, consists in the continuous use over generations of an AI until resistance emerge. After generalization, the use of the AI is stopped and a new AI replaces the previous one, and so on (van den Bosch et al., 2014a; REX Consortium, 2013). Sequence is then similar to alternation in that it relies on temporal heterogeneity but with very long rhythm. Resistance evolution is not delayed in the case of sequence. The practical consequences of resistance generalisation are only avoided thanks to the shift of MoA. From an historical point of view, sequence might correspond to situations where resistance was ignored by stakeholders and where the search for facility and maximal efficacy primed over a longer-term view of resistance management, with the insurance that new AIs would be continuously provided by the industry. For example, *Botrytis cinerea* multifungicide resistance results from a stepwise accumulation of single resistances (Li et al., 2014). *Per se*, sequence facilitates multiple resistance, as resistance to a new AI is selected in genetic backgrounds already resistant to the previous AIs. In case of a fitness cost, the frequency of resistance might decrease while the exposure to the next AI, which may authorize further reintroduction after sufficient time. Unfortunately, in some cases, genetic hitchhiking maintains the frequency of the first resistance allele, because of its close genetic proximity with the second one. For all these reasons, the sequence strategy is not to be considered sustainable.

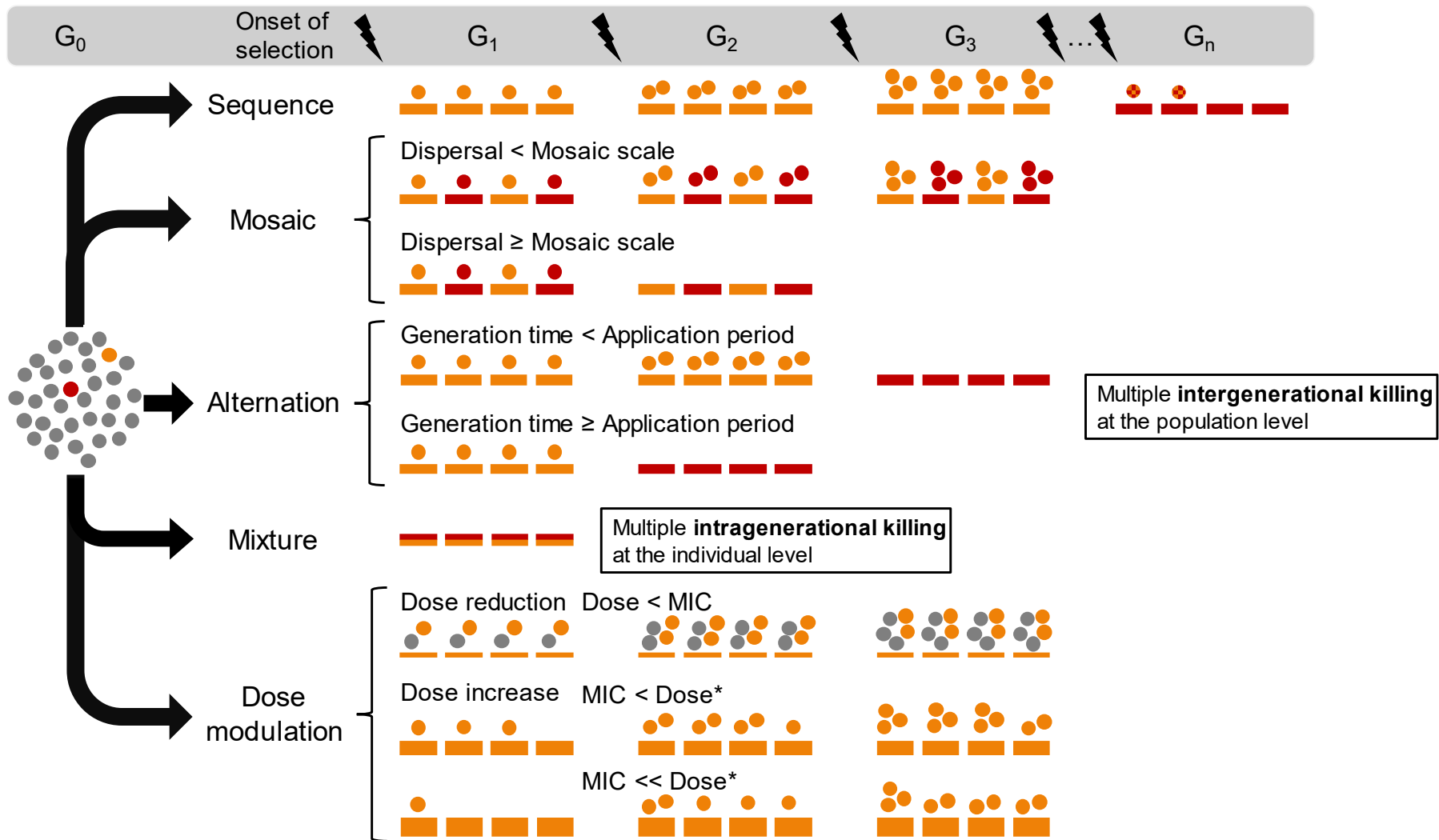


Figure 19. Anti-resistance strategies and their effect on populations of pests and pathogens. Adapted from (REX Consortium, 2013). At each generation  $G_n$ , resistance is selected in several populations of pests or pathogens by molecule 1 (in orange patches), molecule 2 (in red patches), or a combination of both (in orange-and-red patches). The thickness of the patches represents the dose of the molecule. Susceptible individuals are represented in grey, individuals resistant to molecule 1 but susceptible to molecule 2 are in orange; individuals resistant to molecule 2 but susceptible to molecule 1 are in red and individuals resistant to both molecules 1 and 2 (multiple or generalist resistance for example) are represented with orange and red squares.

\* Dose depending on the RF: dose qualitative resistance > dose quantitative resistance.

#### 2.2.2.2.2 *Alternation*

Alternation displays temporal heterogeneity of the selection pressure. The periodical substitution of an AI with another one globally decreases its average exposure time (van den Bosch et al., 2014a; National Research Council, 1993; Raymond, 2019). Alternation is all the more efficient at delaying resistance since the resistant genotypes display a competitive disadvantage, when not exposed to its selective AI. Fitness penalty may then contribute to counter-select resistance alleles when exposed to alternative selection pressure. Otherwise, the frequency of resistant genotypes might stabilize for a few generations, until the next exposure with the AI. Although it might be complicated to establish fitness costs *in natura* as they might vary over time because of compensation or allele replacement (Andersson and Hughes, 2010; Lenormand et al., 2018; Raymond, 2019), some applications of this strategy are currently used. For example in northern Europe, the resistance management to antibacterial drugs is based on alternation (Laxminarayan et al., 2006). Similarly, examples can be found in perennial crops as vineyards or fruit trees for which protection period is long. Pathogens or pests are then exposed during a large timeframe to selection pressures leading to resistance selection. The fungus *Venturia inaequalis* responsible for apple scab, is controlled yearly with about 10 to 20 fungicide interventions and has been concerned by a succession of resistance to almost new fungicide class that was introduced from the 1950's (Cox, 2015). A five-spray program based on alternation with unrelated chemical groups was assessed to be as efficient as a standard 12 fungicide application program (Chatzidimopoulos et al., 2020), underlining the value of such strategy.

Alternated AIs should be chosen carefully. Positive cross-resistance between the alternated AIs, because of generalist resistance or because they share the same MoA, should be avoided. By contrast, negative cross-resistance might enhance the respective susceptibilities of resistant individuals when exposed to the alternative AI and improve the performance of the alternation (Imamovic and Sommer, 2013; Raymond, 2019). The application timing is also essential to control population size and should be defined depending on the duration of the pathogen or pest generation. Indeed, if this application period is higher than the generation time, several generations of individuals will occur before receiving the next selection pressure. In practice, for short generation time organisms (bacteria, virus, fungi for example), it is very unlikely that every generation is treated differently. Depending on the pattern of alternation, several generations

may be submitted to homogeneous selection (*i.e.* no intergenerational killing) and/or heterogeneous selection (*i.e.* intergenerational killing). Adapted rhythm of alternation should create sufficient heterogeneity of the selection between the generation but sufficient duration between treatments based on the same AI to allow the expression of putative resistance cost.

### *2.2.2.2.3 Mosaic and refuges zones*

Mosaic strategy consists in presenting a spatial pattern of applications of at least two AIs preferentially with independent MoAs and lack of positive cross-resistance (REX Consortium, 2013). A fraction of the population is exposed to one MoA while another part is treated at the same time with another MoA. As for alternation, positive cross-resistance, multiple resistance or generalist resistance represent limitations for this strategy. When resistant individuals spatially disperse from a patch to another one treated differently, it is exposed to a distinct selection pressure and is then disadvantaged. The gene flow resulting from dispersal decreases the frequency of resistant genotypes in the local population (dilution effect). Secondly, at a given time, the spatial exposure to diverse MoAs is also preventing the quick spread of resistance from an area (or patient) to another (Raymond, 2019). As for alternation, mosaic relies on multidirectional selection but applied in distinct areas. The choice of the spatial scale is central for mosaic. For example, mosaic was not enough efficient when deployed at a single ward level, whereas it was reducing rates of antibiotic-resistant Gram-negative bacteria at the hospital level (Takesue et al., 2006, 2010). If the grain of the mosaic is greater than the migration capacity of the pest or pathogen, rather homogeneous selection is applied on the same generation of pathogens and pests. If the dispersal capacity of the pathogen is higher than the grain of mosaic, then intergenerational killing occurs on populations. One obstacle to mosaic is the need to coordinate its implementation at multiple scales (in the field or in the hospital) and between stakeholders.

A variant of mosaic is the high-dose refuge strategy, which is a mosaic except that some areas are untreated, instead of being treated with another MoA. Untreated individuals constitute a reservoir of susceptible genotypes that can "dilute" resistance while migrating to the treated areas, or while mating with resistant individuals. For diploid species, the performance of this strategy may rely on the recessiveness of the resistant alleles in heterozygote individuals (Hendry et al., 2011; National Research Council, 1986; REX Consortium, 2013). The high-dose

refuge strategy may have an economic cost on yield, because of the possible damage authorized in the untreated area but optimization of the spatial organization of mosaic can limit this drawback (Vacher et al., 2003). In agriculture, mosaic is implemented to delay resistance of some insects to Bt crops (Gryspeirt and Grégoire, 2012). Refuges zones have been implanted for more than twenty years and slowed down resistance evolution by a factor of ten to one hundred (Gould et al., 2018). In the medical field, this strategy is not achievable for ethical reasons, and regular mosaic is preferred. At last, in practice, mosaic is generally implemented together with alternation (rotational mosaic) in order to increase DTH even more, with both a spatial and temporal heterogeneity.

#### *2.2.2.2.4 Mixture*

Mixture consists in the concomitant use of at least two different AIs, preferentially of different MoA. Its efficacy is based on a multiple intragenerational killing: individuals are controlled in multiple different ways, since the probability for an individual to carry several resistance alleles is unlikely if there is no cross-resistance and dependence between the AIs (van den Bosch et al., 2014a; Raymond, 2019; REX Consortium, 2013). Hence, both the relative fitness of resistant and susceptible individuals with regard to each AI would decrease and therefore limit positive directional selection. This strategy would be more efficient if the initial frequency of resistance is low, if there are independent loci for each mutation providing resistance, with no positive cross-resistance between the AIs and similar persistence for all of them (Raymond, 2019). Synergy between mixture partners (*e.g.* especially in case of negative cross-resistance) increases even more the efficacy of mixtures. Mixture is also particularly efficient in case of recessive resistance in diploid organisms. The efficacy of a mixture may at last vary according to its components. Indeed, dose and ratio of each AI are important drivers of efficacy and then of selection. Some commercial mixtures include full doses of each partner, but synergy may allow the reduction of one or both partners while maintaining field efficacy. Dose decrease in mixture is also an opportunity to reduce the toxicity of the specialties and to keep acceptable costs, then authorizing their use largely, including in emerging countries. Today, their use is common either in agriculture or in human medicine.

The mixture strategy therefore often gets along with a dose modulation. Nevertheless, except when half doses of each AI constitute the mixture, the total amount of pesticides or drugs in a

mixture is regularly greater compared to the solo use of each AI in sequence, mosaic or alternation.

Some AIs are also primarily combined to broaden the spectrum of activity (*i.e.* to control several pests or pathogens at the same time, any AI not being systematically efficient against all of them) and/or to enhance the efficacy of the mixture (*i.e.* mixtures of AIs sharing the same MoA, or mixture between a MoA already concerned by resistance with another one not concerned) (Laxminarayan et al., 2006). Such mixtures have little interest to reduce resistance selection.

#### *2.2.2.2.5 Dose modulation*

##### *Dose reduction*

From a theoretical point of view, dose reduction (below the MIC of the susceptible strain) might slow down resistance selection in susceptible populations by decreasing the selection coefficient (*i.e.* might increase the absolute fitness of the sensible strain). However, it might not efficiently decrease the population size of pests and pathogens, enlarging in return the mutational supply. Moreover, low to moderate doses may allow the emergence, selection and combination of a diversity of low to moderate resistance alleles, consistent with general resistance or polygenic mechanisms. Such mechanisms are possibly associated with weaker decrease of treatment efficacy but with positive cross-resistance between independent MoAs (Blanquart, 2019; Raymond, 2019). Reduced doses could, yet, be considered when implemented with other control methods like in the IPM strategies developed to control of *Phytophthora infestans*, responsible for downy mildew on potatoes (Cooke et al., 2011).

Splitting an initial efficient dose into several lower doses (fractionation) is another interpretation of dose reduction. The evolution of resistance then relies on the balance between the increase of exposure time (all the more important since AIs are persistent) and the reduction of the selection coefficient. Hence, empirical data tend to confirm that resistance selection is accelerated when the total dose is fractionated (van den Bosch et al., 2014a).

##### *Dose increase*

Once resistance has already emerged in the population (particularly in case of qualitative resistance), an increase of the dose, to the maximum permitted, will often not significantly

reduce the fitness of the resistant strain (depending on the resistance factor). Therefore, by reducing only the absolute fitness of the susceptible strain, dose increase might increase the selection coefficient in favour of the resistant individuals. In this case, a majority of studies conclude to a quicker evolution of resistance when the dose is increased (Blanquart, 2019; van den Bosch et al., 2014a). But dose increase may be a relevant strategy in the early phases of the evolution of quantitative resistance and resistances with some generalist mechanisms. Indeed, those situations are characterized by low resistance factors, an increase of the dose could then be enough to control (even partially) the resistant strains. Nonetheless, it may be a risky strategy if the control of the resistant population is not satisfactory (*e.g.* with RF higher than expected for example), as selection might then be promoted (Hendry et al., 2011).

On the other hand, if resistance has not yet emerged in the population, high dose strategy could be efficient to control emerging resistant variants, especially those exhibiting low RFs, and also to prevent the construction of polygenic resistance and keep resistance alleles of diploid organisms under their recessive form (REX Consortium, 2013).

#### *2.2.2.2.6 Designing sound anti-resistance strategies*

As introduced in the previous sections, strategies might not perform equivalently because they rely on distinct drivers. The biology and genetics of resistance may differ according to pests and pathogens on one side, and on pesticides and drugs on the other side, then valuing these drivers differently (Beardmore et al., 2017; Raymond, 2019; REX Consortium, 2013). In particular, the pattern of selection pressures select resistance differently depending on reproduction mode, the type of resistance to be selected (qualitative *vs.* quantitative, specific *vs.* generalist), or the phase of resistance considered (emergence, selection or generalisation). For example, organisms with sex and recombination are advantaged by the linear directional change of an environment or by slow periodic change, whereas asexual reproducing organisms have a higher fitness in randomly fluctuating or rapidly changing environment (*i.e.* higher reproductive rate is more successful than elevated levels of genetic variance) (Bürger, 1999). Consequently, the design of strategies of resistance management should be tailor-made to each situation. The choice of drivers (as those exemplified in Table 2) to be operated may influence DTH. However, their effect on selection is not always well defined (Beardmore et al., 2017; Raymond, 2019). In addition, the multiple forms of selection applied on a given population should



be considered globally to design strategies. For example, in agriculture, seed treatments may exert selection such as foliar treatments for systemic pathogens (Kitchen et al., 2016). Similarly, treatments targeting a primary pathogen may also select indirectly for another one (*e.g.* SDHI used to control stripe rust also select resistance in *Z. tritici* populations) (Hagerty et al., 2020). Altogether, this literature review sets the question of which drivers of strategies are actually the most efficient at delaying resistance evolution in a given situation and if recommending an unequivocal strategy is responsible.

Table 2: Examples of drivers that can be used to mitigate resistance evolution in strategies

Mixture	Alternation(s)	Mosaic	Dose modulation
Exposure time			
Number of different AIs			
AI type: intrinsic resistance risk and efficacy			
AIs interaction (cross-resistance)			Dose choice (increase/decrease)
Total dose	Application period: relative to generation time and AI persistence	Spatial scale (size of plots or cohort): relative to spatial dispersion	
	Rhythm	Number of treated plots or individuals	% of control expected
		Spatial pattern (aggregation)	

## 2.3 Evaluating anti-resistance strategies

Several approaches are available to assess the performance of anti-resistance strategies. They are complementary since they may inform about distinct criteria of performance and might not be operational for all pathogens and pests and at all scales.

### 2.3.1 Empirical approaches

#### 2.3.1.1 Experimentations *in natura*

To assess which anti-resistance strategy is the best to delay the evolution of resistance, the first approach can be experimentation *in natura* intentionally designed for this purpose. Field/clinical trials may be exposed to several treatments reflecting the strategies to be tested. These can be samples characterized for resistance (susceptibility levels, spectrum of resistance, alleles of resistance...) and measurements traducing population size (counting of weeds, insects

in traps, disease leaf area...) (Barzman et al., 2015; Bruno-Murtha et al., 2005; Cooke et al., 2004; Dooley et al., 2016a; Dusfour et al., 2019; Gilligan, 2008; Heick et al., 2017; Hemingway et al., 1997; Ulber and Rissel, 2016). Other criteria such as treatment efficacy (through yield or time needed to cure a patient for example), or economic benefit are sometimes also considered. However, they do not always inform directly on resistance evolution as yield or efficacy of a clinical treatment may not be immediately impacted by low to moderate resistance frequencies. These experiments are often conducted at small or medium spatial and temporal scales: within a field, a hospital, a locality (or between a few of them) and during a few years (between two and five years in general). However, the definition of the scales may vary depending on the pathogen or pest considered and on the strategy tested (*e.g.* a mosaic strategy will depend on the dispersal capacity of the organism) (Dusfour et al., 2019), while the timescale is also dependant from the characteristics of the epidemics or invasion (*e.g.* most of clinical therapies for bacterial infections are lasting a few days whereas it can be months or years for tuberculosis) (Jansen et al., 2013). Theoretically, all strategies can be tested and compared with these approaches. However, mosaic has not been studied that much compared with others, mainly due to ethical reasons for medical field and logistical reasons for agriculture (high coordination necessary for implementation).

The accuracy of field or clinical experiments *in natura* is appreciated as they inform directly on the dynamics and mechanisms of resistance as a response to tested strategies in real conditions. However, they are often heavy experiments, expensive and with limited number of replicates, of generations and parameters to be compared. The main inconvenient is the quite relative short timescale of this approach. Long-term sustainability is hardly explored, and studies focus mainly on selection phase (when resistant alleles are already in the populations) rather than studies focusing on emergence. In addition, environmental conditions cannot be controlled precisely (or precisely followed and described for some traits). Consequently, several factors can blur the effect of strategies. Similarly, the generalization of the findings from one study is hazardous, as the results might be only valid under the specific environmental and population conditions (Hoy, 1998). Furthermore, the ancestral populations cannot always be perfectly characterized and contamination from non-experimental populations can interfere (Fisher and Lang, 2016; REX Consortium, 2010, 2013).

### 2.3.1.2 Observational studies: *a posteriori* studies of empirical data

Empirical data such as frequency of resistance in a population, characterization of strains and efficacy of treatment are regularly obtained from pesticide monitoring programs or epidemiological surveillance cohorts in human health, carried out by multiple stakeholders. Those data might be established at various time or spatial scales and can then be combined to constitute comprehensive datasets useful for the *a posteriori* evaluation of strategies, and the identification of their drivers, even if they were not originally designed for this purpose. Such observational studies present the advantages of randomized controlled trials while avoiding some non-feasibility or ethical issues (Chandrashekhara and Misra, 2013; Song and Chung, 2010). For example, Garnault et al. (2021) used a dataset including the monitoring of the frequency of fungicide resistance carried out over 70 locations during 13 years in *Z. tritici*. Statistical modelling was used to analyse the relative efficacy of dose modulation and mixtures used in annually repeated trials, in field conditions. Such approach carried out for the agricultural weed *Alopecurus myosuroides* at a national scale revealed that herbicide mixtures were more to favour generalist resistance compared to solo applications (Comont et al., 2020). In the medical field, a standardised approach combining patient, laboratory and epidemiological surveillance data have been implemented worldwide since 2017 (Global Antimicrobial Resistance Surveillance System or GLASS) (WHO). This database will probably highly help the management of resistance to antibiotics in the coming years. However, particular precautions should be taken when the data are chosen because this approach is quite susceptible to bias (Chandrashekhara and Misra, 2013).

The inconvenient of such an approach is the necessity to handle big datasets. Experimental design is not always primarily conducted to address the question of strategy comparison, which complicated the *a posteriori* analysis of such data. This approach can be made impossible for minor pathogens or pests or in countries with poor epidemiological surveillance.

### 2.3.2 Modelling approach

Another approach used to assess anti-resistance strategies is mathematical modelling. Such models reproduce in their equations the mechanisms underlying the evolution of re-

sistance in populations and integrate its most relevant drivers. While modulating the parameters of such models, it is then possible to explore the evolution of resistance under different selection regimes and then to compare strategies and study the effect of a particular trait. Two different modelling approaches have been used to compare the performance of strategies: population genetics and epidemiological models (REX Consortium, 2010).

Population genetics models are focusing on the quantitative effect of the use of a pesticide or drug on the evolution of the frequency of resistant (and susceptible) individuals in a population. The epidemiological models may also sometimes estimate resistance frequency but are more interested in the quality and quantity of the disease or pest host or resource (*e.g.* patients, yield, ...). Epidemiological models are constituted of different compartments referring the status of the host and parameters that will impact the epidemiology or the dynamics of the pathogen or pest. Those parameters can for instance characterize pesticide or drug use and their variation allows strategy comparison. Parameters are not necessarily the same between epidemiological and population genetics models (*e.g.* migration might be formalized differently). However, models generally make explicit the biological traits of the pathogen or pest considered. Similarly, outputs of the model vary according to the biology of the studied organism. For example, most of the models are examining final resistance frequencies, while the time to reach a threshold (loss of efficacy for example) is mostly considered for fungicide and herbicide resistances (Hobbelen et al., 2011). The waiting time to appearance, or emergence time, or first detection of resistance is also used in models as a criteria for evaluation (*e.g.* for resistance to antibiotics (Smith et al., 2010) or fungicides (Mikaberidze et al., 2017)). Economic criteria are not often studied, despite it may influence the relevance of strategy on short or long term (Elderfield et al., 2018). The timescale considered in those modelling studies varies depending on the biological parameters. Nonetheless, the effect of anti-resistances strategies is often considered over several years or multiple generations, which is an advantage compared to empirical studies. Distinct spatial scales might be considered in models focusing on the same organism. Epidemiology of the pest or drug resistance may be studied within host or field, or between the hosts or fields from regional to even national scales (Blanquart, 2019; Garnault et al., 2019; North et al., 2019; Somerville et al., 2020). Strategies are not equally explored in models, with variation according to organisms (*e.g.* refuge strategy highly studied for insects' management but rare for fungicide and antibiotic resistance). Generally, mosaic was less studied than

other strategies (including in population genetics models) but some studies exist on drug resistance over space (*e.g.* Débarre et al. (2009)). The implementation of several AIs was more considered over time than space. Mixtures is the most explored strategy for resistance management, while alternation was mainly studied for fungicide resistance (never studied for antiviral drug resistance for example, as not realistic for clinical implementation (REX Consortium, 2010)).

Contrarily to empirical studies, mathematical modelling requires low technical and biological resources and allows the comparison of multiple modalities at large scales. Another benefit of modelling is that even unrealistic strategies (for economical, technical, or ethical reasons) can be investigated which brings perspectives for further development. General and specific models can display different information on anti-resistance strategies. The first can evaluate the strategies regarding to resistance evolution even before the apparition of resistance, while the second often disentangle the drivers at work in critical cases. Non-theoretical models are often dependant from field data to adjust their parameters (Kitchen et al., 2016) and to be accurate (*i.e.* experimental validation of models (Hoy, 1998; Lucas et al., 2015)). In that case, the cost and time needed for the development of such models can increase. Theoretical general models dissect the effect of each evolutionary force on resistance evolution, thus those models are useful to evaluate and compare the different anti resistance strategies or design new strategies (REX Consortium, 2010). However, it should be noted that many studies focus on the evolution of know resistance cases already present with strategies already at work. Therefore the impact of strategies on the emergence phase is less studied than selection (Niewiadomska et al., 2019), and new design of strategies (coupling alternation and mosaic for example) is not highly investigated. The consideration of selection as the main evolutionary force in the models is understandable when dealing with pesticides or drugs. However, the impact of migration, mutations and genetic drift on the evolution of resistance is often minimized (or forgotten) in models, including in population genetics ones. Their omission limits the exploration of some strategies (REX Consortium, 2010). For example, genetic drift would be relevant to test the elimination of a pathogen in some areas or strategies without chemical treatment. Another limitation of models is that many are not considering quantitative resistance, recombination, cross-resistance, and also generalist resistance, whereas those can have high impacts on resistance

evolution (South and Hastings, 2018). The assumption that resistances are monogenic and independent might lead to a biased evaluation of anti-resistance strategies, as multidrug resistance and cross-resistance are not scarce phenomena. Similarly, the resistance of the at-risk AI is often the only one considered which can be misleading when the associated AI may also develop resistance. Finally, fitness cost is very often implemented in models for its ability to counteract selection by pesticide/drug. However, its possible variation in a given environment due to compensatory mutation is generally not taken into account (Lenormand et al., 2018).

### 2.3.3 Experimental evolution approach

Finally, the third approach to study resistance management is experimental evolution (EE) (Box 3). This approach is a conciliation between empirical and modelling studies. EE cultivates in controlled laboratory conditions a given population in several replicates and can study the effect of a specific factor (*i.e.* the variation of one experimental condition) on the evolutionary process (van den Bergh et al., 2018; Jansen et al., 2013; Kawecki et al., 2012). With experimental evolution, adaptation can be observed in real time, with a precise quantification of the variation of population structure, population size, and the characterization of individuals. Not only the dynamics of resistance evolution can be studied but samples can also be collected and stored for further studies on the qualitative aspects of resistance (McDonald, 2019). Genetic variation and composition of the initial population, as well as gene flow and genetic drift can be modulated experimentally. Therefore, the study of a variety of evolutionary parameters and situations is possible, including the impact of anti-resistance strategies on resistance evolution. For example, mixture, alternation and dose modulation of antibiotics have been studied by EE for their effect on resistance evolution (Kim et al., 2014; MacLean et al., 2010; Pena-Miller et al., 2013). To our knowledge, no mosaic strategy has been properly tested. However some studies are taking into account the spatial dimension of antibiotic resistance (*e.g.* Baym et al. (2016)). Depending on the biology of the organism considered, the goal of the study and the selection pressure, EE can run from a few days or weeks to several years. The best-known example is Lenski's experimental evolution who has maintained 12 populations of *Escherichia coli* in a simple laboratory environment for more than 30 years and 70 000 generations now (Lenski, 2017). Some lines have been studied for their susceptibility to antibiotics and dynamics of resistance (Card et al., 2019).

The majority of the experiments on the evaluation of anti-resistance strategies are running only on a few weeks (Lagator et al., 2013b, 2013a). In comparison with empirical data, EE highly reduces the timescale necessary for resistance evolution, and then offers the possibility to study very long-time scale, *i.e.* a greater number of generations. Another advantage of this approach is the possibility to repeat the experiment easily, and to include high numbers of replicates and modalities. This confers a high statistical power but also brings a prediction dimension to this approach (van den Bergh et al., 2018; McDonald, 2019).

However, one limitation is that EE requires the easily cultivation of the pathogen or pest in the lab. Moreover, one of the criticisms of EE is that environment is specific and simplified, and does not reflect field reality and its interactions (Bailey and Bataillon, 2016; Bank et al., 2014; Remigi et al., 2019). For example, EEs may overselect non-synonymous adaptive mutations compared to “natural” evolution (Remigi et al., 2019). Even if several cases of EE were able to identify mutations responsible for resistance in natural populations, the question of the transferability of the results to natural systems is pending. Similarly, sexual reproduction is not always possible in experimental conditions and the exploration of recombination effect on resistance evolution is complex. Yet Fisher and Lang (2016) describe the possibility to construct, quite easily for fungi, artificial recombined mutants and to use them in experiments to bypass such limit. Even if it is possible to carry out EE on a multiple of organisms, this approach remains the most relevant for small size organisms and with short generation time as bacteria. Unfortunately, EE is underused with fungi (Fisher and Lang, 2016).

### ***Box 3: Experimental evolution: purposes and design.***

Experimental evolution (EE) is the study of populations of living organisms for several generations under defined and controlled conditions, in the laboratory or in the field, for the observation of natural selection or the understanding of evolutionary processes (Garland and Rose, 2009; Remigi et al., 2019; Van den Bergh et al., 2018). EE is based on the parallel culture of replicated populations for multiple generations either in a novel environment or in the ancestral one (*i.e.* experimental controls). Environment modifications can be abiotic (*e.g.* composition of the nutrient medium, temperature), biotic (*e.g.* competition, impact of predators), or demographic (*e.g.* population size). Genetic variation and gene flow in the population can also be defined at the onset (Fisher and Lang, 2016; Giraud et al., 2017; Vogwill et al., 2012). Diverse type of experiments can therefore be qualified as experimental evolutions if they respect the following requirements that are the maintenance of control populations, the simultaneous replication of independent lines, the observation over multiple generations and the achievement of programmed genetic, phenotypic or physiologic analyses during or after the experiment (genetic analysis are the most common one). A common type of EE is "laboratory natural selection", in which the environment is altered (*e.g.* temperature change). A variation of this EE type is "laboratory culling", characterized by a lethal (or sublethal) stress (*e.g.* pesticide or drug) applied to the population (Garland and Rose, 2009). In either case, the population evolves freely as no artificial selection is achieved to found the next generation, allowing natural selection over multiple generations. This design differs from "artificial selection", in which phenotype or specific traits are measured regularly, and an intervention is carried out according to their scores to build the next generation.

EE also allows the rapid construction of mutants that can be used *per se* for specific applications (*e.g.* consumption of a given substrate) or for the study of the relationship between fitness, phenotypes, and *de novo* mutations (*e.g.* resistance to drugs or pesticides) (Fisher and Lang, 2016; McDonald, 2019; Remigi et al., 2019; Vogwill et al., 2012). Moreover, the genetic and/or phenotypic study of the whole evolutionary process (at the end of the experiment but also during its course) offers major opportunities to decipher in real time adaptive processes, gene regulation or even host microbiome interaction (Baucom, 2019; Hindré et al., 2012; Remigi et al., 2019; Robbins et al., 2017; Van den Bergh et al., 2018).



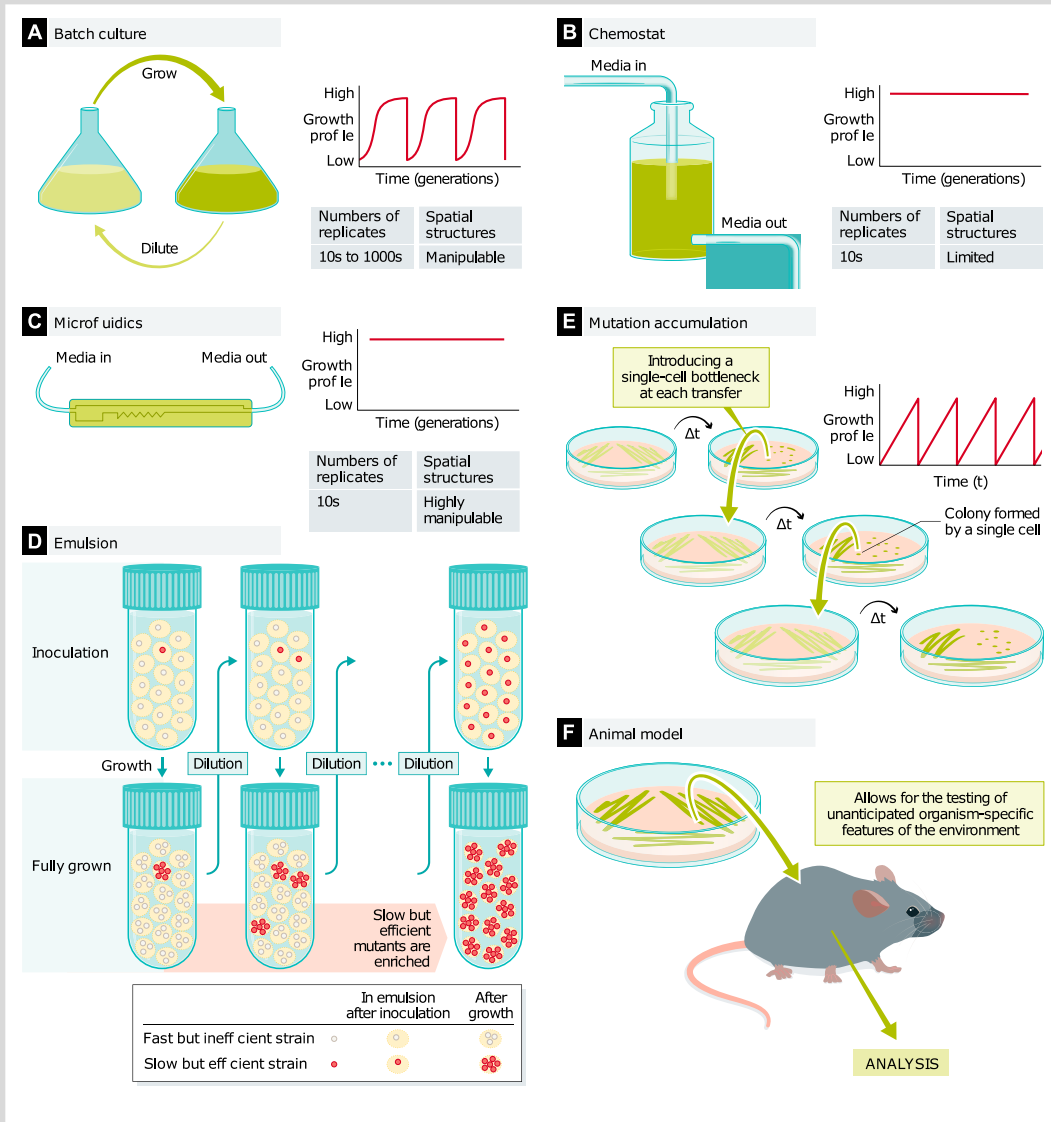


Figure 20: Different set-ups of experimental evolutions (McDonald, 2019).

**(A)** Batch culture requires the regular dilution of culture into fresh media. These experiments are relatively easy to establish, since a range of vessels commonly used in a microbiology laboratory can be used for batch culture. These experiments can be scaled to a large number of replicates, for example when using 96-well plates. **(B)** Chemostat culture systems include mechanisms for the constant supply of fresh medium. This provides the continuous cultures of populations and constant growth without large fluctuations in populations size or growth phase. **(C)** Microfluidics provides the most precise control over the supply of media and supplements to cell cultures. Microfluidics may need to be custom designed, and the number of replicates will be limited. **(D)** Emulsion cultures take advantage of small cell containing vesicles that form when mixing an oil, surfactant and cells. The number of cells in each vesicle is determined by the ratio cell, surfactant and oil. The cells can be mixed back into a single population by vortexing and centrifuging the solution. One advantage of evolving cells in a large number of small populations is that this can select for yield per-vesicle rather than rapid growth. **(E)** Mutation accumulation introduces a regular, single-cell bottleneck into each replicate population. This is achieved by streaking out cells on a Petri dish and then choosing a single colony (founded by a single cell) to streak out the next plate. **(F)** Microbial cultures can be introduced into a model organism, often a plant or a mouse, and left to propagate for a number of generations before it is recovered from the organism. The recovered cells can be analysed or subjected to further propagation in the organism. This mode of experimental evolution allows for the testing of unanticipated organism-specific features of the environment that are difficult to replicate in the laboratory.

Indeed, EE allows testing multiple evolutionary hypotheses such as the dynamics of adaptation, the possible outcomes of adaptation and their repeatability, including in different conditions (Fisher and Lang, 2016; Garland and Rose, 2009; Gutiérrez-Alonso et al., 2017; Lenski, 2017; Zhan and McDonald, 2013). Factors influencing the selection processes can be identified and help the building of predictions (Bailey and Bataillon, 2016; Giraud et al., 2017).

Even if EE can be theoretically carried out with all living organisms, this approach is largely used on microorganisms. Indeed, they provide several advantages: large population size and short generation time are essential for assessing evolution on a large number of generations, and molecular and genomic resources as well as analysis tools are often available for these models (Elena and Lenski, 2003; Garland and Rose, 2009). Moreover, samples of most of these species can be frozen all along the experiment for further analyses and comparisons or to restart the experiment in case of technical problems (Bailey and Bataillon, 2016; Fisher and Lang, 2016; McDonald, 2019). Several set-ups like serial transfer and continuous culturing (*i.e.* in a chemostat) are available and illustrated in Figure 20 (van den Bergh et al., 2018). The most popular and simplest one is the serial transfer and was the one used in this thesis. Populations are grown in batch cultures with dilution transfer between them. This design provides new nutrients and space to the cultures and then ensure the continuous maintenance of lines evolving under natural selection. Population size is not constant (because of population growth during each cycle and the regular bottlenecks associated to transfers) compared to with a chemostat. However, devices are not a limiting factor for replicates.

### 2.3.4 The debate on the performance of anti-resistance strategies

All the anti-resistance strategies that have been previously described globally display some efficacy to delay the evolution of resistance to pesticides or drugs, compared to the homogeneous selection over time and space with a single AI (REX Consortium, 2013). From a general point of view, because of its multiple intra-generational killing, mixture is predominantly acclaimed as the best anti-resistance strategy in modelling and experimental studies (Table 3) (Brooks and Brooks, 2014; Elderfield et al., 2018; Raymond, 2019; REX Consortium, 2013). Furthermore, its use is facilitated by commercial formulations in which the different AIs

are pre-mixed and do not need coordination between stakeholders. A similar overview of published studies on the performance of nine different strategies (referred as tactics) was established specifically for the management of fungicide resistance management (van den Bosch et al., 2014a).

Table 3 : Pairwise comparison of strategies in terms of their relative efficacies for delaying or preventing resistance in multiple taxa. (REX Consortium, 2013).

Strategy		Theoretical studies					Empirical studies			
1	2	n <sup>a</sup>	1>2	1=2	1<2	Condi-tional <sup>b</sup>	n	1>2	1=2	1<2
Combination	Responsive alternation	14	11	0	0	3	10	8	2	0
Combination	Periodic application	16	14	0	1	1	8	2	5	1
Combination	Mosaic	7	5	0	1	1	1	1	0	0
Periodic application	Responsive alternation	7	3	4	0	0	9	7	2	0
Periodic application	Mosaic	11	2	3	5	1	3	2	0	1
Mosaic	Responsive alternation	3	2	1	0	0	2	1	0	1

<sup>a</sup> n, number of comparisons in all theoretical and empirical studies.

<sup>b</sup> the ranking of the strategies depends on the setting for one or several input or output parameters.

However, it is important to note that among approaches presented above, mixture was also the most studied anti-resistance strategy. Consequently, some bias may be resulting, as under-explored strategies might also be relevant if studied equivalently. Additionally, the number of studies, especially many empirical, realising direct, like-for-like comparisons between anti-resistance strategies is not that important when we consider all the confounding factors and stochastic effects potentially affecting the results (Beardmore et al., 2017; Blanquart, 2019; Lucas et al., 2015; Schmid et al., 2019). At last, if the mixture strategy seems predominant in a majority of situations, it is not anyway a universally efficient strategy: some factors may drive its performance in each particular situation and those, as well as their interplay, are not made explicit.

Consequently, more proper comparisons of anti-resistance strategies, covering the range of their inner diversity, are necessary for a larger range of specific conditions, with the aim of

improving their practical use dealing (Elderfield et al., 2018; REX Consortium, 2013). In particular, more information on the drivers influencing each strategy is needed, as well as their respective efficacy at managing resistance. Beyond the establishment of a universal ranking between strategies which are in fact each intrinsically very diverse, a sound understanding of their advantages and limits and of the reasons of their performance in a given situation would be a first step to their smart tailoring and combination.

### 3 *Zymoseptoria tritici*, a relevant model to explore the durability of anti-resistance strategies

#### 3.1 *Zymoseptoria tritici* as the main pathogen affecting wheat

##### 3.1.1 Economic impact of STB

Wheat (mainly bread wheat, *Triticum aestivum*, and to a lesser extent, durum wheat, *Triticum turgidum ssp. durum*) is the second most important cereal in the world (after maize) and represents around 30% of the worldwide cereal production (Fones and Gurr, 2015; Passion Céréales, 2021; USDA, 2021). In the European Union, it is the first produced cereal (European Commission, 2021): its cultivation represents more than 10% of the global wheat surface and 20% of the world wheat production (*i.e.* about 140 million tonnes). EU exported more than 35 million tonnes during the campaign 2019-2020 (European Commission, 2021). Consequently, yield and quality losses due to phytopathogenic fungi are a big concern for this economically important crop in the EU.

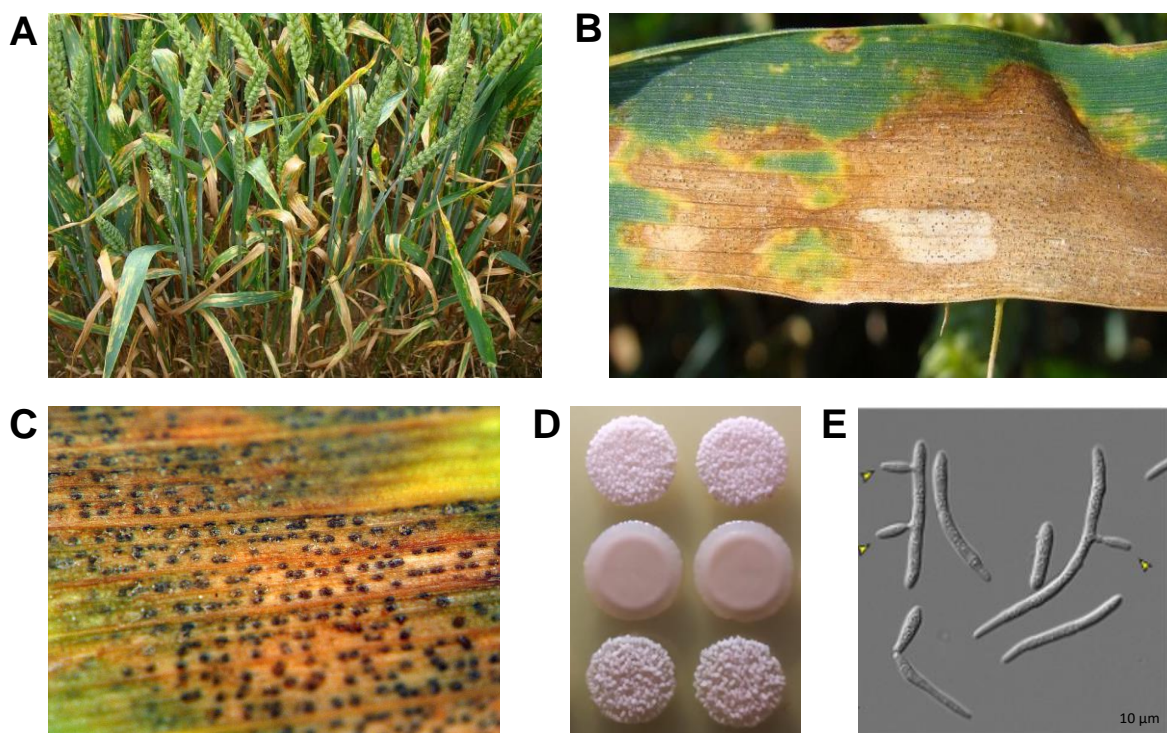


Figure 21: Symptoms of *Septoria tritici* blotch on wheat and forms of *Z. tritici*. **A.** Symptoms of STB on adult wheat plants (Arvalis), **B.** Wheat leaf infected by *Z. tritici*, showing necrotic areas and pycnidia (Arvalis), **C.** Pycnidia at the surface of a wheat leaf. Pycnidia are structures producing the asexual pycnidiospores, ejected as spore-forming jelly or cirrhes (Arvalis). **D.** In vitro growth of the yeast form of *Z. tritici* on solid YPD medium (Ballu, 2021). **E.** Germinated pycnidiospores of the *Z. tritici* strain IPO-323 (Steinberg, 2015).

*Septoria tritici* (leaf) blotch (STB), mostly caused by *Zymoseptoria tritici*, is to date the most damaging disease for bread and durum wheat in EU (Fones and Gurr, 2015). It causes symptoms of chlorosis and later of necrosis on leaves (Figure 21), which greatly affects photosynthesis, and therefore yield. Indeed, in France, yield losses are estimated to about 17 dt/ha in average but can reach up to 50% of the potential yield in the worst cases. The management of *Z. tritici* with fungicides has been estimated to cost about €1bn annually (Arvalis - Institut du Végétal, 2013; Fones and Gurr, 2015; Torriani et al., 2015). Losses due to STB for UK, France and Germany are higher than €1,400 million yearly, even though control methods are deployed (Fones et al., 2016) (detailed in 3.2).

### 3.1.2 Biology of *Zymoseptoria tritici*

*Z. tritici* (formerly *Mycosphaerella graminicola*, teleomorph, and *Septoria tritici*, anamorph; Quaedvlieg et al. 2011) is an ascomycete from the class of the Dothideomycetes and the Mycosphaerellaceae family. It is considered a hemibiotroph, *i.e.* a pathogen with a biotrophic phase without symptoms induction before a switch to a necrotrophic phase (O’Driscoll et al., 2014; Sánchez-Vallet et al., 2015; Steinberg, 2015)(Figure 22). Recently, due to new insights from genomic data, it is now classified as a mesotroph (necrotroph with a long latent period) (Hane et al., 2020).

The disease cycle of *Z. tritici* encompasses both asexual and sexual reproduction (Figure 23). Primary infections may occur at late autumn-early winter, from ascospores, pycnidiospores or mycelium and potentially chlamydospores. These forms overwinter on plant material such

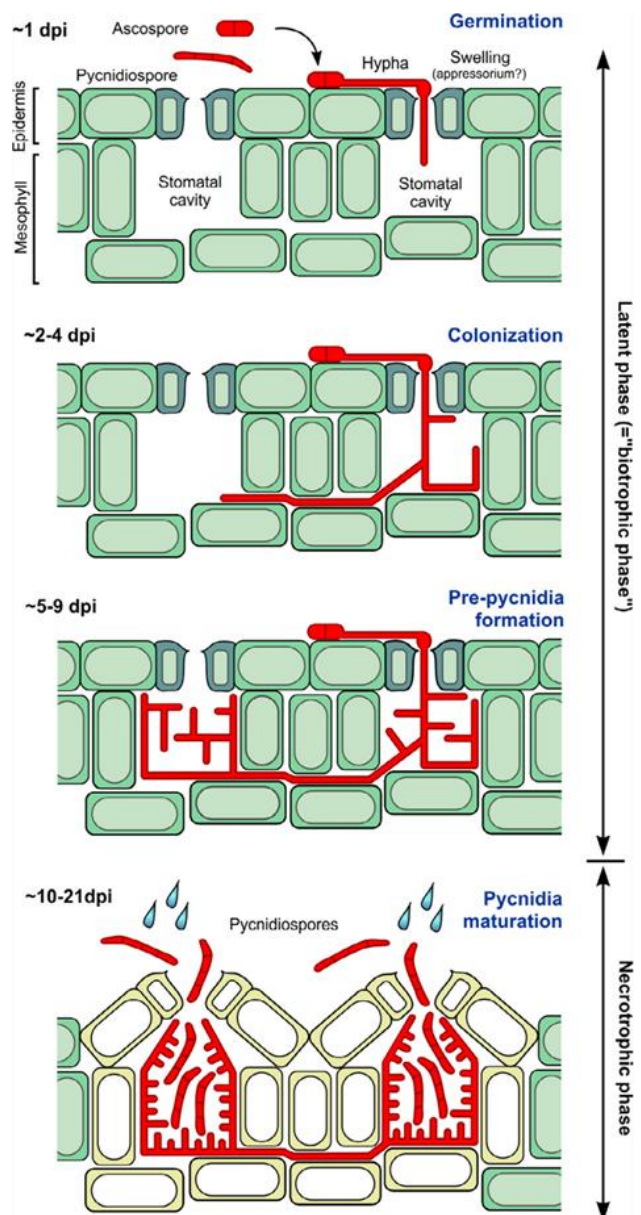


Figure 22: Plant infection stages of *Z. tritici*, and formation of asexual pycnidiospores. (Steinberg 2015).



as crop debris, stubbles, seeds, wheat volunteers and grass alternative hosts (Francisco et al., 2019; Suffert et al., 2011). However, ascospores, resulting from the sexual reproduction on wheat debris constitutes the principal source of inoculum for the early stages of STB epidemics. The first phase of the infection is asymptomatic and lasts for about 9 days post inoculation (dpi) (Steinberg, 2015) (Figure 22). When environmental conditions are favourable (temperatures above  $-2^{\circ}\text{C}$ , optimum at  $15\text{--}20^{\circ}\text{C}$ , and high humidity due to highly wet days or a succession of rainy days (Fones and Gurr, 2015), ascospores or pycnidiospores quickly germinate and enter into the leaf *via* stomata. Then, fungal hyphae colonize the intercellular space of the mesophyll. In a first instance, asexual reproduction is leading the epidemics.

Pycnidia are the asexual fruiting bodies producing the asexual pycnidiospores. Pycnidia formation starts at the end of the colonization stage. At 10 to 12 dpi, a switch occurs, and the necrotic phase begins. It is characterised by host-programmed cell death allowing pycnidia maturation and spore release, leading to symptoms apparition. Pycnidiospores are disseminated towards upper and nearby leaves by water splashing during rain events. Pycnidiospores are produced throughout the cropping season and contribute to disease multiplication *via* asexual cycles (about 5-6 depending on environmental conditions) on upper leaves as long as the conditions are favourable (Suffert et al., 2017). A long time (around 30-50 days) after the first production of pycnidia on a leaf layer, some perithecia (or pseudothecia) finally appear. Perithecia are the sexual fruiting bodies which result from the sexual reproduction of two opposite mating types (MAT1-1 and MAT1-2) infecting the same plant (Suffert et al., 2011, 2019). They produce ascospores that can remain on the senescent leaves and debris. Ascospores are smaller than pycnidiospores and therefore can be wind-dispersed over hundreds of kilometres. They constitute a new source of inoculum for early infections of the next wheat culture. More generally, *Z. tritici* displays a variety of morphotypes, which is of great interest for its maintenance in laboratory conditions (Figure 24; Box 4)

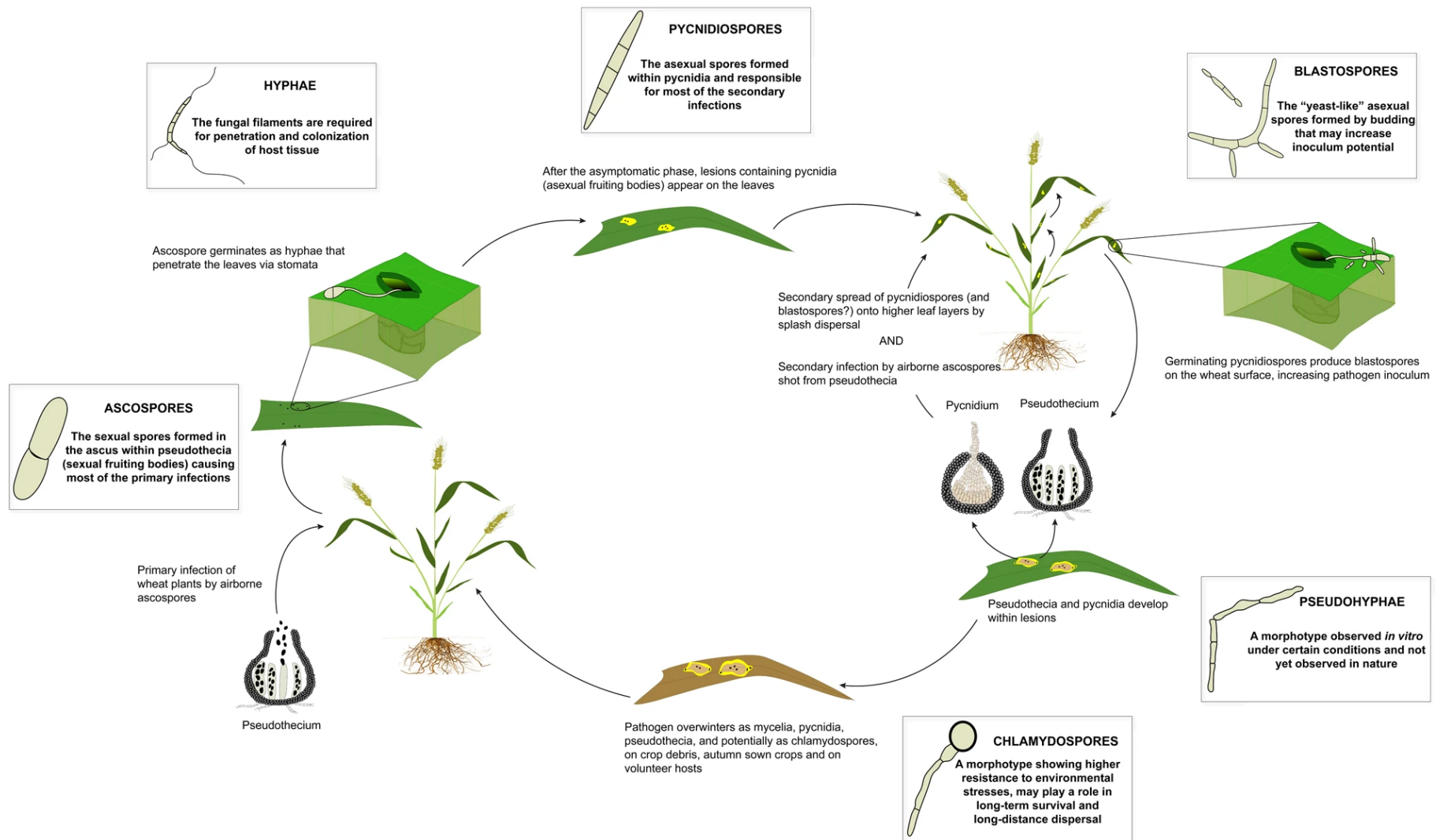


Figure 23: Disease cycle showing the functions of the different cell morphologies of *Z. tritici* in STB epidemics. (Francisco et al., 2019).



#### Box 4: *Zymoseptoria tritici* a pathogen suitable for experimental evolution

The morphology of *Z. tritici* can be very diverse depending on the infection and epidemic stage (Francisco et al., 2019; Steinberg, 2015). Morphotypes change can be induced by environmental conditions (*e.g.* nutrient conditions, temperature). Blastoporulation can be generated by high nutrient availability and temperatures between 15°C and 18°C. Consequently, the culture of blastospores (also called “yeast-like” form) in laboratory is easily handled either in liquid or solid medium and is the most common form cultivated in laboratory. The culture of *Z. tritici* as blastospores is well adapted for experimental evolution as it allows the formation of large populations over a relatively short time scale (6-7 generations per week in our conditions) in small containers. Its growth under controlled conditions is easily available and many replicates are possible. This gives the opportunity to study the evolution of *Z. tritici* in response to multiple factors over a few weeks.

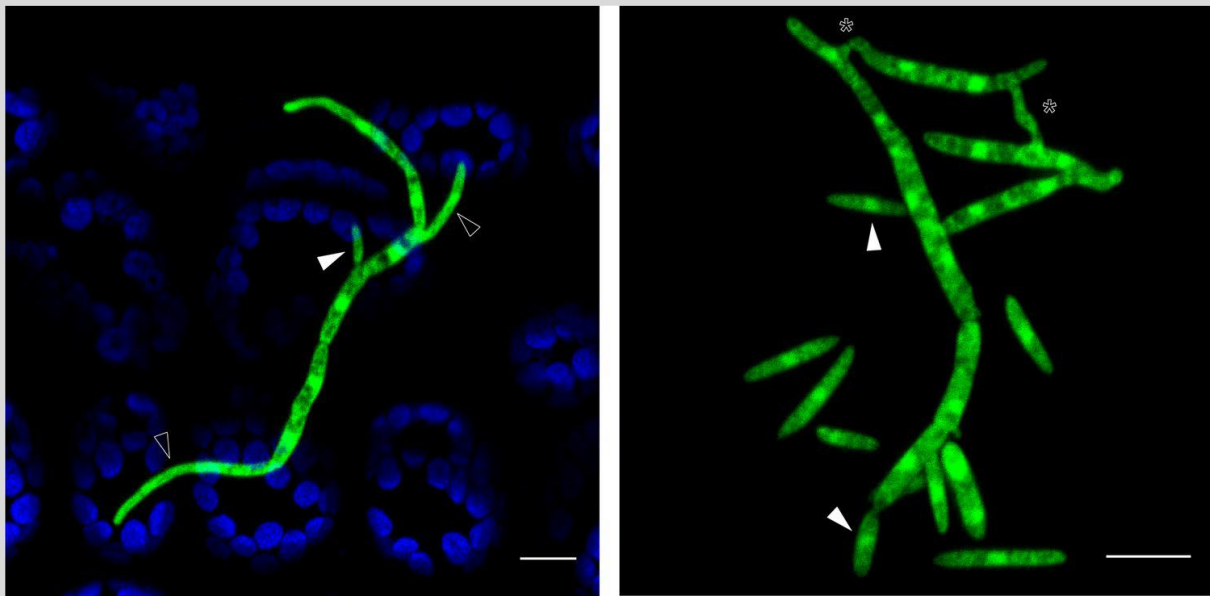


Figure 24: Blastospores (marked by a white triangle), produced by germinated pycnidiospores of *Zymoseptoria tritici*. (Francisco et al. 2019)

### 3.1.3 *Z. tritici* as a model to study the adaptation to fungicides

The high adaptation capacity of *Z. tritici* first results from the original characteristics of its genome. It measures about 40 Mb and is sometimes called a “two-speed” genome (Goodwin et al., 2011; Stukenbrock et al., 2010). Indeed, it encompasses thirteen core chromosomes, which carry the essential genes and evolve slowly and up to eight accessory chromosomes

containing unique or (often silenced) redundant genes. The polymorphism of these accessory chromosomes (also called dispensome) is highly variable as these can be lost and rearranged during the mitotic propagation. But the influencing factors are hardly known (Möller et al., 2018). The dispensome is enriched in repetitive elements and the high rate of mutation, recombination, insertion and deletion events may explain the rapid evolution observed in the dispensome. Moreover, about 17% of the whole genome are repeated and these redundant parts often include class 1 transposable elements (TE). These retrotransposons count for 70% of the repetitive content in *Z. tritici*, which can alter genome structure, create new genes and modulate the function of some existing ones (Dhillon et al., 2014; Fones and Gurr, 2015). TEs contribute to genome expansion and are more frequent in accessory chromosomes than in core chromosomes, providing one explanation why size variation is more important in the dispensable rather than in the rest of the genome (Oggenfuss et al., 2021). This plasticity of the genome is prone to promote the emergence of new alleles, whose maintenance and dispersion is favoured by the biology of this species.

*Z. tritici* is recognized for its large effective population size Zhan and McDonald (2004) estimated larger than 24,000 and with a mutation rate (for Restriction Fragment Length Polymorphism markers) of  $4 \cdot 10^{-5}$ . The bigger the population, the more easily mutations can arise and be maintained by selection (Fones et al., 2016). The one (or two, in some regions) annual cycles of sexual reproduction, generating ascospores that can represent up to 30% of the population at the end of a growing season (Eriksen et al., 2001), are also source of consequent gene flow. New combinations of alleles can be created yearly, amplified by asexual reproduction or dispersed through long distance by ascospores (Fones and Gurr, 2015; Fones et al., 2016; Zhan and McDonald, 2004). Sexual reproduction and recombination are considered the main drivers of adaptation, before the dynamics of TEs (Grandaubert et al., 2019).

Altogether, these characteristics contribute to make *Z. tritici* a highly adaptive pathogen and explain the worldwide relevance of STB. Over the years, adaptation to fungicides and host resistance, for which sexual reproduction highly contributes, are commonly observed in *Z. tritici* populations (Kema et al., 2018). As another example, Boixel et al. (2019a) highlight the high plasticity and variation of individuals in adaptation to temperatures at contrasted spatiotemporal scales, relevant in a context of global warming. In particular, adaptation to temperature was observed during as short-term as within a cultural season. Indeed, sporulation intensity

would be enhanced during winter while, in spring, *Z. tritici* would display a shorter latency period (Suffert et al., 2015). Consequently, this high evolution capacity makes STB management even more complex and challenging.

## 3.2 Methods to control *Septoria tritici* blotch

As mentioned before in § 2.2.2.1, Integrated Pest Management (IPM), encouraged for a better use and a reduction of pesticides, should use all the methods, economically and environmentally available, to fight against phytopathogenic fungi (European Commission, 2017). Various actions can be set up for fighting STB, some even before the implementation of the crop in the field.

All methods described in this section should not be considered as antagonist. Indeed, they can be used in a complementary fashion. They also induce concomitant selection pressures and are therefore susceptible to face pathogen's adaptation and to be overcome. IPM promotes the use of all the available resources in order to increase the overall heterogeneity of the selection pressure and provide a successful and sustainable control of the disease.

### 3.2.1 Prophylactic actions to reduce epidemic intensity before vegetative development

#### 3.2.1.1 Agronomic preventive practices to reduce inoculum

Several agronomic decisions can reduce the number of spores and their dispersal. Late sowing is beneficial to prevent STB. Indeed, as temperatures and humidity are less favourable to *Z. tritici* under colder conditions in case of late sowing, winter wheat escapes some contaminations, which reduces inoculum the following spring (Suffert et al., 2011). Similarly, a high sowing density and nitrogen fertilisation will induce higher disease pressure, even if this effect is irregular (Arvalis - Institut du Végétal, 2020). Then, a compromise between disease pressure and yield promotion must be found. Crop rotation is recommended to interfere with the pathogen cycle, as continuous wheat growing favours the maintenance of the inoculum on debris and volunteers (Arvalis - Institut du Végétal, 2020; Eyal, 1987; Pedersen, 2009). Tillage may be also a useful practice as wheat debris on the surface are source of primary inoculum. It was

used on 41% of bread winter wheat growing area in France in 2017 (Agreste, 2020). However, as ascospores are dispersed over long distances and inoculum is unlimited, tillage efficacy might be reduced if residues remain in nearby unploughed fields (Arvalis - Institut du Végétal, 2020; Eyal, 1987).

All these practices reduce inoculum size but do not prevent the disease. The most efficient prophylactic practice is the use of tolerant or resistant varieties, as detailed hereafter.

### 3.2.1.2 Varietal choice: promoting wheat genetic resistance

Breeding wheat cultivars resistant to STB is another option to control this disease. Susceptibility to STB is not homogeneous within common wheat varieties. In a set of 335 elite European wheat varieties naturally inoculated by STB (likely airborne ascospores), some had a density of pycnidia about 250 times higher than others (Karisto et al., 2017). This study illustrates the large continuum range of STB infection intensity, spore production and symptoms that can be observed in wheat varieties and reveals variable wheat susceptibility to STB. Breeding programs for wheat varieties focus on obtaining varieties with low susceptibility to biotrophic aggressors and maximising yield which can result in a trade-off between those 2 traits (Vyska et al., 2016). To this purpose, crosses between different wheat varieties or wild relative species resistant to pathogens are generally carried out (Eyal, 1987; Torriani et al., 2015).

A significant part of resistance variation in *T. aestivum* can be explained by the presence of qualitative resistance genes, *i.e.* *Stb* genes (Brown et al., 2015). Twenty-one *Stb* genes have been identified so far. Complete resistance to *Z. tritici* has never been described until now but qualitative resistance is generally strong, even if often specific to a few avirulent pathogen genotypes (Duba et al., 2018). Indeed, qualitative resistance is often monogenic and controlled by a gene-for-gene interaction between the plant and the fungus (Flor, 1971). This qualitative resistance is effective all along plant life. However, among the twenty-one qualitative resistance genes described, to date, the gene-for-gene relationship has been established only for *Stb6*. It is also the only *Stb* gene of wheat cloned so far (Saintenac et al., 2018). Recently, a new resistance gene, *Stb16q*, has been identified and characterized (Saintenac et al., 2021). The only avirulence gene functionally validated in *Z. tritici* is *AvrStb6* which encodes a small secreted protein (Zhong et al., 2017).

Genetic resistance of wheat towards *Z. tritici* can also be partially explained by quantitative resistance. In this case, resistance variation is controlled by quantitative resistance traits that exhibits partial efficacy. Several genes are involved in this polygenic quantitative resistance and each of them has a low to moderate contribution to plant resistance. This resistance is less specific than the qualitative one and therefore often efficient, but not always, on multiple genotypes of *Z. tritici*. Contrary to qualitative resistance, quantitative resistance can be specific to some growth stages of the plant. In 2015, eighty-nine quantitative trait loci (QTL) or meta-QTL have already been described and new ones will surely be identified (Brown et al., 2015).

In 2020, four out of ten most used winter wheat varieties in France were considered as “quite resistant” or “not very susceptible” to STB. Those four varieties were representing more than 25% of the wheat surface in France (Arvalis - Institut du Végétal, 2020; FranceAgriMer, 2020). The benefit of the use of resistant varieties instead of susceptible ones is highly variable, depending on the year and the disease pressure. In trials in 2019, a difference of 6 dt/ha was estimated between a very susceptible variety and a resistant one (Arvalis - Institut du Végétal, 2019). However, disease resistance genes exert a selective pressure on pathogen populations and also contribute to their adaptation. An erosion of the resistance of varieties, and especially of the most cultivated ones, is frequently observed. The speed of resistance breakdown depends on the genetics of plant resistance. Indeed, quantitative resistances are considered more durable than qualitative ones (Brown et al., 2015; Mundt, 2014). This might be due to the number of genes involved in quantitative resistance but also to the fact that the selection coefficient should be smaller for resistance with smaller effect or that specificity is lower in quantitative resistance than in qualitative one. The evolution of virulence in some *Z. tritici* populations can rapidly overcome the resistance of some varieties. For example, *Z. tritici* population in the Willamette Valley of Oregon became fully virulent to the cv. Gene in 5 years and this adaptation is still generalized even after the use decrease of this cultivar (Cowger et al., 2000). A survey carried out between 2013 and 2017 revealed that the avirulent isoforms of *AvrStb6* was universally present in field isolates sampled from major wheat-growing regions of the world (Stephens et al., 2021). Annual monitoring is therefore regularly carried out to follow the evolution of the ability of varieties to resist the pathogen and help farmers in their varietal choice. Gene pyramiding (*i.e.* the combination of several resistance genes in the same cultivar) may enhance varietal resistance as the probability that the pathogen mutate to virulence for each gene is

low (Brown et al., 2015; Mundt, 2014). This has been an efficient strategy for about 40 years to fight against wheat stem-rust. Varietal mixtures are efficient to reduce STB progress (Gigot, 2013; Vidal, 2017) and may even constitute a strategy to delay adaptation of *Z. tritici* to varietal resistance (as resistance gene rotation) (Mundt, 2014; Orellana-Torrejon et al., 2021). The mechanisms involved in mixtures efficacy to control diseases might be a dilution effect, a barrier effect, a premunition effect, a compensation effect or a disruptive selection (Borg et al., 2018). In 2020, varietal mixtures were representing about 12% of bread wheat acreage in France (FranceAgriMer, 2020).

### 3.2.2 Use of plant protection products during the growing season

Another option to protect wheat from STB is to spray antifungals, from natural or synthetic origin.

#### 3.2.2.1 Biocontrol of STB

In agreement with IPM, some biocontrol products might be considered in first intention compared to synthetic chemicals when treatment against STB is needed. Biocontrol agents or products are defined as “part of integrated pest management using natural mechanisms that can be either macro-organisms or phytopharmaceuticals products including micro-organisms, chemical mediators [...] or natural substances of plant, animal or mineral origin” (Code rural et de la pêche maritime Article L253-6 (Légifrance, 2020)). Biocontrol agents either have a direct antifungal effect or are plant defence inducers.

In France, few biocontrol solutions are available to control STB. Laminarin (Vacciplant®) is a plant defence elicitor extract from the alga *Laminaria digitata*, active on both dicots and monocots such as wheat (Stadnik and Freitas, 2014). Laminarin induce typical defence responses as the activation of protein kinase,  $Ca^{2+}$  influx, oxidative burst and alkalinization of extracellular media. They also increase chitinase and  $\beta$ -1,3- glucanase activities and phytoalexins production. A preparation of *Pseudomonas chlororaphis* (strain MA 342) is also registered in France, probably acting through the secretion of antifungal secondary metabolites (Hernandez-Jerez et al., 2020). However, it is a seed treatment against *Septoria nodorum* (leaf spot disease) and not a foliar one usable against *Z. tritici*. Sulphur is a mineral substance used for a long time on

wheat to control powdery mildew. It has been authorized in 2019 to control STB. Its multisite activity has since been recognized and sulphur conveniently replaces the synthetic multisite inhibitor chlorothalonil withdrawn in 2020. It is now the most used biocontrol product on cereals. Indeed, the offer for disease biocontrol is still narrow and efficacy is partial. (Arvalis - Institut du Végétal, 2020) estimates that four applications of sulphur alone give good results (although not as good as two conventional treatments), but the economic impact associated to those four treatments is higher. The use of sulphur alone is relevant when there is a low disease pressure or with varieties carrying some resistance to STB. In practice, sulphur is mostly used for the first application in association with a triazole, to enhance efficacy and disease spectrum (action on yellow rust).

In the last decades, many investigations on biocontrol have been carried out because it may help reducing the use of conventional pesticides. For example, Kildea et al. (2008) described the high potential of *Bacillus megaterium* to control STB, as it synthesises multiple antifungal compounds. Similarly, Lynch et al. (2016a) studied antifungal *Lactobacillus* strains. However, to date, neither are authorized. From screening in laboratory to efficacy in the field, there is often a gap, and product formulation is not always easy to optimize. The next products on the market will be potassium phosphonates that are currently under registration process as plant defence inducers and also direct antifungals such as natamycin. Those may be available in 2022.

The biocontrol market is globally rising (+8.5% sales performance in France between 2018 and 2019 (IBMA, 2020)) but concerning STB control, it is mainly thanks to sulphur use.

### 3.2.2.2 Chemical control of STB

Despite the implementation of the methods described before, STB may still escape control and chemical treatments often appear necessary. Today, chemicals remain the principal method used to control STB in France, as in the whole world. In 2015, Torriani et al. (2015) and Fones and Gurr (2015) estimated that about 70% of the €1.3 bn annual budget for fungicides in the EU were actually targeting *Z. tritici*.

Five different fungicide modes of action are available to control STB. They either target only one biochemical site (unisite inhibitors) or multiple ones (multisite inhibitors) (Figure 25).

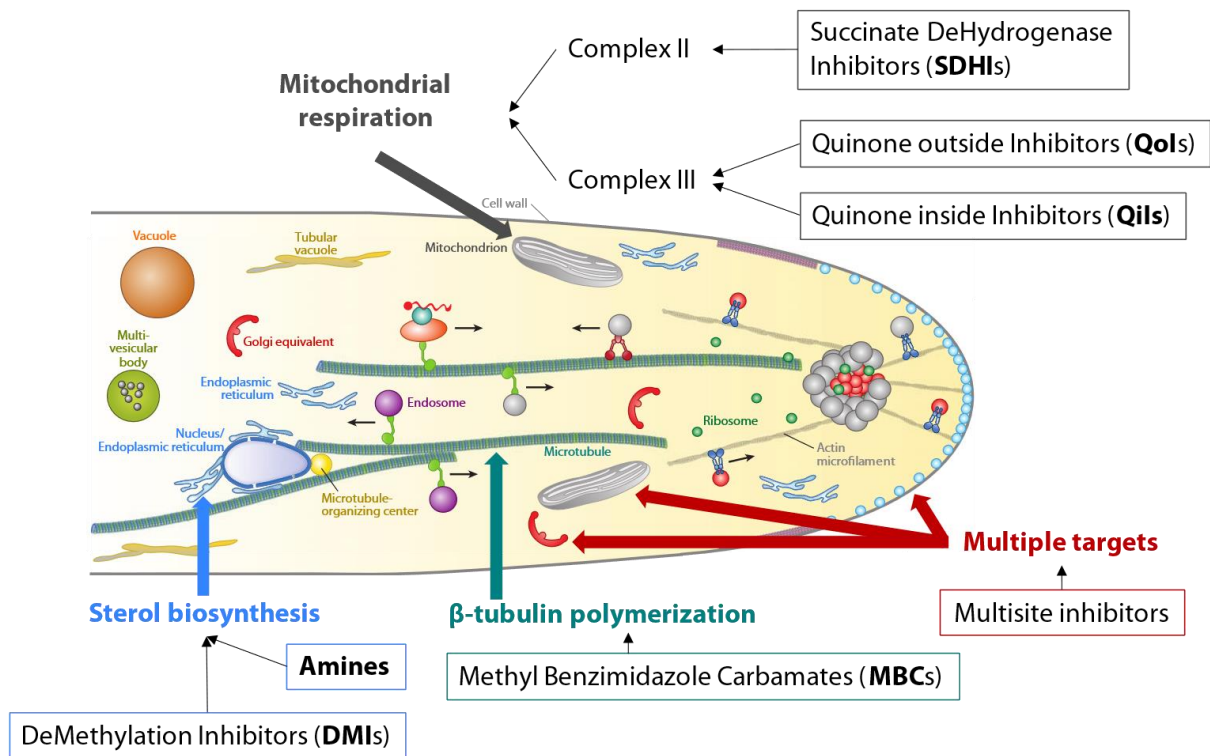


Figure 25: Main biological pathways targeted by fungicides inhibitors used to control STB. Adapted from (Riquelme, 2013).

### 3.2.2.2.1 Multisite inhibitors

Multisite fungicides may affect simultaneously the fungal respiratory, lipidic and glucidic pathways due to the interaction with many chemical groups, and the thiol groups in particular (Leroux, 2003). As thiol groups are not present only in fungal proteins, multisite fungicides are often unspecific, affecting many different organisms. Additionally, the intrinsic activity and the persistence of those contact fungicides is quite low which implies the regular applications of high doses. Consequently, ecotoxicological problems are often associated to those AIs. As an example, the massive use copper in vineyards induced important environmental issues due to contamination of soils and water, as well as toxicity for non-target organisms (Peña et al., 2018).

Chlorothalonil (chloromethylmercaptans) has been the most popular multisite inhibitor on cereals for about 40 years, largely recognized for its efficacy against STB in Europe. Yet, it has been removed from the EU in 2020 for toxicological reasons (Arvalis - Institut du Végétal, 2020). Similarly, the commercial authorization of mancozeb (dithiocarbamates) has been withdrawn by the EU in 2021. A few multisites are still authorized: sulphur has already been mentioned before as a biocontrol product, and the registration of folpet has recently been extended for



use on *Z. tritici*. Folpet is a nonspecific multisite inhibitor affecting energy production and cell membrane (Waard and Nistelrooy, 1984).

#### *3.2.2.2.2 Inhibitors of $\beta$ -tubulin*

The Methyl Benzimidazole Carbamates (MBCs) were released on the market in the 1970s, with the benzimidazoles class being the most popular. These fungicides quickly seduced farmers with their broad spectrum of activity including various fungi (on ascomycetes and basidiomycetes), their systemic activity, and their high efficacy. Benzimidazoles target the  $\beta$ -tubulin (encoded by *tub2*) constituting microtubules, resulting in an inhibition of its polymerisation, and thus a default in cell division and hyphal growth (Olaya and Geddens, 2019; Velasco-Corcía et al., 2018). Today, this mode of action has been removed because of resistance issues and lower efficacy, compared to more recent fungicides. Carbendazim is the most famous benzimidazole used against STB. It was withdrawn in 2009, while its pro-drug thiophanate-methyl has just been removed in 2020.

#### *3.2.2.2.3 Inhibitors of sterol 14 $\alpha$ -demethylation*

DeMethylation Inhibitors (DMIs) inhibits the sterol 14 $\alpha$ -demethylase (a cytochrome P450 encoded by *cyp51*) within the sterol biosynthesis pathway and therefore affect the fungal cell wall (Leadbeater, 2011; Leroux and Walker, 2011; Leroux et al., 2007; Torriani et al., 2015). Indeed, ergosterol biosynthesis is disrupted, whereas it is involved in membrane permeability and fluidity. Among DMIs, the most active fungicides against *Z. tritici* are triazoles (*e.g.* metconazole, tebuconazole, mefentrifluconazole) but some imidazoles (*e.g.* prochloraz) and triazolinethiones (*e.g.* prothioconazole, which is the pro-drug of prothioconazole-desthio) are also registered. The first DMIs were introduced in the market in the 1970s. They displayed a large spectrum as well as good curative activity for many AIs and high persistence. They are still largely used despite the generalization of a quantitative resistance in European populations. Many have now been withdrawn for being endocrine disruptors (Zhang et al., 2019).

#### 3.2.2.2.4 Inhibitors of $\Delta 8 \rightarrow \Delta 7$ isomerase and $C_{14}$ reductase

As DMIs, amines disrupt the biosynthesis of ergosterol while targeting the  $\Delta 8 \rightarrow \Delta 7$  isomerase and the  $C_{14}$  reductase but their spectrum of action is narrower (Leadbeater, 2011). Fenpropidin (piperidine) is authorized to control *Septoria spp.* but is not really used against STB in practice because of its weak activity compared to other unisites. It is mostly used against powdery mildew in cereals.

#### 3.2.2.2.5 Inhibitors of respiration complex III

Quinone outside Inhibitors (QoIs) were released at the end of the 1990s on many different crops to control a large range of fungi. Strobilurin fungicides (*e.g.* azoxystrobin) are derived from a natural fungal toxin and represent the wider and most famous class of QoIs. QoIs interfere with the energetic metabolism of the fungus by blocking electron transfer in the mitochondrial complex III of the respiratory chain. Precisely, the fungicide operates in the fixation site of ubiquinone on the outer face of cytochrome bc1 (encoded by *cytb*), which makes those fungicides highly site-specific and therefore at high resistance risk (Fernández-Ortuño et al., 2010; Siah et al., 2014). When they first appeared on the market, QoIs were very attractive because of their wide spectrum, their high efficacy and their "greening effect" (delayed leaf senescence), increasing yield on wheat. However, their efficacy quickly decreased when the first resistances emerged just a few years after their introduction (see below § 3.3.1.4).

#### 3.2.2.2.6 Inhibitors of respiration complex II

The second generation of Succinate DeHydrogenase Inhibitors (SDHIs) were released to control STB in the 2010s. Despite their relative higher cost compared with other fungicides, they became popular because of their high level of activity and their broad spectrum (on ascomycetes and basidiomycetes), associated with the fact that they offered expected new solutions in some places. From the release of boscalid in 2006, several pyrazole-carboxamides extended this MoA, the most recent ones being benzovindiflupyr and fluopyram (2016). SDHIs target the complex II of mitochondrial respiration by blocking the fixation of ubiquinone on the succinate dehydrogenase (SDH) enzyme. The SDH is composed of four subunits, encoded by the *sdhA*, *sdhB*, *sdhC* and *SdhD* genes. The binding site of SDHIs is composed of SdhB, SdhC

and SdhD. As for QoIs, the target site is quite specific in the mitochondrial complex. These fungicides have been categorized with a high to medium fungicide resistance risk (Hagerty et al., 2020; Sierotzki and Scalliet, 2013). Indeed, resistance is nowadays developing in Europe.

#### *3.2.2.2.7 Recent and future unisite modes of action*

Mefentrifluconazole (DMIs) has been launched in 2020. It seems less affected by cross-resistance compared to the older AIs or to prothioconazole and epoxiconazole because positive cross-resistance is generally weak for many, but not all *cyp51* genotypes (Ishii et al., 2021; Jørgensen et al., 2020).

Quinone inside Inhibitors (QiIs) were released in France in the spring 2021 campaign with the launch of fenpicoxamid. Like QoIs, QiIs target the cytochrome *bc1* of complex III of respiratory chain but binds at the inner quinone binding site, making them an independent mode of action (Owen et al., 2017). Though, strains exhibiting the G143A change (leading to QoI resistance) do not exhibit cross-resistance with QiIs. However, QiIs resistance risk is probably similarly high as QoIs.

Metyltetraprole is a new QoI (newly referred as QoI-A) that should be launched in the market in the following years.

According to Suemoto et al. (2019), it may not present cross-resistance with the actual QoIs and should therefore be interesting to control strains carrying the G143A change.

Pydiflumetofen is a new SDHI (sub class of stretched heterocycle amide SDHIs (SHA-SDHIs)) that is still under registration (maybe will be authorized in 2022?). It may display higher intrinsic activity compared the former or actual SDHIs (Steinhauer et al., 2019).

#### *3.2.2.2.8 Control of STB with fungicides in France*

Fungicides targeting STB are generally applied preventively, before symptoms apparition. The main goal of such interventions is to protect the two upper leaves which have a major impact on future yield (contributing to about 35% of the yield in healthy conditions (Wazziki et al., 2015)). In France, the number of fungicides sprays on wheat varies from 1 to 3 during the growing season, depending on the climatic conditions and the varietal sensibility. In 2020, only 14% of wheat fields were sprayed three times (23% in 2019), while the proportion of fields

sprayed only once represented 42%. When disease pressure is low, the first treatment is bypassed. This illustrates the recent switch in spraying strategies, explained by lower disease pressure in the very recent years and greater availability of resistant cultivars.

The first treatment (31-37 on the BBCH scale) is often composed of a multisite (sulphur, folpet) associated or not with a triazole, while the third (60-69 on the BBCH scale) is composed of one or a mixture of DMIs (triazole + imidazole for example) or even a mixture of a triazole and a QoI. Note that this last treatment does often not only target STB but other fungi such as rusts or *Fusarium spp.* The most important intervention is the one close to the flag leaf stage (usually the 2<sup>nd</sup> treatment, 39-59 on the BBCH scale) that is mostly composed of the association of an SDHI and a triazole (and sometimes also a multisite). Almost the whole wheat area received at least one SDHI during the growing season in 2020 (Arvalis - Institut du Végétal, 2020), which highlights the economic importance of this mode of action. QiIs provide a new mode of action that could be interesting in association during the second treatment.

#### *3.2.2.2.9 Piloting treatments with decision support tools in France*

In addition to the visual observation of symptoms, some decision support tools (DST) have been developed to help farmers determine when an intervention (conventional chemical or biocontrol) is necessary. Indeed, the decision to spray a fungicide (biosynthetic or natural) depends on weather conditions, on the infection status of the field, on the estimated loss of yield induced and on treatment cost (economical and time). In particular, preventive fungicides might be delicate to spray at the appropriate timing, before symptoms are observed. In a context of pesticides reduction, those tools are helpful to determine the relevance of an intervention and its optimal timing. In France, many programs have been developed as for example Taméo or Septo-LIS from Arvalis or ATLAS-xarvio field manager (BASF), Météus (ISAGRI), Opti-Protech, Farmstar (non-exhaustive list), based on Arvalis models. For a precise piloting, they take into account the crop, the bio-aggressor epidemiology and its harmful threshold (in the area considered), as well as the climatic conditions at local scales, before eventually recommending a treatment and its optimal timing. To encourage the use of such tools, a phytopharmaceutical products savings certificate can be delivered from a subscription to this type of programs (Ministère de l'agriculture et de l'alimentation, 2020). In 2019, it was estimated that those DST were used on 10% of wheat area in France (Contrat de Solutions, 2019).

### 3.3 Fungicide resistance in *Zymoseptoria tritici*

Up to date, all unisite inhibitors are concerned by fungicide resistance in *Z. tritici*. The resistance mechanisms associated to each mode of action are listed in Table 4. The resistances mechanisms occurring in *Z. tritici* are described in Figure 26.

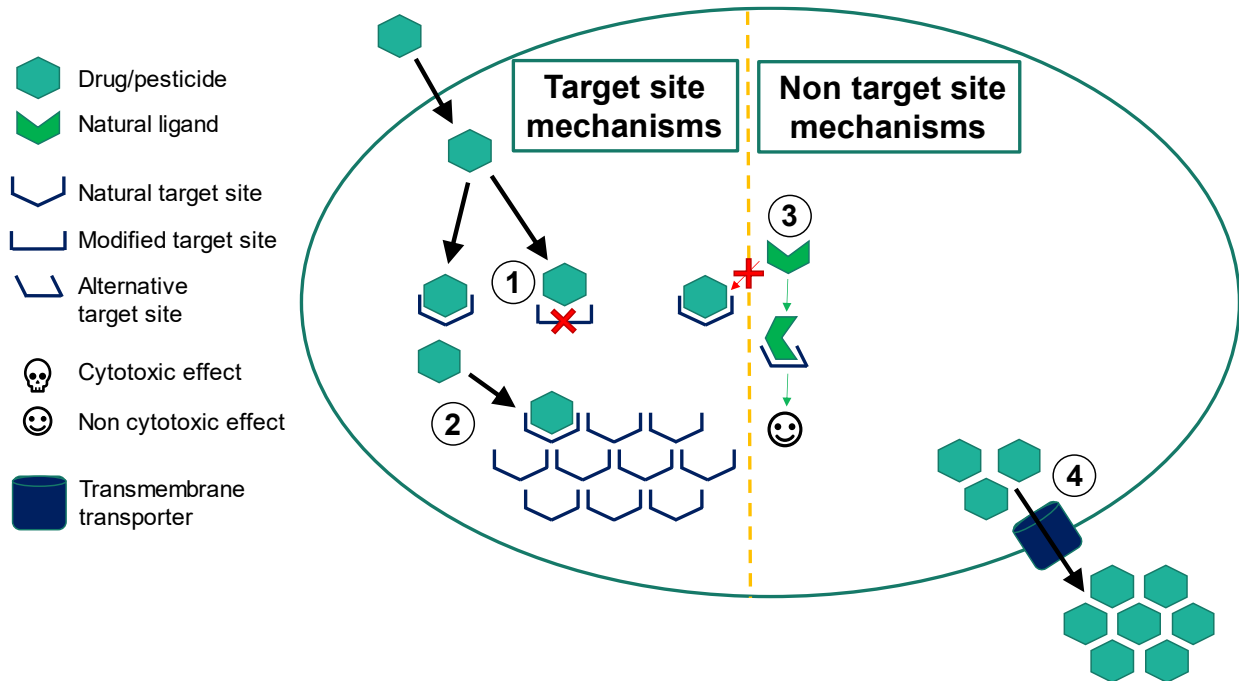


Figure 26: Fungicide resistance mechanisms described in *Z. tritici*. Adapted from (R4P Network, 2016). Target Site Resistance (TSR): 1) Target site structural alteration decreasing the affinity for the fungicide; 2) Target site overexpression. Non-Target Site Resistance (NTSR): 3) Bypass of the target site function by an alternative pathway; 4) Enhanced efflux of fungicide outside the cell.

Table 4 : Fungicides used to control *Z. tritici* in France and associated resistance phenomena. Adapted from (Garnault et al., 2019).

Fungicides											Resistance <sup>a</sup>					
Biochemical mode of action <sup>s</sup>			Chemical structures <sup>s</sup>		Active ingredient <sup>†</sup>	Autorisation in France <sup>‡</sup>			Codes		Resistance phenotype abbreviation	Target site resistance		Non target site resistance		
Main proces-ses	Target site and mode of action	Abbreviation	Main class	Secondary class		Registration	First year of use	Removal	R4p <sup>§</sup>	FRAC <sup>†</sup>		Target alteration	Target overexpression	Compensation	Enhanced efflux (MDR)	
Mitochondrial respiration	Complex II or succinate dehydrogenase Binding site of ubiquinone involving the subunits SdhB, SdhC and SdhD.	SDHI	Carboxamides	Benzamides	Fluopyram	2016	2017	-	A2a	C2/7	CarR	+	[LR] SdhB: N225I, R265P, T268A/I SdhC: T79N, W80S, N86A/S R151M/S/T, I261S, V166M, T168R SdhD: R47W, I50F, M114F [MR-HR] SdhC: T79I, H152R	-	-	+ [LR when alone]  MFS1 promoter: types I, II or III inserts
				Nicotinamides (syn. Pyridine-carboxamides)	Boscalid	2006	2007	-								
				Pyrazole-carboxamides	Benzovindiflupyr	2016	2017	-								
					Bixafen	2011	2011	-								
					Penthiopyrad	2014	2014	-								
				Fluxapyroxad	2011	2011	-									
	Stretched heterocycle carboxamides	Pydiflumetofen	-	-	-											
	Complex III or cytochrome bc1. Binding site of ubiquinone to cytochrome b at the "o" center (proton output) in the heme bl proximal domain	QoI (or QoI-P)	Synthetic strobilurins and analogs	Methoxy-acrylates	Azoxystrobin	1996	1997	-	A5a	C3/11	StrR (6-12)	+	[LR] Cytb: F129L  [HR] Cytb: G143A	-	+ [LR] AOX over-expression	+ [LR when alone]  MFS1 promoter: types I, II or III inserts
					Picoxystrobin	1999	2000	2018								
				Methoxy-carbamates	Pyraclostrobin	2000	2000	-								
Oximino-acetates				Kresoxim-methyl	1996	1997	2013									
				Trifloxystrobin	2001	2002	-									
Oximino-acetamides				Dimoxystrobin	2009	2010	-									
Fluoxastrobin	2006	2006	-													
Tetrazolinone	Metyltetraprole	2022?	-	-												

	Mitochondrial complex III or cytochrome bc1 - fixing area for the ubiquinone on the inner face of the cytochrome b	QiI	Carboxamides	Picolinamides	Fenpicoxamid	2020	2021	-	A3a	C4/21	QiR (10-13)		[HR] Cytb: G37V (lab mutant)	-	-	+ [LR when alone] MFS1 promoter: types I, II or III inserts
					Florypicoxamid	-	-	-								
Sterol biosynthesis	Binding to sterol 14 $\alpha$ -demethylase (syn. CYP51)	DMI or SBI-DM	Heterocyclic compounds	Imidazoles	Prochloraz	1980	1980?	-	E2	G1/3	TriR (6,14-16)	+	[LR] Cyp51: one change (e.g. Y137F, Y459C/D/H, G460D/S, Y461H/S, $\Delta$ Y459/G460) or two changes (V136A+Y461H/S or $\Delta$ Y459/G460) [MR] Cyp51: two changes including A379G/I381V+Y459S/D/N, Y461H/S or $\Delta$ Y459/G460) [HR] Cyp51: three to six changes including those reported for TriLR and TriMR phenotypes, combined with D107V, D134G and/or S524T.	+ [LR when alone] Cyp51 promoter: 120 bp or 1000 bp inserts	-	+ [LR when alone] MFS1 promoter: types I, II or III inserts
				Triazoles	Bromuconazole	1994	1995	-								
					Cyproconazole	1987	1987	-								
					Difenoconazole	1988	2011	-								
					Epoxiconazole	1992	1992	2020								
					Fenbuconazole	1991	1992	-								
					Fluquinconazole	1997	1998	2016								
					Flusilazole	1985	1986	2013								
					Flutriafol	1983	1984	2015								
					Hexaconazole	1990	1990	2007								
					Metconazole	1993	1994	-								
					Propiconazole	1980	1980?	2019								
					Tebuconazole	1988	1989	-								
					Tetraconazole	1991	1991?	-								
				Triadimenol	1987	1988	2014									
Mefentrifluconazole	2019	2020	-													
Triazolinethiones	Prothioconazole	2006	2006	-												

Cell division: microtubules of the mitotic spindle	Binding to $\beta$ -tubulin	BMC	Benzimidazoles and precursors	Benzimidazoles	Carbendazim	1972	1972?	2009	K2b	B1/1	BenR (6,17)	+	[HR] Tub2: E198A	-	-	-
				Thiophanates	Thiophanate-methyl	1973	1980?	2021								
Multisite activity	Fungicide acting non-specifically on multiple targets, especially respiratory enzymes	MSI	Dithio-carbamates	Akylene-bis-dithiocarbamates	Mancozeb	1999	1999?	2021	W11	M3	NC	NC	NC	NC	NC	NC
					Maneb	1988	1988?	2017								
			Chloronitriles	Phthalonitriles	Chlorothalonil	1980	1980?	2020	W14	M5						
			Halogenated alkyl-thio compounds	Phthalamides	Folpet	2003	2003	2009 (reintroduced in 2020)	W10	M4						
	Sulphur-based mineral substance	-	Elemental sulphur	1942	1942?	-	W4	M2								

? Approximate date; NC: Not concerned.

<sup>§</sup> According to the R4P classification (<http://www.r4p-inra.fr>).

<sup>†</sup> According to the FRAC classification (<http://www.frac.info>).

<sup>‡</sup> Legal authorisation of the active ingredient in France. Year the molecule was registered in France, year it was first used in the field and year it was removed from French registration (<http://www.ephy.anses.fr>).

<sup>¶</sup> Phenotypes are described in § 3.3. [LR]: low resistance levels (<25); [MR]: moderate resistance level (25<RL<100); [HR]: high resistance level (>100).

References: 1) (Torriani et al., 2015), 2) (Dooley et al., 2016b), 3) (Rehfus et al., 2018), 4) (Scalliet et al., 2012), 5) (Yamashita and Fraaije, 2018), 6) (Lucas et al., 2015), 7) (Fernández-Ortuño et al., 2010), 8) (Torriani et al., 2009), 9) (Stammler et al., 2008), 10) (Miguez et al., 2004), 11) (Wood and Hollomon, 2003), 12) (Omrane et al., 2017), 13) (Fouché et al., 2020), 14) (Leroux and Walker, 2011), 15) (Cools and Fraaije, 2013), 16) (Huf et al., 2018), 17) (Griffin and Fisher, 1985).



### 3.3.1 Status of fungicide resistance in French populations of *Z. tritici*

Most of the MoAs allowed for STB control are facing resistance (Figure 27). These cases will be successively developed in this section.

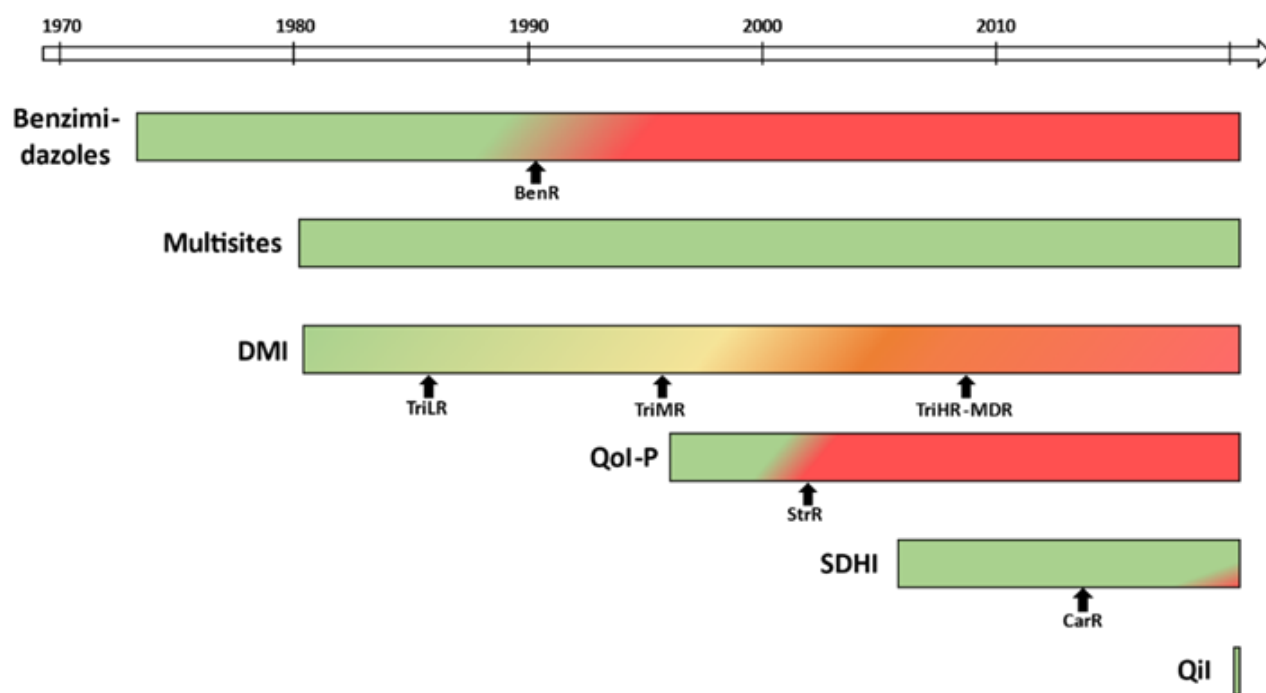


Figure 27 : Evolution of the status of resistance of French *Z. tritici* populations to the MoAs used to control STB, from their introduction to nowadays. Green represents susceptible population, and red resistant populations (with high RFs). Quantitative resistance to DMIs is marked with colours varying from green to red.

#### 3.3.1.1 Susceptibility of multisite inhibitors

Multisite fungicides are considered as low resistance risk AIs as multiple mutations in genes encoding their various target proteins might be lethal. Nonetheless, NTSR might be expected for these fungicides. As examples, some resistance to organomercurials have been observed in *Pyrenophora avenae* (Noble et al., 1966; Sheridan et al., 1968)(unknown mechanism). In bacteria like *Pseudomonas aeruginosa*, resistance to copper has been described (Bondarczuk and Piotrowska-Seget, 2013). The mechanisms involved are extra- and intracellular sequestration, enzymatic detoxification, and efflux outside the cell *via*  $\text{Cu}^+$ -ATPase. In practice, specific resistance towards multisite inhibitors has never been observed in *Z. tritici* and is then supposed unlikely but not impossible. The popularity of multisites arises from their low resistance risk rather than from their intrinsic activity. Arvalis tested in recent trials their ability to decrease resistance selection to multiple unisite MoAs and multidrug resistance (a generalist mechanism described in § 1.1.4) (Arvalis - Institut du Végétal, 2019, 2020). Despite some claims from chemical companies, Arvalis could not highlight the reduction of the frequency of TriHR and MDR strains after multisite-based applications. Consequently, multisite

may not modify population structure, but they are certainly an asset to keep efficacy, as they are not concerned by resistance.

### 3.3.1.2 Resistance to inhibitors of $\beta$ -tubulin polymerization

Resistance to benzimidazoles is qualitative and associated to the unique E198A change in  $\beta$ -tubulin, which confers high resistance levels (RF>1000) to *Z. tritici* (Griffin and Fisher, 1985; Lucas et al., 2015). Many others mutations in *tub2* have been described in other species (multiallelic resistance) and are usually associated with variable resistant factors and cross-resistance patterns (Hawkins and Fraaije, 2016). Positive cross-resistance between benzimidazoles (and also to zoxamide which is a benzamide) has been described in the presence of the E198A change, but it is also associated with a negative cross-resistance to N-phenylcarbamates (*e.g.* diethofencarb, withdrawn in 2007). This resistance is generalized in Western Europe populations since the 1980s. In France, the frequency of this resistance is stabilized in populations at around 90% (Garnault et al., 2019). Benzimidazoles became quickly inefficient to control STB and are therefore no longer used. The absence of frequency decrease over the years despite the minimal use of benzimidazoles in the last decades probably reveals a lack of fitness penalty for this resistance in *Z. tritici* (Figure 30).

### 3.3.1.3 Resistance to inhibitors of respiration complex III

Similarly to benzimidazoles, the qualitative resistance to QoIs is associated to the single monoallelic change G143A in the mitochondrial cytochrome b of *Z. tritici* (Fernández-Ortuño et al., 2010; Lucas et al., 2015). Positive cross-resistance between all QoIs (except with metyltetraprole (Suemoto et al., 2019)) has been described for this genotype. Resistant strains exhibit very high resistance factors (>250) and got generalized in Western Europe only 4-5 years after the registration of this MoA. Phylogenetics studies demonstrated that this G143A change in *cytb* appeared independently at least four times in Europe (Torriani et al., 2009). The high selection pressure induced by QoI use selected those resistant genotypes that were then wind-dispersed from western to eastern Europe. In France, the progression of this resistance was also observed from north to south and its speed was estimated at around 120-145 km/year between 2004 and 2008 (Garnault et al., 2019) (Figure 28). QoIs are inefficient to control STB when resistance frequencies are high as it is now in France (>95%) and in western Europe. Consequently, their use highly decreased (about half their use when compared to the 2000s) and their residual use targets rust and Fusarium head blight. The

stabilisation of the resistance frequency can result from the lack or weak resistance cost and the remaining selection pressure induced by the use for other diseases (Figure 30).

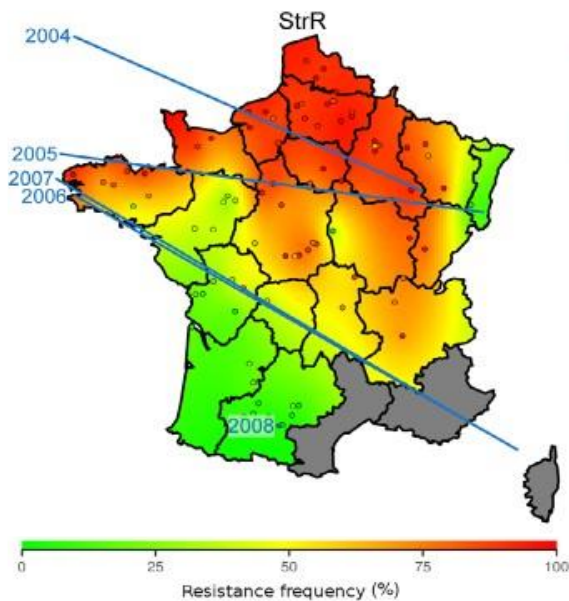


Figure 28: Spatial progression over time of the QoI resistant phenotypes (StrR) in France. Background map and points are representing the status of resistance in 2006. From (Garnault et al., 2019)

QiIs, released only in 2021 are not yet concerned by resistance. However, resistance risk is assumed to be medium to high. Indeed, predictive experiments suggest that the cytb G37V change, affecting the Qi site of cytochrome b, should be the most probable resistance mechanism (Fouché et al., 2020) and might be selected even in G143A backgrounds.

Moreover, it should be noted that the activation of the alternative oxidase (AOX) allows bypassing complexes III and IV and therefore the inhibition of complex III by QoIs and QiIs in *Z. tritici* (Miguez et al., 2004; Wood and Hollomon, 2003). The activation of the AOX pathway leads to an energy loss

(only about 40% of normal energy production is maintained (Wood and Hollomon, 2003)) which is however sufficient for a certain mycelial growth in the plant despite fungicide application. This lower energy is insufficient for the early stages of infection, but AOX activation could increase the probability to select target-site resistance.

### 3.3.1.4 Resistance to inhibitors of respiration complex II

SDHIs, registered in the late 2000s, also select for qualitative resistance but an increasing diversity of alterations is being recorded in either subunits B, C or D of SDH (Dooley et al., 2016b; Sierotzki and Scalliet, 2013). Indeed, almost every year new mutations associated with reduced susceptibility are discovered in field isolates (Hagerty et al., 2020). Resistant strains exhibit one single change, very rarely two, in different subunits. These changes are associated to low to high RFs, and diverse cross-resistance patterns between AIs (Hagerty et al., 2020; Steinhauer et al., 2019; Torriani et al., 2015; Yamashita and Fraaije, 2018). For example, strong cross-resistance only between fluopyram and isofetamid has been recorded (Yamashita and Fraaije, 2018). The first resistant strains have been collected in 2012 and were bearing the C-T79N change in France and C-W80S one in UK. These

substitutions are associated with a low to moderate decrease of SDHIs susceptibility *in vitro*. Moreover, the C-H152R change, associated to high resistance factors towards all SDHIs, has been discovered in 2015 in Ireland and later in other countries (Dooley et al., 2016a). Its frequency is still low in France and might be associated with a fitness cost (Scalliet et al., 2012) but its presence is regularly detected and then preoccupying (Garnault et al., 2019). Currently, the most frequent substitutions found in Europe are the C-T79N and C-N86S ones (Rehfus et al., 2018). Altogether, SDHI resistant phenotypes represented 13% of the French population in 2019 to 18% in 2020 (which was a year of low STB pressure) (Figure 30) (INRAE, Anses, Arvalis - Institut du Végétal, 2021; A.-S. Walker, unpublished). As SDHIs are usually used in mixtures with other MoAs, they still perform as expected in most situations (Arvalis - Institut du Végétal, 2020; Torriani et al., 2015). Yet, resistance to SDHIs is much more evolved in UK and in Ireland, where some weak alleles are present at high frequencies and C-H152R may reach more than 10% in some areas. This population structure is consistent with the erosion of efficacy in these areas (FRAC, 2020; Hellin et al., 2020).

In 2019, the identification of a single dispensable gene paralog of SdhC (called ZtSdhC3), coding for an alternative SdhC, has been correlated to resistance to the subclass of SHA-SDHIs (Steinhauer et al., 2019). About 20-30% of the European tested strains of *Z. tritici* exhibits this ZtSdhC3 paralog. The resistance levels associated to this SHA-SDHI resistance is not only correlated to the presence of ZtSdhC3 but also to its expression level and its alternative splicing. The use of the SHA-SDHIs like fluopyram should therefore induce a strong selection pressure on those resistant genotypes when used alone (but currently only sold in mixture with bixafen).

### 3.3.1.5 Resistance to inhibitors of sterol 14 $\alpha$ -demethylation

By contrast with the previous modes of action, resistance to DMIs is quantitative. A large diversity of phenotypes has been observed since the late 1970s, concomitantly with RFs increase. They are referred as TriLR, TriMR or TriHR for low, medium or high resistance phenotypes, respectively and each category includes several genotypes. DMI resistance can be polygenic. The first mechanism that emerged in field strains was the alteration of the target protein, the 14 $\alpha$ -demethylase encoded by *cyp51*. Up to 11 changes, leading to more than 33 *cyp51* genotypes when combined were recently recorded in a collection of 331 European isolates (Huf et al., 2018). But globally more than 30 different mutations and 100 haplotypes have been identified (Cools and Fraaije, 2013; Huf et al., 2018; Lucas et al., 2015). These genotypes are associated to varying RFs and partial cross-resistance, depending on the DMI considered (Cools et al., 2011; Heick et al., 2020; Leroux and

Walker, 2011; Lucas et al., 2015). For example, the changes V136A and I381V are respectively providing low and high RFs to tebuconazole and conversely for prochloraz (Huf et al., 2018; Leroux and Walker, 2011). Patterns of incomplete cross-resistance are also quite apparent. For example, tebuconazole, difenoconazole and mefentrifluconazole are displaying positive cross-resistance and are constituting one group distinct from the one composed of epoxiconazole, cyproconazole and prothioconazole (Heick et al., 2020; Huf et al., 2018; Ishii et al., 2021). Cross-resistance may then impair the efficacy of new AIs from the same chemical class.

The overexpression of *cyp51* is a possible second resistance mechanism, contributing to DMIs resistance, with a ten-times reduction of susceptibility in some isolates (Cools et al., 2011; Leroux and Walker, 2011). Indeed, the overexpression of *cyp51*, due to possible possible inserts in the promoter of this gene, counteracts fungicide effect by greater concentration of 14 $\alpha$ -demethylase. DMIs resistance is also often associated with enhanced efflux (multidrug resistance or MDR) but will be discussed in a next section.

DMI resistance is present everywhere in Europe, with some common haplotypes, and some specific to a few countries. Huf et al. 2018 found that 85% of their collection were actually composed of only 9 *CYP51* haplotypes. A global gradient of azole susceptibility is observed across Europe from less susceptible populations in North-West to more susceptible in South/-East (Jørgensen et al., 2018a). Indeed, in UK and Ireland haplotypes carrying the S524T substitution are present in more than 50% of the studied populations (Huf et al., 2018), and sometimes upper than 70% in some areas (Hellin et al., 2020). Those haplotypes are currently the ones most affecting the susceptibility of DMIs (Cools et al., 2011; Huf et al., 2018; Leroux and Walker, 2011). These spatial differences can probably be explained by the contrasted use of fungicides (due to the higher disease pressure and the intensive production of wheat in the west of Europe compared to east) (Fones and Gurr, 2015; Huf et al., 2018). DMIs resistance probably emerged in North-West of Europe and spread to South-East *via* ascospores dispersal, helped by the continuous selection pressure promoting the less susceptible genotypes (Lucas et al., 2015). The variation of *cyp51* haplotypes is not only spatial, but also temporal. Indeed, French monitoring described a recent counterselection of TriLR (<1% today) and TriMR phenotypes (27% in 2019 against 21% in 2020) while the frequency of TriHR strains is still increasing in populations (47% in 2019, 55% in 2020) (Garnault et al., 2019; A.-S. Walker, unpublished) (Figure 30). Those data are consistent with the observed erosion of DMIs efficacy in Europe for the last decades, illustrated for example by a recent significant increase of EC<sub>50s</sub> for epoxiconazole, prothioconazole and metconazole while the substitution S524T emerged in populations (Heick et al., 2020; Jørgensen et al., 2020). At a regional scale, a shift in susceptibility is also observed from north to south of France that may

be explained by the higher presence of TriHR genotypes (with the *cyp51* V/C136A and D134G changes) in the north (Garnault et al., 2019). The continuous emergence of new and more complex haplotypes associated with higher RFs and their sequential spread through Europe should explain the high diversity of haplotypes observed over time and space (Cools and Fraaije, 2013; Garnault et al., 2019). Field efficacy of DMIs in France is globally partial and decreasing, but their use is not completely compromised and highly depend on the haplotypes present in the population and the DMIs considered. This explains why DMIs are nowadays mainly used in mixtures with other MoAs.

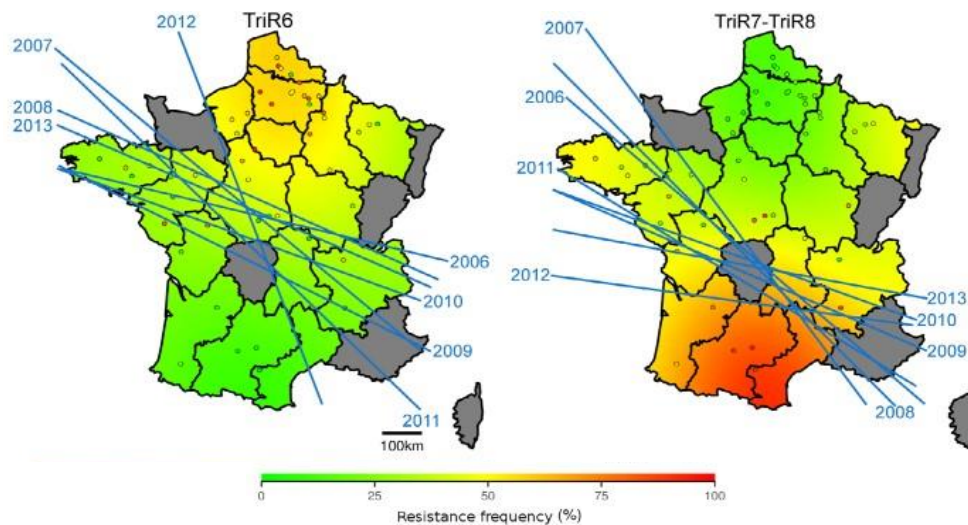


Figure 29: Spatial progression over time of the TriR6 and TriR7-TriR8 phenotypes (TriMR phenotypes) in France. TriR6 or TriR7-TriR8 2013 background map. Lines show the spatial partition of resistance frequency for these phenotypes over time and points are representing the status of resistance in 2013 sampling sites. From (Garnault et al., 2019).

### 3.3.1.6 Multiple and Multidrug resistance in *Z. tritici*

The various MoAs active on *Z. tritici* were successively introduced over time (Table 4 and Figure 30) and population resistance emerged and evolved consequently in the last decades, with different dynamics as described previously. As a result, new resistances were selected in already resistant genetic backgrounds, like the actual population structure shows. This explains why most of the strains in France display multiple resistance to BMCs, QoIs, DMIs, and possibly to SDHIs.

In addition, a generalist mechanism, leading to NTSR, was detected in French populations in the late 2000s. Multidrug resistance (MDR) can regulate the accumulation of DMIs, QoIs, SDHIs (and possibly also of future fungicides) into cells by a rise of active efflux (Leroux and Walker, 2011). This mechanism results from the overexpression of the membrane transporter MFS1, from the major facilitator superfamily (Omrane et al., 2015). Its overexpression results from the presence of an insert in the MFS1 promotor (Omrane et al., 2017). At least three MDR types were distinguished according to the

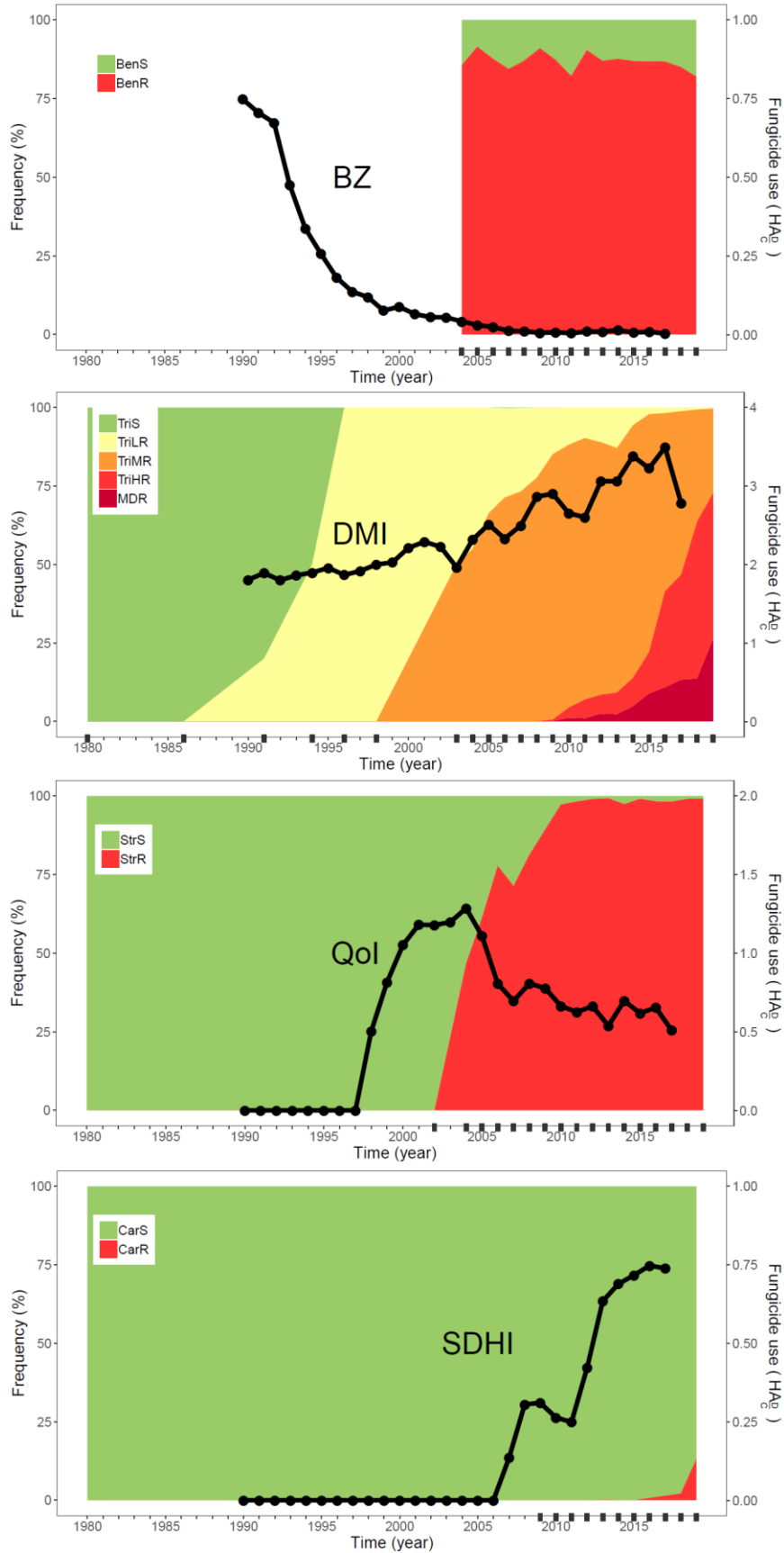


Figure 30: Changes in resistance frequency in *Zymoseptoria tritici* populations (left axis) and fungicide use (right axis) in France for four fungicide MoAs. Thick ticks along the x-axis indicate years for which resistance frequency was assessed by monitoring. Lighter ticks indicate that the information was obtained or extrapolated from sources other than the Performance database. BZ, benzimidazoles (antimicrotubule agents); DMI, sterol demethylation inhibitors; QoI, inhibitors of respiration complex III; SDHI, inhibitors of respiration complex II. From (Garnault et al., 2019).



length and the insertion site of the inserts in the promoter region. Types I and II display transcription factor binding sites. A fourth promotor insert has recently been found but is not associated with MFS1 overexpression (Mäe et al., 2020). If this mechanism alone is generally of low intensity (low resistance factors), it significantly contributes to increase RFs when associated to TSR (Gazeau and Walker, unpublished), as for DMIs and SDHIs. MDR currently represents more than 40% of the French population.

*To conclude, several independent resistance mechanisms can lead to SDHI, QoI or DMI resistance. Those can recombine (or unlink) due to sexual reproduction, contributing to the large panel of phenotypes actually described in populations.*

### 3.3.2 Actual fungicide resistance management for *Z. tritici*

The main advantages and limits of anti-resistances strategies have been discussed in a general manner in § 2.2.2. Here, I will focus on specific aspects concerning anti-resistance strategies for the particular control of *Z. tritici* and the blurred areas that might remain.

van den Bosch et al. (2014a) measured the impact of dose modulation on resistance evolution in *Zymoseptoria tritici*. Assuming that resistance was emerged in populations, five out of the six identified studies concluded that an increase in the applied dose (of two DMIs and of a QoI) was associated with an increase in resistance selection and one (with a DMI) revealed that dose increase had no effect on selection. van den Bosch et al. (2017) nevertheless distinguished the performance of dose modulation for qualitative *vs.* quantitative resistance in *Z. tritici*. For the DMI quantitative resistance, increasing the dose to its maximum permitted value could be optimal from a financial point of view because of efficacy increase, but may be less valuable for long-term resistance management. By contrast, both resistance management and financial optimization can be reached while decreasing the fungicide dose in some cases, for qualitative resistance. Concerning emergence time, a similar distinction was made in a mathematical model between total (*i.e.* qualitative) and partial (*i.e.* quantitative) resistance (Mikaberidze et al., 2017). Over a range of pathogens possibly representative of *Z. tritici*, emergence time decreased monotonically with increasing the dose. But, in some cases with partial resistance and within a limited parameter range, emergence may decelerate at higher doses. Conversely, for 3 different DMIs splitting the dose (*i.e.* the total dose is not reduced but split into different timings) also increased resistance selection of *Z. tritici* because exposure duration was expanded (van den Bosch et al., 2014a).



According to a modelling study, mixtures extend the effective life of the to-be-protected fungicide all the more since the dose of the mixing partner is high and its resistance risk low (van den Bosch et al., 2014b). But the benefit of mixtures was not always proved under field conditions (van den Bosch et al., 2014a). In situations where resistance has emerged, four empirical studies concluded that mixtures had no impact on resistance selection in *Z. tritici* populations when a mixing partner was added (DMIs with QoIs, or QoIs with DMIs or a multisite), and only one experiment showed a decrease in resistance selection when such partner was added. By contrast, mixing a single site fungicide with a multisite may delay the emergence of resistance in *Z. tritici* to the unisite fungicide (Hobbelen et al., 2014).

Finally, as developed in § 2.2, the dose of the to-be protected fungicide is often similar, whereas it is used a solo compound, in alternation or mixed with a partner. Therefore, the total dose of fungicides applied in each strategy might strongly differ in mixture *vs.* alternation or sequence and the subsequent comparison is unfair. Modelling studies studying the evolution of resistance in *Z. tritici* conclude that mixtures perform better than alternations (or at least equally well) in most cases depending on the fraction of each fungicide used in the mixture (Hobbelen et al., 2013). The direct comparisons between alternation and mixture at equivalent doses (*i.e.* the dose being halved when used in a mixture) showed that mixture gave better performance in a model when considering the lifetime yield as a metrics, but that both strategies could be optimal when using other criteria for assessing performance (Elderfield et al., 2018). When compared to half dosage mixtures, van den Bosch et al. (2014a) concluded that alternation and mixtures could not be categorically hierarchised, as half of the studies ranked mixtures above alternation for delaying resistance, a few placed alternation first due to a weaker resistance selection and finally others didn't find a difference between them because of the balance between redundant killing and dose reduction in mixtures.

The recommendations for the management of resistance in *Z. tritici* that result from those insights of literature and experimentations are the following. In 2020, the Fungicide Resistance Action Committee (FRAC) recommended using fungicides in mixtures on cereals, including for the control of STB. Even if mixtures with similar MoA are used mainly to increase the efficacy or to widen the spectrum of activity rather than to manage resistance, mixture partners should not exhibit cross-resistance and should provide effective disease control on their own (Hagerty et al., 2020). Similarly, alternation with distinct MoAs is also recommended for DMIs and MBCs to decrease resistance development. FRAC also advocate to apply the fungicides as early as possible in order to rely on their preventive rather than curative action. It also discourages splitting treatment and dose below manufacturers recommendations that would not provide good disease control and may accelerate resistance selection. A

maximum number of applications for each MoA per growing season is recommended in Europe: two for QoIs, one for picolinamides (QiIs) and two for SDHIs. When DMIs are used alone, multiple applications should be limited within a cultural season in areas where disease pressure is high (FRAC, 2021). Hahn (2014) recalls that in many countries, most SDHIs are only sold as mixtures. This fits with recommendations on resistance management. However, it reduces the possibility to design tailor-made fungicide programs.

In France, recommendations for resistance management are published annually in the « Note commune - INRAE, Anses, ARVALIS -Institut du Végétal». Globally, experts equally recommend diversifying MoAs through time and space in order to limit the selection of the specific resistances, possibly associated to MDR. However, SDHIs, QoIs and QiIs should be used only once a year and applied in alternation or in mixture with another MoA also efficient on the same target pathogen. SDHIs may be associated with a mixture partner (DMI for example) providing efficient disease control. Concerning DMIs, they should be associated with a multisite, a SDHI or a QiI and alternated as much as possible within a cropping season. If several DMIs are to be used in a year, AIs should differ to take benefit of their respective partial efficacy. Even if dose splitting is highly appreciated by farmers because of their better efficacy, this strategy is not recommended as it may select more highly resistant strains (high resistance to DMIs and also MDR). For STB control, the « Note commune » also recommend using multisite inhibitors when it is possible, to maintain efficacy. French recommendations are then more restrictive than those of the FRAC and are generally followed by farmers and advisors. This is acceptable because disease pressure is lower than in other European countries. As a result, almost all French wheat surface received one SDHI spray (only 3% with two SDHI applications) while two applications are permitted, demonstrating that recommendations of the “Note commune” is largely respected. Mixture is still the most used anti-resistance strategy in France.

*With the integration of IPM in many French agricultural systems, the control of STB does not rely anymore entirely on chemicals but also allies agronomic preventive practices, varietal choice or decision support tools. Yet, despite these improvements in STB control and the regular publication of local recommendations for PPP use, resistance to SDHIs, DMIs and MDR are still increasing in *Z. tritici* populations. Therefore, resistance management of *Z. tritici* still needs improvement. The consequences of resistance consequences are still a big concern for farmers who are expecting new answers in the coming years. Studies unravelling how the efficacy of anti-resistance strategies can be improved are welcome and new approaches might be useful to contribute to this still on-going*

debate. Validating that recommended strategies are equivalently efficient on the diversity of populations actually described in Europe would also be of particular interest (Heick et al., 2017).

## 4 Thesis project

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### 4.1 Challenges and questions of the thesis

Maintaining wheat production with high yield, at acceptable costs without damaging the environment is highly challenging, even more in the context of reduction and better use of plant protection products, of loss of diversity of the modes of action available and while facing growing resistance issues. Resistance prevention and management are burning issues and are highly relevant for *Z. tritici* affecting wheat. There is no clear consensus brought by theoretical and experimental studies on which anti-resistance strategy most maximises the sustainability of the available modes of action in diverse situations. This thesis project intends to contribute to the resolution of the practical and scientific issues dealing with resistance management (as defined in Figure 17) by proposing a complementary and new approach in the field of fungicide resistance *via* experimental evolution. Through this approach, we hope to provide different insights and answers to questions previously addressed with theoretical and empirical approaches. Exemplifying resistance of the fungal pathogen *Z. tritici* to multiple fungicide MoAs, we aimed in particular at answering the following questions:

- « How can we enhance the performance of anti-resistance strategies and what are the drivers of their optimization? »

To address this question our objectives were:

- ➔ To characterize the qualitative and quantitative performance of the mixture, alternation and dose modulation strategies, in comparison to sequence
  - ➔ To disentangle the relative impact of strategy key drivers and understand their interplay
  - ➔ To combine previous results to maximize selection heterogeneity, as a first step towards sound strategy tailoring.
- A second question in this PhD was: « How the initial status of resistance modulates the performance of anti-resistance strategies? »

For this part, the objectives were:

- ➔ To characterize the impact of multiple resistance (*e.g.* resulting from recombination or sequential selection) on the sustainability of heterogeneous strategies
- ➔ To discern the ability of different strategy drivers to delay resistance selection in population differing in their initial structure regarding resistance

This approach lies in the development of an original experimental protocol aiming at quantifying the sustainability of anti-resistance strategies and identifying the key drivers of resistance evolution. This methodological development represented the first challenge of this work.

## 4.2 Scientific approach and thesis organization

### 4.2.1 General comments

In this PhD, we used *Z. tritici* to explore the previous questions, as it is recognized a highly adaptive pathogen of great agronomic relevance. We also used experimental evolution, as a promising tool to shed the light on new aspects of anti-resistance strategies performance. In particular, our assumptions were : (1) the performance of a strategy is positively correlated with the degree of heterogeneity of the exerted selection pressure; (2) selection heterogeneity results from the interplay between components of strategies; (3) heterogeneous selection pressures, as intended in sustainable strategies, may favour generalism, *i.e.* a broad-spectrum resistant phenotypes of possibly low RFs, consistent with NTSR; (4) understanding the interplay between the components of strategies can help their sound design.

Our experimental evolution protocol used either an ancestral strain susceptible to all fungicides (Parts III-V) or artificial populations composed of susceptible and resistant individuals (both simple and multiple). In the first case, we allowed *de novo* mutations responsible for fungicides resistance to emerge and be amplified by selection. In the second case, we mostly studied the selection dynamics of the introduced mutants. In all experiments, selection regimes were designed to mimic strategies of contrasting complexity and to study the relative influence of their components on global performance. Optical density enabled us to assess resistance dynamics whereas strain isolation and phenotype characterization enabled to infer resistance characteristics and mechanisms. Our experiments were based on these common technical features. In particular, we tested whether alternation, mixtures and dose modulation were relevant strategies to limit resistance evolution (in populations where resistance has emerged or not), and whether the resistance mechanisms selected were distinct than with a sequential selection.

The manuscript is organized as detailed below.

## 4.2.2 Setting up experimental evolution to analyse the performance of anti-resistance strategies (Part II)

Experimental evolution protocol needed several preliminary adjustments, either for the implementation of the experiment itself (culture conditions, selection pressure, characterization of the strains used) or for the analysis of collected data (measurement of population size, characterisation of evolved phenotypes). The different preliminary experiments necessary to clarify those parameters and choices are described in part III. The optimized technical settings were then used similarly for all the experiments in the following parts.

## 4.2.3 Performance of alternation and categorization of its components (Part III)

The strategy of alternation is based on temporal heterogeneity of the selection pressure. In this part, we used three fungicides (benzovindiflupyr, carbendazim and prothioconazole-desthio) representative of different MoAs and then of intrinsic resistance risk. Selection regimes allowed us to study three components:

- The number of AIs. We hypothesized that the more active ingredients (AIs), the most efficient was the strategy.
- The inherent resistant risk of AIs. We supposed that the alternation of AIs of low resistance risk was more efficient to delay resistance evolution than strategies using AIs with higher resistance risks.
- The rhythm of alternation, reflecting the duration of continuous exposure with a single AI over generations. We assumed that long alternation rhythms should favour the expression of potential fitness costs, providing longer control of the population.

-

## 4.2.4 Performance of mixture and dose modulation strategies and impact of their components (Part IV)

This part was designed to disentangle the respective performance of redundant killing (as expected in mixture strategy) and of selection intensity (as expected in dose modulation strategy) in delaying resistance evolution, as these strategies are often combined in commercial products. Like for the previous part, using the same three fungicides, we also characterized the drivers that set selection regimes. Therefore, we tested the relative impact of:

- The number of AIs on resistance growth. In this experimental evolution, we worked at similar selection pressure in all selection regimes and tested two- and three-way mixtures in comparison to the AIs used directly. We expected mixtures to be more efficient in delaying resistance compared with a solo fungicide and presumed that the more AIs were mixed together, the better the performance. We also assumed that increasing the number of AIs would favour generalism in evolved lines.
- The inherent resistant risk of AIs. We assumed that mixture with fungicides of low and medium resistance risk would perform better to delay resistance evolution than mixture with fungicides at higher resistance risks.

#### 4.2.5 Maximising the heterogeneity of selection as a proof of concept for sustainable anti-resistance strategies (Part V)

In this last part, we aimed at validating the findings from parts III and IV. We compared mixtures and alternations regimes to assess the relevance of MoAs diversity within *vs.* between generations. The strategy components included in this final experiment were the number of AIs (1, 2 or 3), the intrinsic resistance risk associated to a given MoA and the diversity of AIs used in the strategy (AIs from the same MoA: intra-MoA diversity *vs.* AIs from different MoAs: inter-MoA diversity). Second, we have taken care to provide an educational perspective to our findings while using AIs used in current commercial products used to control STB, representing part of the available panel. We also constituted artificial populations including susceptible strains and resistant ones, associated to genotypes actually found in European population. As those mutants were introduced at low frequency in population from the start, we studied resistance evolution only in the selection phase. At last, to assess the impact of sexual reproduction on resistance evolution, which could hardly be achieved *in vitro*, we compared the evolution of single *vs.* double mutants, thus mimicking the effect of recombination or of sequential selection, such as the one occurring *in natura*.

## Part II.

# Setting up an experimental evolution to analyse the performance of anti-resistance strategies



# Setting up an experimental evolution to analyse the performance of anti-resistance strategies

Experimental evolution is a relevant tool to address fundamental questions in evolutionary biology such as the dynamics of adaptation and the underlying mechanisms (see BOX 3 in the introduction). Yet, until then, it has been underused to study the evolution of fungi, especially of plant pathogens (Fisher and Lang, 2016). Using a living organism to simulate in controlled conditions the evolution experienced in the field integrates a significant part of the complexity of the involved biological processes. These cannot be captured by modelling (Garland and Rose, 2009). Nonetheless, a diversity of experimental evolution protocols is available, and the latter must be correctly set up to address the specific research question. Organisms with large population size and a short generation time are preferred for evolutionary studies conducted in a short time scale. As described previously (Box 4 of the introduction), *Z. tritici* in its yeast like form perfectly fulfils these requirements. The type of EE chosen was a batch culture (serial transfer) for practical issues (van den Bergh et al., 2018). The protocols detailed in this part were used for all the experiments conducted in parts III, IV and V, unless otherwise stated.

## 1 General design

### 1.1 Culture conditions

Our laboratory culture conditions were optimized in order to maximise *Z. tritici* growth in its yeast form. In particular, these conditions were chosen so that nutrients and oxygen supply were not limiting for the fungal growth throughout the duration of the experiment. The basic requirements for optimal growth (*i.e.* container size, medium, incubation conditions) were established during the M2 internship of Baptiste Vancostenoble, before the start of this PhD. More particularly, conditions were defined so that they reached a trade-off between optimal fungal growth and miniaturization and handling simplicity of culture containers, in order to facilitate large-dimension experiments and minimize manpower.



Based on these requirements, 50 mL borosilicate Erlenmeyer flasks containing 25 mL of medium were recognized as the optimal containers. Smaller containers (*e.g.* vials or microtiter plates) did not allow a sufficient number of generations in a reasonable time span, by contrast to what is established in literature for bacteria, for example. Erlenmeyer flasks were capped with carded cotton wool and sterilized before use (Figure 1). YPD (Yeast extract Peptone Dextrose, Broth Traditional Formulation with Peptone powder from USBiological, composed of 20 g/L of dextrose, 20 g/L of peptone and 10g/L of Yeast Extract) was preferred as it is a rich liquid medium generally suitable for all *Z. tritici* cultures. This medium enables rapid and uniform growth of the yeast-like form of *Z. tritici*, without hyphae pellets formation in our conditions. Light is known to improve the growth of *Z. tritici* yeast-like form (Goodwin et al., 2011) but as we didn't have the technical possibility to light a large number of cultures uniformly, they were grown in the dark in a culture chamber.

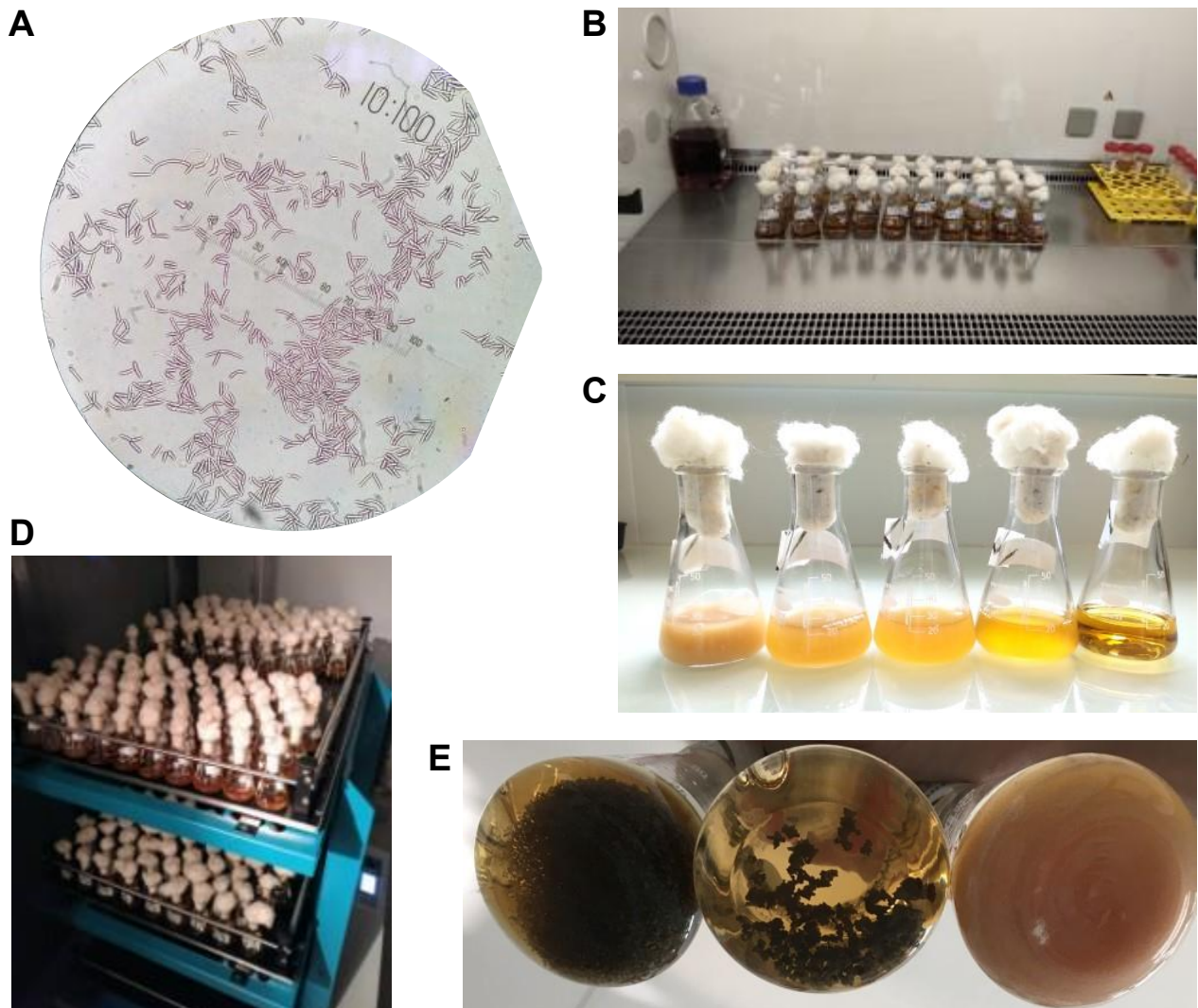


Figure 1: Cultures conditions of the experimental evolution. A: Yeast like form of IPO323 (optical microscope x400 magnification), B: filling of Erlenmeyer flasks in sterile conditions, C: Erlenmeyer flasks containing gradual concentrations of spores (high to low from left to right), D: multi-layer shaker filled with Erlenmeyer flasks stirred at 150 rpm, E: pellets and melanised phenotypes of *Z. tritici* (left and middle) that should be avoided, to the benefit of smooth cultures (right).

The hyphal form of *Z. tritici*, visible as mycelium pellets in liquid cultures (Figure 1), is enhanced by temperatures between +25°C and +28°C, as well as by CO<sub>2</sub> tension, oxidative stress or poor-nutrient medium (Fagundes et al., 2020; Francisco et al., 2019; Nagy et al., 2017). Therefore, culture conditions of the climatic chamber were adjusted to favour blastospores (*i.e.* yeast-like form) cultures. In addition to the nutrient rich medium YPD, the ambient temperature was set at 18°C in a culture chamber with a 70% relative humidity. Erlenmeyer flasks were shaken at 150 rpm in the dark in a single three-stage shaker, to allow constant oxygenation and standardize cultures.

## 1.2 Ancestral strains

Each strain used in our experiments was previously tested in our culture conditions (both liquid and solid) to check their regular growth. In particular, in liquid medium, we preferred isolates which were not forming pellets (*i.e.* little induction of the filamentous form). Indeed, such pellets can prevent pipetting and may also bias the measurement of optical density (OD). The absence of melanisation at 7 days was also an important criterion as it would also distort the measurement of optical density.

The most used strain was IPO323, which is already laboratory-adapted and a worldwide reference for *Z. tritici* (Goodwin et al., 2011). Therefore, its adaptation to laboratory environmental conditions should not interfere in our experiments. Additionally, its genome is well known and its possible cryopreservation in glycerol at -80°C opens a wide range of possibilities for further analyses. IPO323 is susceptible to all known fungicides and was used alone for parts III and IV, and in association with other isolates in part V.

In part V, besides the use of IPO323, three other strains have been used. Those additional strains were carrying field mutations (detailed in the part V) and were obtained by Gwilherm Gazeau (INRAE BIOGER) from a cross between IPO323 and three field strains. Consequently, the three mutant isolates used in part V are not fully isogenic but share part of IPO323 genetic background as representative of its progeny.

For all these strains, we verified the absence of contamination microscopically. We purified them when necessary and established a cryopreserved stock from a single pure culture.

### 1.3 Initial population size and cycle duration

The size of the initial founding population was chosen as a compromise between material and space constraints, the carrying capacity of the medium volume and the expected mutation rate. The population had to be large enough to ensure mutation emergence (principally for parts III and IV) but without reaching the carrying capacity of the 25 mL YPD medium used in these seven-day cultures. *Zymoseptoria tritici* global mutation rate is estimated at  $4 \cdot 10^{-5}$  (Zhan and McDonald, 2004). Then, we fixed the size of the founding population at  $10^7$  spores in our 25 mL liquid cultures (*i.e.* a  $4 \cdot 10^5$  spores.mL<sup>-1</sup> concentration), to overcome this average mutation rate. Preliminary experiments with the ancestral strain IPO323 showed that an initial population of  $10^7$  spores reached a plateau from 7 days (Figure 2).

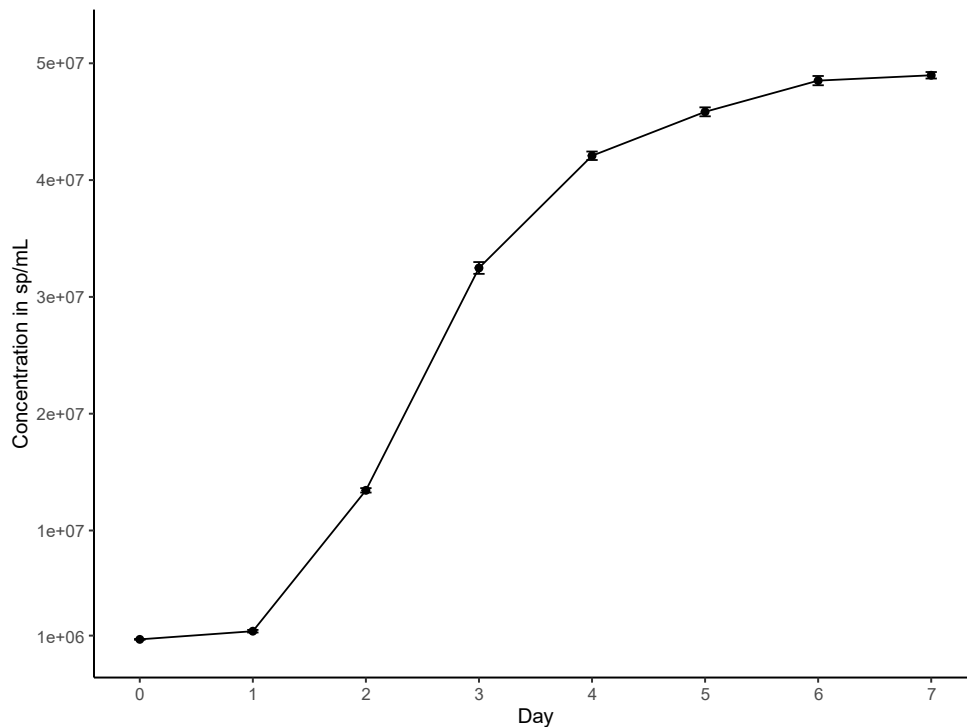


Figure 2 : Growth of the IPO323 reference isolate over time. Five experimental repeats with four biological replicates and 2 technical measurement replicates each. Culture conditions: one-week incubation; initial concentration of  $4 \cdot 10^5$  sp.mL<sup>-1</sup> in 25mL YPD in 50 mL Erlenmeyer flasks, 18°C, humidity 70%, stirring 150 rpm.

The cycle duration was then fixed to 7 days, to keep cultures in their exponential phase, to prevent reaching the carrying capacity and also for practical experimental organization. In these conditions, the population size is roughly multiplied by a factor 125 in one cycle. Assuming that each cell divides asexually into two sister cells, the number of generations,  $n$ , occurring for one week is:

$$n = \frac{\log(125)}{\log(2)} = 6.96, \text{ i.e. 6 to 7 generations per week.}$$

## 1.4 Duration of the evolution experiment

When *de novo* mutations conferring resistance are expected to rise after mutation of the fully susceptible ancestral strain (parts III and IV), the duration of the whole experiment (*i.e.* the number of cycles to be carried out) was designed so as to fulfil two conditions. First, experiment duration had to allow the fair comparison between strategies. In particular, populations exposed to different selection regimes had to receive similar numbers of fungicide applications or the same total amount of fungicide or if not, this had to be corrected in data analysis. As an example, alternation regimes with two fungicides had to last for an even number of cycles. The second condition was to allow resistance emergence, selection and generalization in at least control straight lines without extending the total duration of the experiment unnecessarily, which is always subject to technical and working-time constraints, as well as the ever-present possibility of contamination.

For experimental evolution where resistance was present from the start, the decision rule was to stop a line when the Malthusian growth (see below) represented at least 95% of the Malthusian growth of the control line during three consecutive cycles.

## 1.5 Transfer and immigration rate

The basic tenet of a serial experimental evolution is to use an aliquot of the cultures from the end of cycle  $n$  to inoculate cultures of cycle  $n+1$ . This results in a strong population bottleneck, which may favour genetic drift and the loss of emerging resistance alleles or its generalisation in the population. The transfer rate between cycles was previously optimized (B. Vancostenoble, M2 internship) and a 2% transfer rate was found as an ideal compromise in our culture conditions to speed up resistance emergence from IPO323 as the ancestral strain, while providing convenient growing conditions to start the following cycle.

In cases where this 2% transfer volume did not reach the threshold of  $10^7$  spores for founding the next population, we resorted to « immigration ». We supplemented the 2% transfer volume with spores from the control line in order to always start cycle with the same initial concentration. Preliminary tests showed that most lines conducted without immigration extinguished within the four first cycles and little evolved resistance (B. Vancostenoble, unpublished). Indeed, immigration provides new mutation supply material when susceptible strains are negatively selected in the presence of fungicides (MacLean et al., 2010). An increase of immigration in bacterial populations is correlated with an increase of the evolution of antibiotic resistance (MacLean et al., 2010; Perron et al., 2008). In our studies, immigration was important during the first phases of resistance emergence. However,

once adaptation had started, immigration volumes decreased until being unnecessary, as it would otherwise interfere with resistance generalisation by dilution effect (Vogwill et al., 2012).

## 1.6 Prevention of contamination

Even when manipulating in a sterile environment, it was challenging to carry out experimental evolution over a long time in culture conditions adapted to contaminants growth (rich liquid medium, adequate temperatures, ...). To prevent contamination, we first added systematically to our medium mixture of streptomycin and penicillin (100 mg.L<sup>-1</sup> each), amended at a 0.25% of the final volume. Penicillin is mainly targeting Gram positive bacteria while streptomycin provides a good control over Gram negative bacteria. Then, using this mixture prevents a large spectrum of potential bacterial contamination. Their effect on *Z. tritici* growth has been tested in our culture conditions and was found acceptable (final concentration after 7 days 4.98\*10<sup>7</sup> sp.mL<sup>-1</sup> without antibiotics and 4.95\*10<sup>7</sup> sp.mL<sup>-1</sup> with them) even close to be significant at a 5% confidence level (t test,  $P = 0.073$ ). We considered that the benefit of antibiotics was greater than the slight associated delay in growth culture. Additionally, all cultures, (whatever their selection regimes) were exposed similarly to antibiotics. Therefore, the use of antibiotics was designed to minimize bias in our conditions.

Second, we also regularly verified microscopically the absence of contamination (bacteria or yeast) in our cultures, in particular before the beginning of every experiment and in immigration sources. Moreover, at the end of each evolution experimental cycles, an aliquot from each Erlenmeyer flask was spread on a solid YPD medium, and its visual aspect was checked after five or six days, in order to detect potential contamination.

## 1.7 Sample storage

At the end of each cycle, 2 mL of every line culture was conserved (sometimes in duplicates) in a solution of glycerol at 25%. Samples were stored at -80°C. Those were used for further analyses or served as backup in case of interruption or contamination of specific lines.

## 2 Design of fungicide selection pressure in experimental evolution

### 2.1 Maintenance of solvents and fungicide solutions

Several solvents (*e.g.* ethanol, DMSO and their mixture of 50% each) were compared in preliminary experiments for their ability to dissolve high concentrations of the various fungicides and for their low toxicity for the IPO323 ancestral strain at their usual dose in test conditions (Table 1).

Table 1: Effect of various organic solvents inoculated at 0.5% of the final culture volume on the growth of *Z. tritici*. Concentration in  $\text{sp.mL}^{-1}$  with standard error measured after 7 days in our usual experimental conditions; 4 biological replicates.

	<b>Control</b>	<b>EtOH 80%</b>	<b>DMSO</b>	<b>½ EtOH 80% / ½ DMSO</b>
Final concentration ( $\text{sp.mL}^{-1}$ )	$4.95 \cdot 10^7$ $\pm 2.49 \cdot 10^5$	$5.03 \cdot 10^7$ $\pm 8.70 \cdot 10^5$	$4.99 \cdot 10^7$ $\pm 2.78 \cdot 10^6$	$5.00 \cdot 10^7$ $\pm 2.79 \cdot 10^5$

Ethanol 80% was found an ideal compromise in our experimental conditions since selection doses were easily dissolved and ethanol slightly increased the growth of *Z. tritici* growth when added at 0.5% of the final medium volume (emmeans comparisons of control *vs.* treatment based on a linear model, tukey method adjustment,  $P=0.11$ ). Ethanol was generally preferred to DMSO, which may also have fulfilled our technical requirements, because of its lower toxicity for the operators.

Technical-grade fungicides were dissolved in ethanol 80% and prepared as 4 000  $\text{mg.L}^{-1}$  stock solutions, kept at 4°C in ground glass vials, except for chlorothalonil that was dissolved in DMSO for solubility reasons. To ensure consistency of the selection pressure over cycles, serial dilutions of these stock solutions, corresponding to the selection doses, were prepared once at the beginning of the experiment and kept in similar conditions. In long-term experiments, controls solvent were always added to detect a potential effect of the latter on larger timescale.

### 2.2 Establishment of the selection doses

Dose-response curves were established for each single fungicide with the susceptible IPO323 strain in Erlenmeyer flasks in our experimental conditions. Several concentrations of fungicides were tested and amended to the media, and fungal population growth was measured after seven days as a response to fungicide exposure (*e.g.* Figure 3). These curves were repeated in a minimum of two

independent experiments, with at least three technical replicates each time. Dose-response curves were also re-established when new fungicide solutions were prepared, *i.e.* when starting a new experiment.

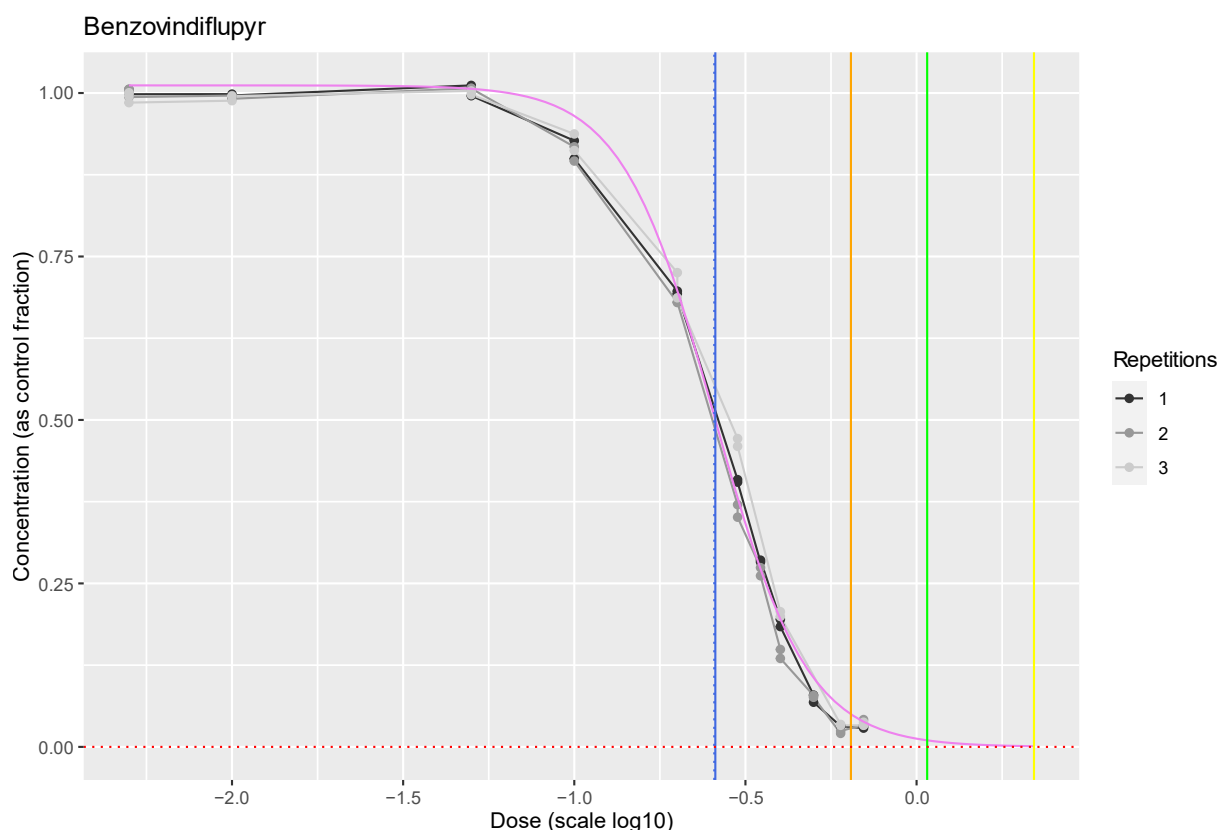


Figure 3: Dose-response curve established for benzovindiflupyr. The three experimental repeats are represented in shades of grey. In pink: modelled curve. Model estimates in other colours: EC<sub>50</sub> (blue), EC<sub>95</sub> (orange), EC<sub>99</sub> (green) and EC<sub>99.9</sub> (yellow).

Growth data (*e.g.* spores' concentration) from treated conditions were normalized with the spores concentration measured in the control. These normalized data were modelled using a logistic regression (logistic nonlinear mixed effects model, with the function SSlogis, Self-Starting NIs Logistic Model, available in the R package "stats"), with the dose as the fixed effect and our biological repetitions as a random effect. In order to exert a selection pressure that could be comparable to the one received by natural populations in the field, we first intended to determine and use the Minimum Inhibitory Concentration (MIC), which is the minimal concentration inhibiting 100% of the growth (EC<sub>100</sub>) for each fungicide. However, complete inhibition was hardly reached for all tested fungicides. The model was then used to calculate the minimal concentration inhibiting 95% of the growth (EC<sub>95</sub>). The selection pressure exerted by the EC<sub>95</sub> doses was therefore similar between all the fungicides. The final selection doses established for the different chapters are detailed in Table 2.



Table 2 : Overview of the selection doses used for single fungicides.

Selection doses (mg.L <sup>-1</sup> )	Part III	Part IV	Part V
Benzovindiflupyr	0.5	0.5	0.7
Carbendazim	0.1	0.2	-
Prothioconazole-desthio	0.005	0.005	0.005
Fluxapyroxad	-	-	0.7
Mefentrifluconazole	-	-	0.002
Chlorothalonil	-	-	5.5

When two fungicides had to be used as 1:1 mixture in further selection regimes, we established dose-response curves similarly, except that the range of tested concentrations for the mixture was a fraction of the EC<sub>95</sub> established previously for the same fungicides used alone (*i.e.* from  $1 \cdot (\text{EC}_{95\text{-fungicide A}} + \text{EC}_{95\text{-fungicide B}})$  down to  $0.1 \cdot (\text{EC}_{95\text{-fungicide A}} + \text{EC}_{95\text{-fungicide B}})$ ).

After estimating EC<sub>95se</sub> from the modelling of dose-response curves, we validated these selection doses experimentally. Indeed, the preparation of large volume of fungicide solutions may induce slight variation in fungicide concentration. Validation was achieved while amending the target dose in a culture of IPO323 in our usual conditions and measuring growth after seven days. A 5% growth as compared to the control was tolerated, otherwise adjustments with close doses were made if necessary.

## 2.3 Selection regimes

We defined a selection regime as a pattern of fungicide applications mimicking a strategy. For example, in the part III, the application of different patterns of fungicides were designed to simulate alternations. The absence of strategies (*i.e.* the continuous application of a given fungicide alone at every cycle) was referred as straight or sequential use. Those selection regimes will be detailed more precisely in their dedicated chapter. To ensure fair and rigorous comparison of strategies, fungicides were used at their EC<sub>95</sub> when alone. In mixture strategies, we used the EC<sub>95</sub> of the mixture (as a fraction of the EC<sub>95</sub> of its components) (part IV), to allow proper comparison with sequence. However, for comparison between alternation and mixtures strategies (part V), we designed mixture as half the EC<sub>95</sub> of both components, in order to use exactly the same amount of each fungicide for the two strategies at the end of the experiment.



### 3 Design of the measurement of population size

In our experimental setup, the evolution of resistance can be observed and measured through the increase in population size (*i.e.* measured as spore concentration) subjected to fungicide pressure. Spore quantification was also needed to calculate the weekly immigration rates, so as to always start new cycles with  $10^7$  spores in each line. Therefore, we had to develop an accurate, fast and operational spore quantification method in liquid medium usable in routine in a large number of populations.

Three equipments (*i.e.* haemocytometer, particle counter and spectrophotometer) were available in our laboratory. Their accuracy and detection limits were compared in preliminary experiments using serial dilutions of spore suspensions. Their characteristics are detailed in Table 3. Those materials differed greatly for their operability (and then for the number of samples to be analysed simultaneously) and for the range of spore concentrations that can be analysed.

The spectrophotometer was more adapted for our large numbers of samples (to be organized in 96-well plate), provided weekly by experimental evolution, but the output (optical density measurements) does not directly indicate on population size, which constitutes a severe limitation. Therefore, we established the correlation between OD measurement at 405 nm and spore concentration, as measured by the particle counter. We tested several correlation models and established the following one (Figure 4) for its superior quality (max  $R^2_{\text{adjust}} = 0.91$ ; min bias =  $-3.1 \cdot 10^6$ ; min RMSE =  $1.5 \cdot 10^7$ ), after cross-validation:

*Concentration (spores/mL)*

$$= e^{12,698804 + 8,945015 \cdot \ln(\text{OD}_{405\text{nm}}_{\text{standardized}} + 1) + (-3,926577) \cdot (\ln(\text{OD}_{405\text{nm}}_{\text{standardized}} + 1))^2}$$

with  $\text{OD}_{405\text{nm}}_{\text{standardized}}$  as the OD measurement at 405nm normalized with the OD of the liquid medium alone.

Measured optical densities were converted into population size estimates routinely using an automated R script analysing raw data from the spectrophotometer (at least 2 wells read for each sample).

Table 3: Methods enabling to estimate population size in liquid cultures of *Z. tritici*.

	<b>Haemocytometer</b>	<b>Particle counter</b>	<b>Spectrophotometer</b>
<b>Principle</b>	Visual counting of spores in a standardized grid under a microscope	Automatic counting of spores by a high-definition video camera, flowing in a liquid between metal plates	Measurement of optical density under several possible wavelengths
<b>Available equipment</b>	Malassez CE, 0.0025mm <sup>2</sup>	Flowcell FC200S+HR, Occhio	SpectraMax M2 (Molecular Devices), micro-titer plate TC Plate 96 well (Sarstedt), sealing film Breathe easy® (Diversified Biotech)
<b>Detection range (sp.mL<sup>-1</sup>)</b>	10 <sup>4</sup> → 2.10 <sup>6</sup> (dilutions necessary for higher concentration)	0 → 3.2 * 10 <sup>6</sup> (dilutions necessary for higher concentration)	3.5 * 10 <sup>5</sup> → 10 <sup>9</sup> (higher concentration unreached here)
<b>Operationality</b>	Dilution needed Reading: easy and quick for a few samples but time-consuming for large numbers Poor repeatability (user-dependent) ≈ 15 µl culture used No computer interface	High dilution needed Reading ≈ 4 mn per sample Time-consuming for large numbers High repeatability (user-independent) ≈ 1 mL culture used Computer interface	No dilution needed Fast reading ≈ 10/15 mn per plate (96 samples at a time) High repeatability (user-independent) ≈ 200 µL used Computer interface
<b>Specificity</b>	Expert operators detect contaminations.  Phenotypes can be distinguished.	Detects contaminations of different shape/size.  Phenotypes can be distinguished.	Doesn't detect contamination. Susceptible to phenotypic changes (melanisation, pellets)
<b>Output</b>	Spore concentration	Spore concentration, size and distribution	Optical Density

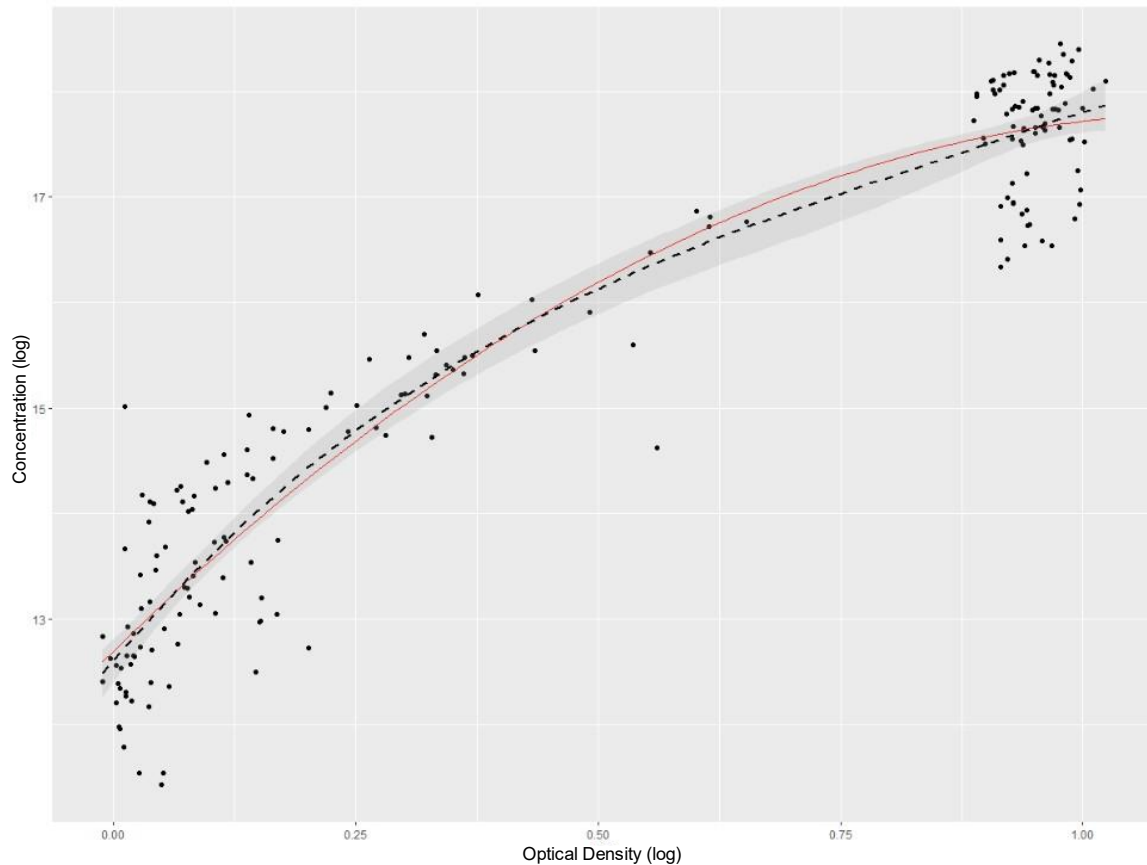


Figure 4: Correlation between optical density (unit scale:  $\ln$ ; measured with spectrophotometer) and spore concentration (unit scale:  $\ln$  of  $\text{sp.mL}^{-1}$ ; measured with particle counter). Regression curve in red and tendency as the black dotted line.

## 4 Characterization of evolved strains and populations

Complementary analysis, aiming at describing the phenotype of strains or populations were carried out either before the experiment, or directly after each cycle or even after recovery, and possibly isolation, from stored samples. Depending on the purpose of the experiment, three tests were developed.

### 4.1 Dose-response curves in microtiter plate

In order to characterize the  $\text{EC}_{50}$  of several strains in routine for a variety of fungicides, we designed a protocol aiming at establishing dose-response curves in 96-well microtiter plates. Indeed, such protocols generally minimize time and manpower compared to similar tests conducted in Erlenmeyer flasks. To avoid any border effect in microtiter plates (slightly starting from 5 days in our conditions), we chose the fifth day as the time for the final OD measurement. We also saturated humidity in the agitator and disposed non-amended medium in the border wells. These wells were not measured. Two strains could be tested in one plate with this design, with a starting concentration of  $2.5 \times 10^5 \text{ sp.mL}^{-1}$  per well. This initial concentration had been previously determined with growth

curves as being the concentration allowing an exponential growth for five days without reaching the carrying capacity of the medium. The layout presented in Figure 5 allows testing a range of 10 concentrations and determining  $EC_{50}$ , using a model of logistic regression as described previously. For solubility reasons, we used DMSO at 0.5% of the final volume (1  $\mu$ L in 200  $\mu$ L medium).

This test was used to characterize the phenotypes of the four ancestral strains used in part V.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	YPD	Dose1	Dose2	Dose3	Dose4	Solvent	Dose5	Dose6	Dose7	Dose8	Dose9	Dose10
C	YPD	Dose1	Dose2	Dose3	Dose4	Solvent	Dose5	Dose6	Dose7	Dose8	Dose9	Dose10
D	YPD	Dose1	Dose2	Dose3	Dose4	Solvent	Dose5	Dose6	Dose7	Dose8	Dose9	Dose10
E	YPD	Dose1	Dose2	Dose3	Dose4	Solvent	Dose5	Dose6	Dose7	Dose8	Dose9	Dose10
F	YPD	Dose1	Dose2	Dose3	Dose4	Solvent	Dose5	Dose6	Dose7	Dose8	Dose9	Dose10
G	YPD	Dose1	Dose2	Dose3	Dose4	Solvent	Dose5	Dose6	Dose7	Dose8	Dose9	Dose10
H												

Figure 5: Layout of microtiter plates used for  $EC_{50}$  determination in *Z. tritici*. The range of 10 doses was adjusted depending on the fungicide tested. The  $EC_{50}$ s of two strains could be determined in a plate (strain 1 in green and strain 2 in blue).

## 4.2 Fungicide resistance profile in microtiter plate

Experiments from part III needed to easily determine the population resistance profile of each line at the end of every cycle. This aimed at quantifying population adaptation and its evolution over time. We then designed a microtiter plate layout (Figure 6) where population growth could be measure under discriminatory doses of multiple fungicides, in comparison to non-amended controls. This layout also enabled to measure fitness cost while comparing the growth of evolved and strains in fungicide-free wells. Tested fungicides were those used in the experimental evolution. Discriminatory doses were designed in preliminary experiments to allow the growth of resistant strains but not of the susceptible ancestral one. The fungicide last used in a given alternation strategy was added at its  $EC_{95}$ , 1.5 times its  $EC_{95}$ , and 2 times its  $EC_{95}$ , in order to observe a potential sensibility shift in the population. The culture conditions were the same as described in the precedent section.

Unfortunately, the setup of this test was not reliable enough, as the discriminatory doses were not able to differentiate the susceptible ancestral strain from a population with weak resistances such as obtained in the early cycles from part III. Moreover, the implementation of such tests was highly time-consuming. Consequently, this semi-quantitative test was abandoned to the benefit of droplets

tests. In this work, I will then not present data established with this test. The fact remains that with more precise development and additional time, this test could serve as a powerful and compact tool that could be adapted to many situations and provide more quantitative data than the droplet tests (next section).

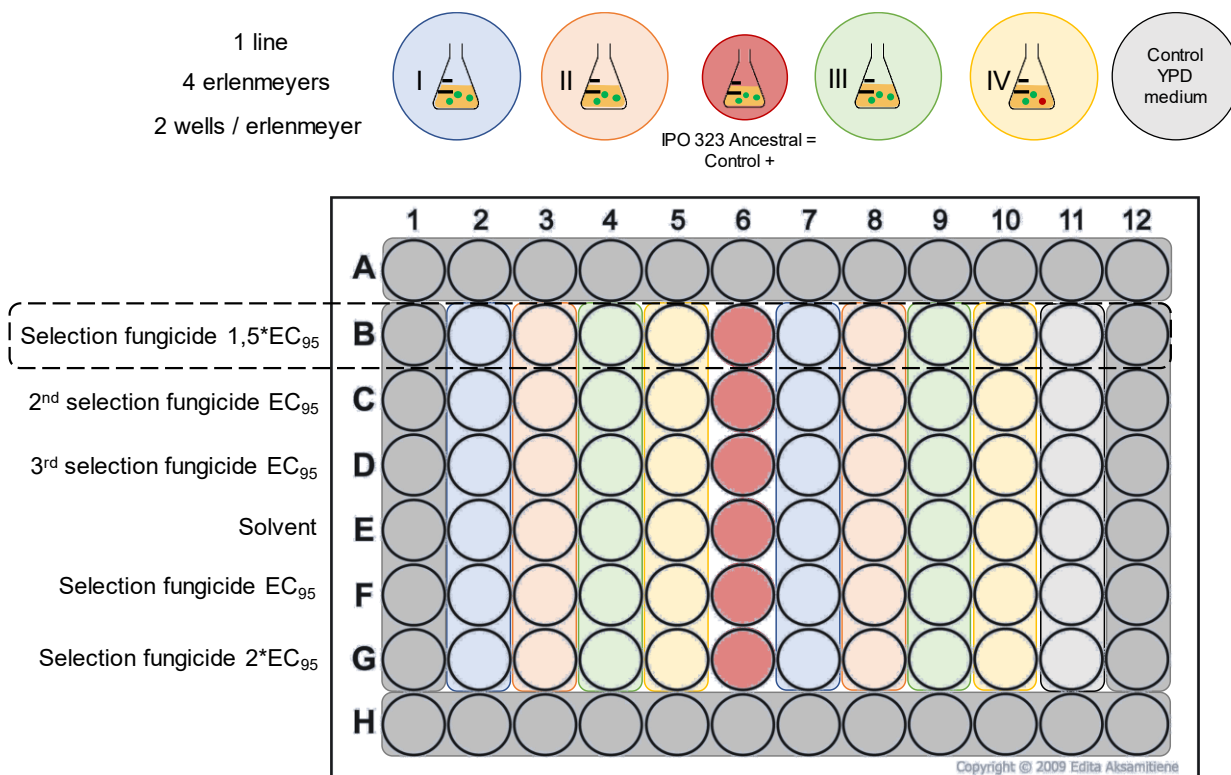


Figure 6: Layout of the microtiter plate test used to establish resistance profile and fitness of evolved populations and strains.

### 4.3 Fungicide resistance profile in droplet test

A droplet test was designed with the view of establishing resistance phenotype profile of the individuals or populations selected in experimental evolution. A droplet of spore suspension is deposited on a square Petri dish (12\*12 cm) with YPD solid medium, amended with a discriminatory dose of fungicide (0.5% final volume), or with the solvent only in the control modality. Four concentrations of spores are tested ( $10^7$ ,  $10^6$ ,  $10^5$  and  $10^4$  spores.mL<sup>-1</sup>; Figure 7).

The ancestral strains (including IPO323 susceptible to all fungicides), and several field strains, displaying resistance to the targeted fungicide or MDR, were systematically included in each plate to verify the efficacy of the discriminatory dose. Fungal growth was observed after six days (or five in part V) and was scored according to the scale detailed in Figure 7.

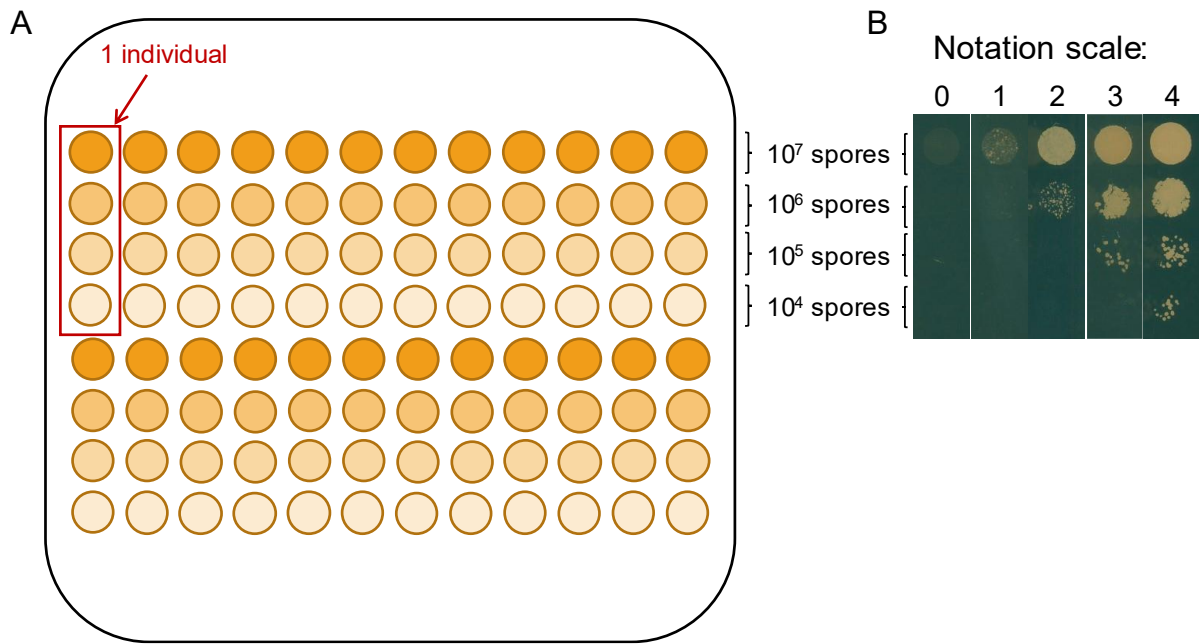


Figure 7: Droplet test used to establish fungicide resistance profiles. A: Layout in square Petri dish. YPD solid medium contained discriminatory doses of testing fungicides. B: Associated scoring scale, including notations observed for the various concentration of spore suspensions.

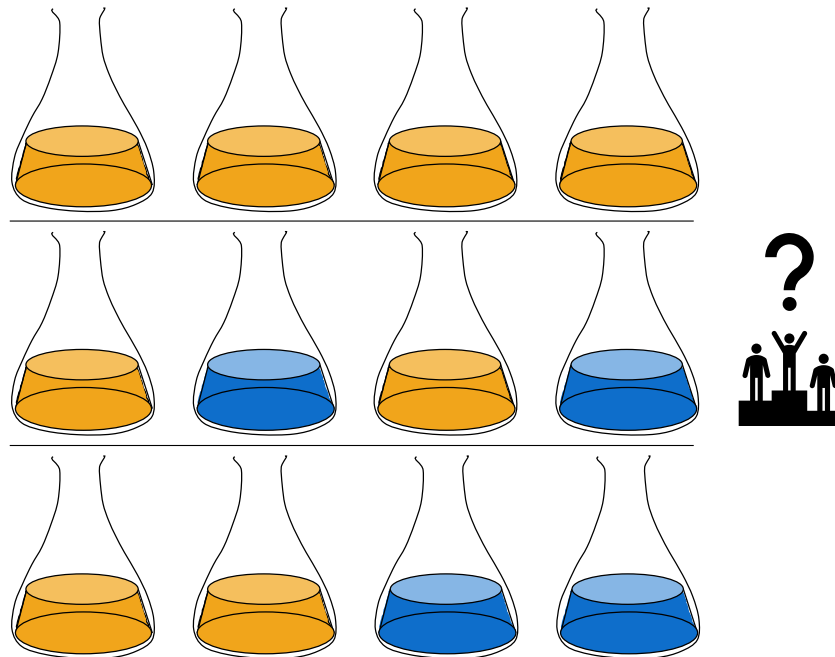
The score was ranging between 0 (susceptible) to 4 (resistant if >0), according to the number of dilution droplets for which growth was observed. The resistant profile corresponded to the concatenation of the independent scores obtained for the tested modalities. This test was finally used to describe the qualitative outcome of anti-resistance strategies in parts II, IV and V.

### Final remarks

Experimental evolution had never been used to study anti resistance strategies with a phytopathogenic fungus before. Protocol adaptation from the few literature sources and many new developments were therefore essential to answer our research questions with such approach. We discovered that *Zymoseptoria tritici* highly responds to environmental changes and therefore its use for experimental evolution was challenging. Technical rigorousness and precision were necessary to obtain meaningful results, either quantitative or qualitative.

## Part III.

# Performance of alternation and categorization of its components



# 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 intrinsic risk of resistance of fungicides probably underlies this trade-off, more so than 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; *Zymoseptoria tritici*.

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## 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<sup>1, 2, 3</sup>. 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<sup>4</sup>. The costs engendered by the evolution of resistance in pathogens, arthropods and weeds amount to billions of US dollars each year<sup>5, 6</sup>, and the development of resistance may lead to the loss of millions of lives<sup>7, 8</sup>, and entail hidden costs, due to greater pesticide use<sup>9</sup> and impacts on biodiversity<sup>10, 11</sup>. 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<sup>12, 13</sup>. 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<sup>14, 15, 16, 17, 18</sup>. The alternation (also known as cycling, rotation, or periodic application) of unrelated active ingredients (AIs) was first proposed by Coyne in 1951<sup>19</sup> and provides multiple means of intergenerational killing, maximizing the probability of killing the offspring of resistant individuals<sup>13</sup>. 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<sup>13, 20, 21, 22</sup>. 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<sup>23, 24</sup>. Nevertheless, the diversity of resistance mechanisms observed in microbes, animals and plants is considerable. The evolutionary origins of these mechanisms may also differ<sup>25</sup>. 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<sup>26, 27, 28, 29</sup>. Theory, with support from experimental evolution experiments, suggests that specialists evolve in homogeneous environments, whereas generalists are more likely to evolve in heterogeneous habitats<sup>30</sup>. 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<sup>31</sup>. In the clinical

environment, generalist resistance has also been shown to be more frequently selected in situations in which antibiotics are alternated<sup>32</sup>. Overall, these studies highlight the importance of treatment frequency relative to the pathogen life cycle<sup>33, 34</sup>. 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<sup>35, 36</sup> — 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<sup>37, 38, 39, 40, 41, 42</sup>. The resistance of *Z. tritici* is determined by various specific target-site

mutations, but also by overexpression<sup>43, 44, 45, 46</sup> or enhanced efflux, a generalist mechanism causing weak multidrug resistance (MDR) when not associated to TSR and affecting at least three modes of action<sup>44, 47</sup>. We made use of the haploid yeast-like easily-culturable form of this pathogen<sup>48</sup>, in an experimental evolution approach<sup>49</sup> 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<sub>95</sub> selection doses (Fig. 1A and 1B). Mean normalized Malthusian growth  $M_{it}^n$  (referred to hereafter as the global resistance evolution rate  $\rho$ ) 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),  $\rho$  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  $\rho$ ) 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.

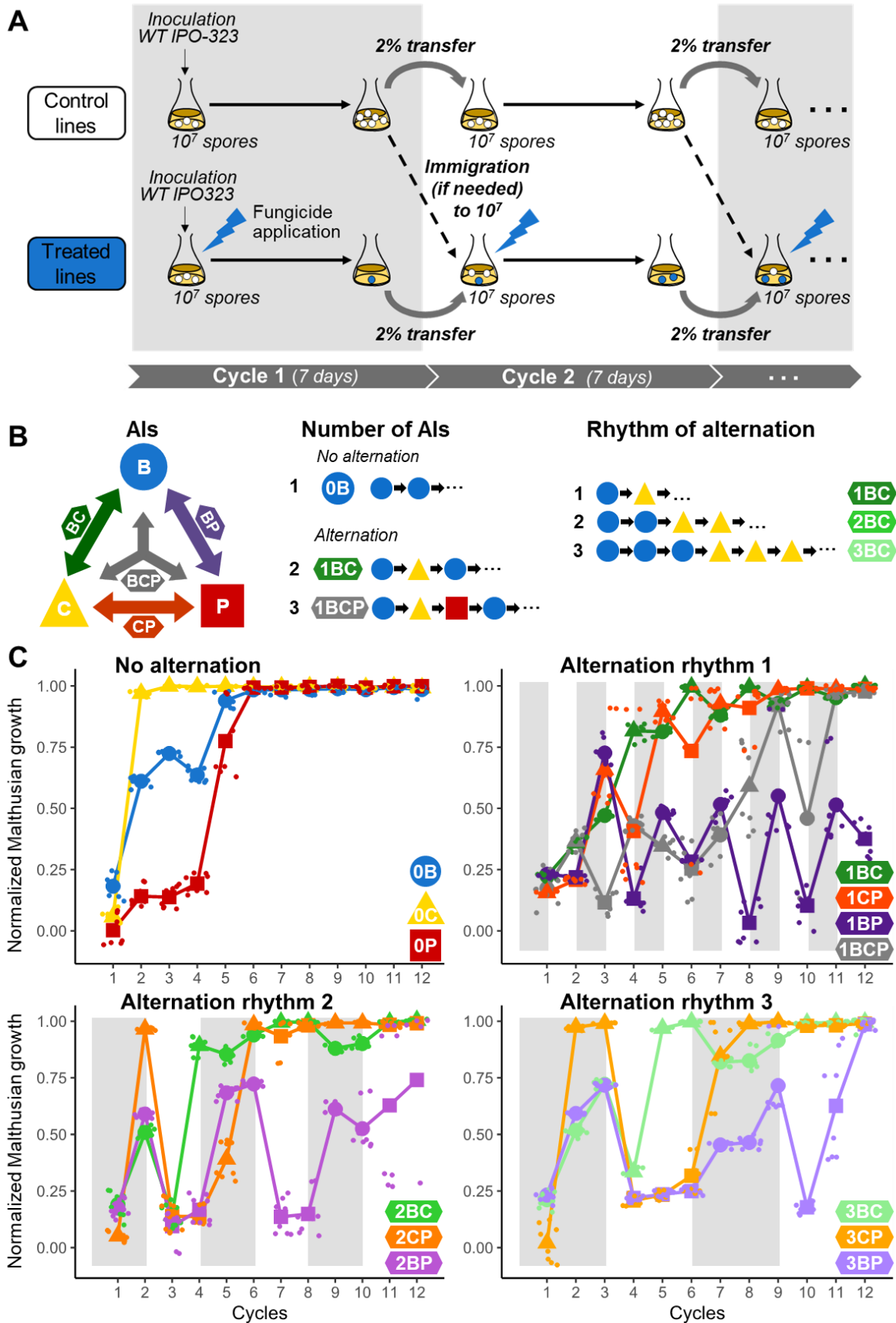


Figure 1: Experimental evolution of fungicide resistance under treatments with contrasting temporal heterogeneities. (A) The susceptible strain IPO-323 of *Z. tritici* was used to found 56 lines, with each Erlenmeyer flask inoculated with  $10^7$  spores. Fungicides were added to the treated lines to mimic 14 patterns of selection. After seven days, 2% of the population was used to inoculate fresh medium to start the next cycle, supplemented,

**(Fig. 1 continued)** 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  $\rho$ , 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  $\rho$  only for time segments corresponding to fungicide  $f$  exposure ( $\rho_f$ ), 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<sub>405</sub> in our experimental conditions), a selection phase (here, resistance rates between 15% and 90%) and a generalization phase (here, over 90%)<sup>16</sup>. 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

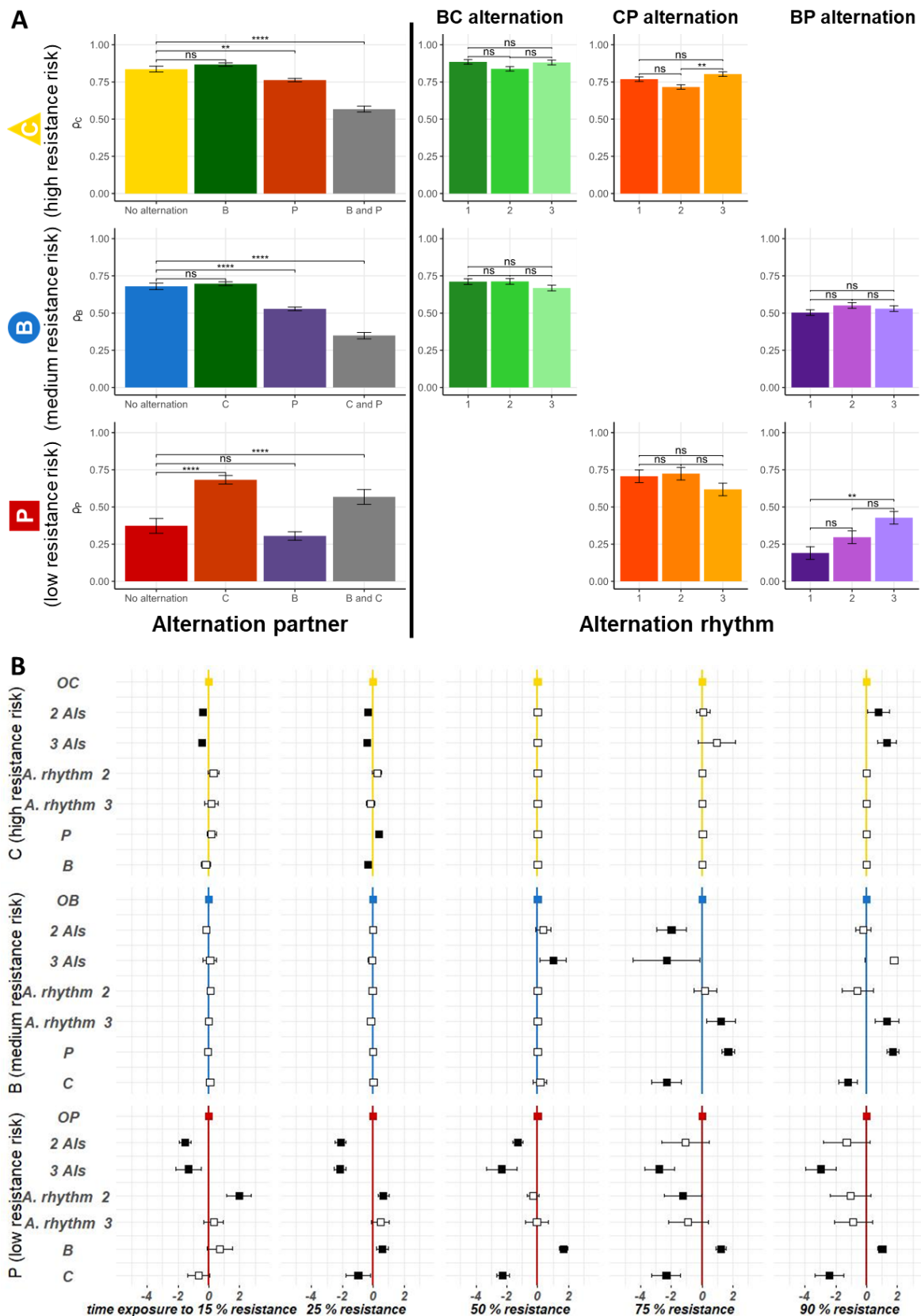


Figure 2. Effects of alternation components on the evolution of resistance. (A)  $\rho_f$ , the mean rate of resistance evolution for time of exposure to fungicide  $f$ , is shown as a function of alternating AIs on the left, and for each alternation pair, as a function of alternation rhythm on the right. 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  $M_{it}^a$  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 error 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.

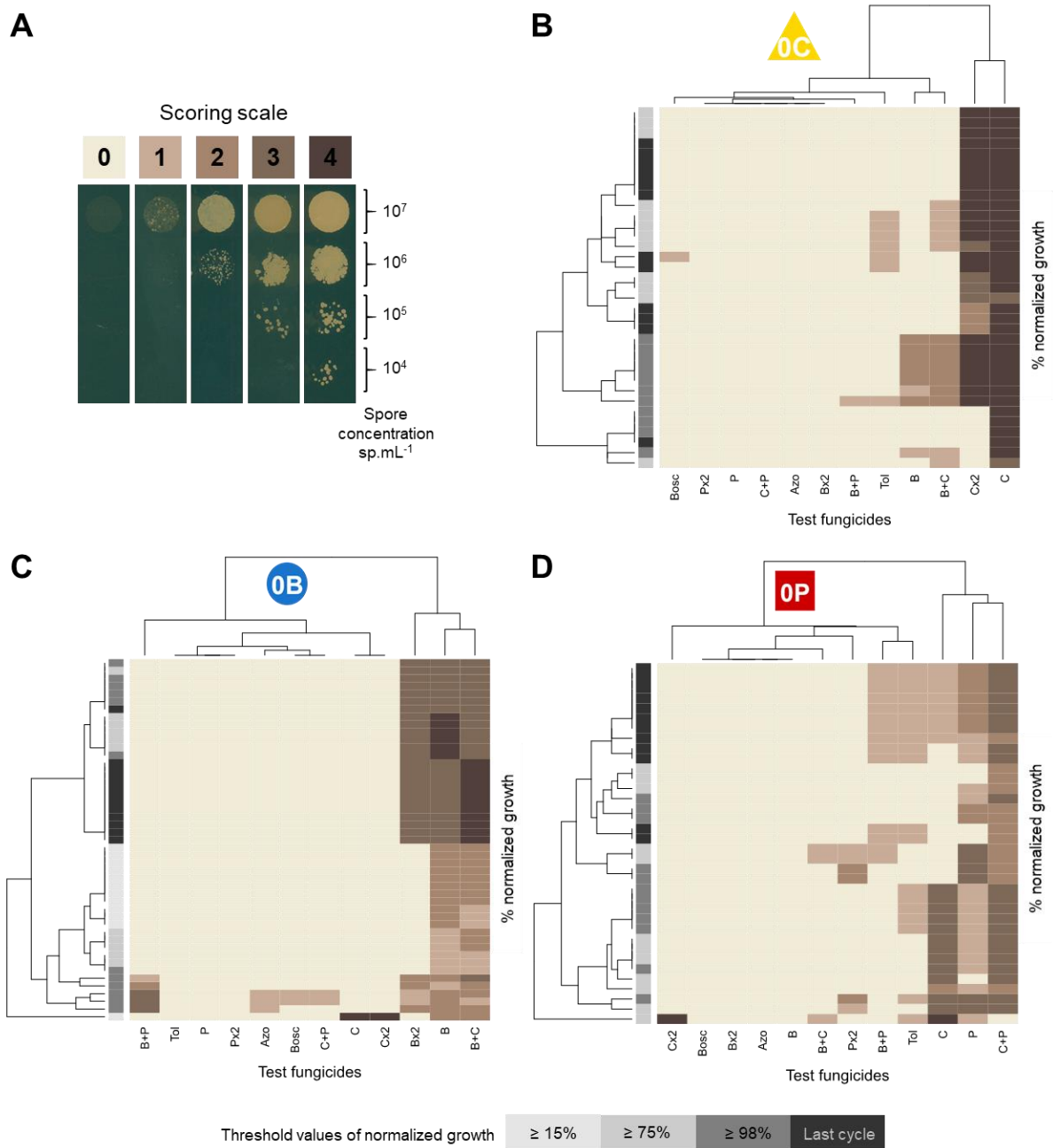
case of alternation with the partner of lowest possible risk (delayed by 1.70 and 1.01 exposure cycles, respectively), with the high-risk partner decreasing the time required to reach the generalization phase (by 1.22 and 2.42 exposure cycles, respectively). By contrast, alternation with a partner with 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<sup>50</sup>, 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 OB, OC and OP; 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<sup>44</sup>.

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, toltaftate). 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 ( $\geq 15\%$  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  $\beta$ -tubulin was detected in a few isolates evolving under direct carbendazim selection. This target site mutation has been reported to

## Discussion

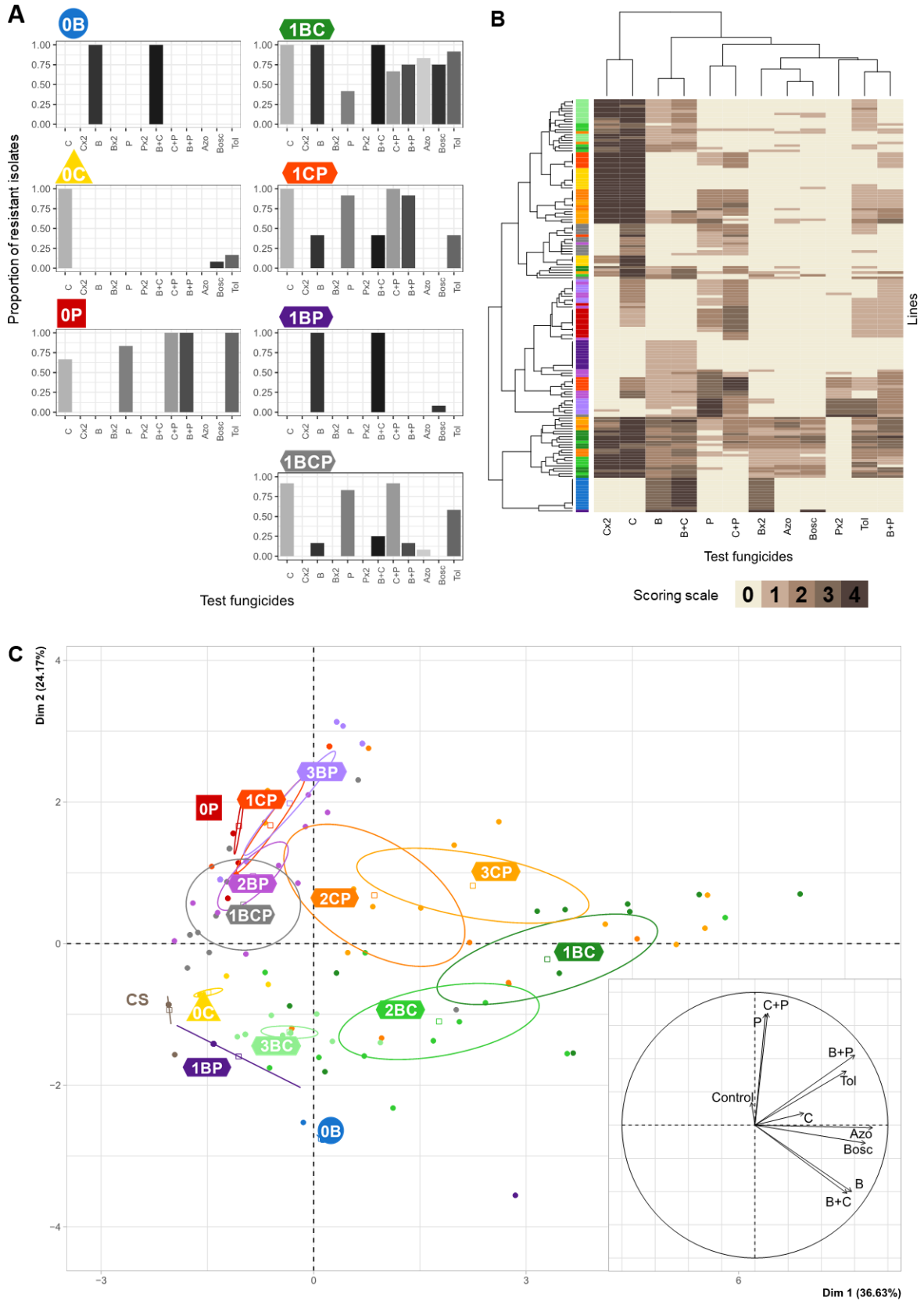
This study confirms the value of experimental evolution as an approach for investigating conceptual issues in temporal adaptation, such as the factors affecting the adaptive differentiation of local populations<sup>49, 54, 55</sup>. Here, we used an experimental evolution approach and the economically 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<sup>13, 20</sup> and mediate generalism in evolved individuals<sup>30, 56</sup>, as previously demonstrated for weeds, insects and bacteria<sup>15, 32, 57, 58, 59, 60</sup>.

Specifically, using three different fungicides, we found that alternation was either neutral or

confer a high degree of resistance to benzimidazoles in field isolates of 12 fungal species, and in nematodes<sup>51, 52</sup>. 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 Rf's *per se* but causing cross-resistance to unrelated compounds in *Z. tritici*<sup>44</sup>. In field strains, it is determined by insertions in the promoter region of the MFS1 transporter gene<sup>53</sup>. 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.

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<sup>24</sup>. However, this would suggest that resistance evolution rate due to exposure to a given fungicide ( $\rho_f$ ) 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  $\rho_f$  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 ( $\chi^2=36.5$ ;  $df=2$ ;  $P=10^{-8}$ ) 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 ( $\chi^2=4.91$ ;  $df=2$ ;  $P=8 \times 10^{-3}$ ) and alternation rhythm ( $\chi^2=3.85$ ;  $df=2$ ;  $P=0.022$ ). 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 ( $F=70.17$ ;  $df=1,225$ ;  $P=6 \times 10^{-15}$  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<sup>61, 62</sup>. 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<sup>63</sup>.

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<sup>64</sup>. 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<sup>32, 65</sup>. 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<sup>66, 67, 68</sup>. 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<sup>69</sup>. 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*<sup>55</sup>. 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<sup>70</sup>. 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

## 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

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<sup>62</sup>. 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<sup>24</sup>, 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. 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<sup>71</sup>.

YPD plates (20 g.L<sup>-1</sup> dextrose, 20 g.L<sup>-1</sup> peptone, 10 g.L<sup>-1</sup> yeast extract, 20 g.L<sup>-1</sup> 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<sup>-1</sup> 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<sup>72</sup>.

Population size was routinely determined by measuring optical density at 405 nm (or OD<sub>405</sub>) 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  $3.5 \times 10^5$  sp.mL<sup>-1</sup> in the experimental conditions used. The OD<sub>405</sub> of the fungal cultures was normalized against that of the non-amended medium. Spore concentration was estimated from the OD<sub>405</sub> 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<sup>-1</sup> 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<sup>7</sup> spores of IPO-323 were used to inoculate YPD in an Erlenmeyer flask, which was then incubated for seven days before OD<sub>450</sub> measurement. OD<sub>405</sub> data were modelled by logistic regression, to calculate, for each fungicide, the EC<sub>50</sub> (concentration inhibiting 50% of growth), EC<sub>95</sub> (concentration inhibiting 95% of growth) and MIC (minimum inhibitory concentration) values. We finally chose to use

the EC<sub>95</sub> 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<sup>-1</sup> for benzovindiflupyr, 0.1 mg.L<sup>-1</sup> for carbendazim and 0.005 mg.L<sup>-1</sup> 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<sup>73</sup>. 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  $2 \times 10^7$  sp.mL<sup>-1</sup> and each Erlenmeyer flask was inoculated with 10<sup>7</sup> spores (500 µL). The resulting starting concentration of  $4 \times 10^5$  spores.mL<sup>-1</sup> 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<sub>405</sub> was measured at the end of each cycle and transformed into population size, as described above. The OD<sub>405</sub> of the treated lines was normalized with the mean OD<sub>405</sub> 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<sup>7</sup>, 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<sup>7</sup>. 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

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

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  $\rho$ ) 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,  $\rho_f$  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.  $\rho$  and  $\rho_f$  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 OB lines, from cycles 2, 3 and 12 for OC lines, and from cycles 5, 6 and 12 for OP 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<sup>-1</sup> 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<sup>7</sup>, 10<sup>6</sup>, 10<sup>5</sup> and 10<sup>4</sup> spores.mL<sup>-1</sup> 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<sup>-1</sup>, respectively) and twice their selection doses (0.2, 1 and 0.01 mg.L<sup>-1</sup>, 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<sup>-1</sup> azoxystrobin, 2 mg.L<sup>-1</sup> boscalid and 2 mg.L<sup>-1</sup> 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*<sup>44</sup>. 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 OC, OB and OP 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<sup>74</sup>. 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<sup>53</sup>.

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*),  $\beta$ -tubulin (*tub2*) and 14 $\alpha$ -demethylase (*cyp51*), respectively<sup>37, 39, 42, 43</sup>. 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 *cyp51* sequences were obtained.

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## Data availability

Data are available on Dryad repository under DOI <https://doi.org/10.11...01/2021.07.11.451819>.

Scripts were deposited in the Zenodo repository under accession <https://doi.org/10.5281/zenodo.5106475>.

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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
<b><i>Tub2</i></b>			
Tub5	CAGTCACTTGCAAACGGGTA (F)	63.7	This work
Tub14	GAAGCCTCCTGGTACTGCTG (R)	63.9	-
Tub15	GTCGACCAGGTTCTCGATGT (F)	64.1	-
Tub13	AACCGACCATGAAGAAGTGG (R)	63.8	-
<b><i>cyp51</i></b>			
CypFra2r	TCCTTCTCCTCCCTCCTCTC (R)	64	Brunner <i>et al</i> (2008)
EBI112	TTCAGCACGCTCGCCATCCTCC (F)	76.5	Brunner <i>et al</i> (2008)
Cyp9	TCTATACTGCCAGCCGATCA (R)	63.3	This work
Cyp12	CACTAACAGGCCAACTGCAA (F)	63.8	-
NB1	CACTCTTCATCTGCGACCGAGTC (R)	69.4	Leroux <i>et al</i> (2007)
<b><i>sdhB</i></b>			
Mg SDHB	TTCGGCACGTCGAGTTTCTGGCCATGCTGGCGG (F)	89.2	This work
Mg SDHB Rv	CCACAATGCCGCAAATCGGCACAACCTGCTCCC (R)	86.1	-
Mg SeqSDHB Fw	ACATCTACCGATGGAACCC (F)	61.2	-
Mg SeqSDHB Rv	TGTGGCATCGGTACAAGC (R)	63.5	-
<b><i>sdhC</i></b>			
Mg SDHC Fw	TCTCTTCGTCGACATCACCA (F)	64.7	This work
Mg SDHC Rv	ACGCACTCGCAACTCA (R)	64.7	-
<b><i>sdhD</i></b>			
Mg SDHD Fw	ACAGCTTCCGAGGTTTCGCG (F)	70.8	This work
Mg SDHD Rv	GCCATCTGTATATACACGCC (R)	63.0	-
<b><i>mfs1</i></b>			
MFS1_consensus_2	GCAAGGATTCGGACTTGACG (F)	66.9	Omrane <i>et al</i> (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 susceptibility 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 <i>Taq</i> polymerase (50X)	0.5
Deionized H <sub>2</sub> O	41
Total volume	50

DNA polymerase: Titanium *Taq* (Takara Bio)

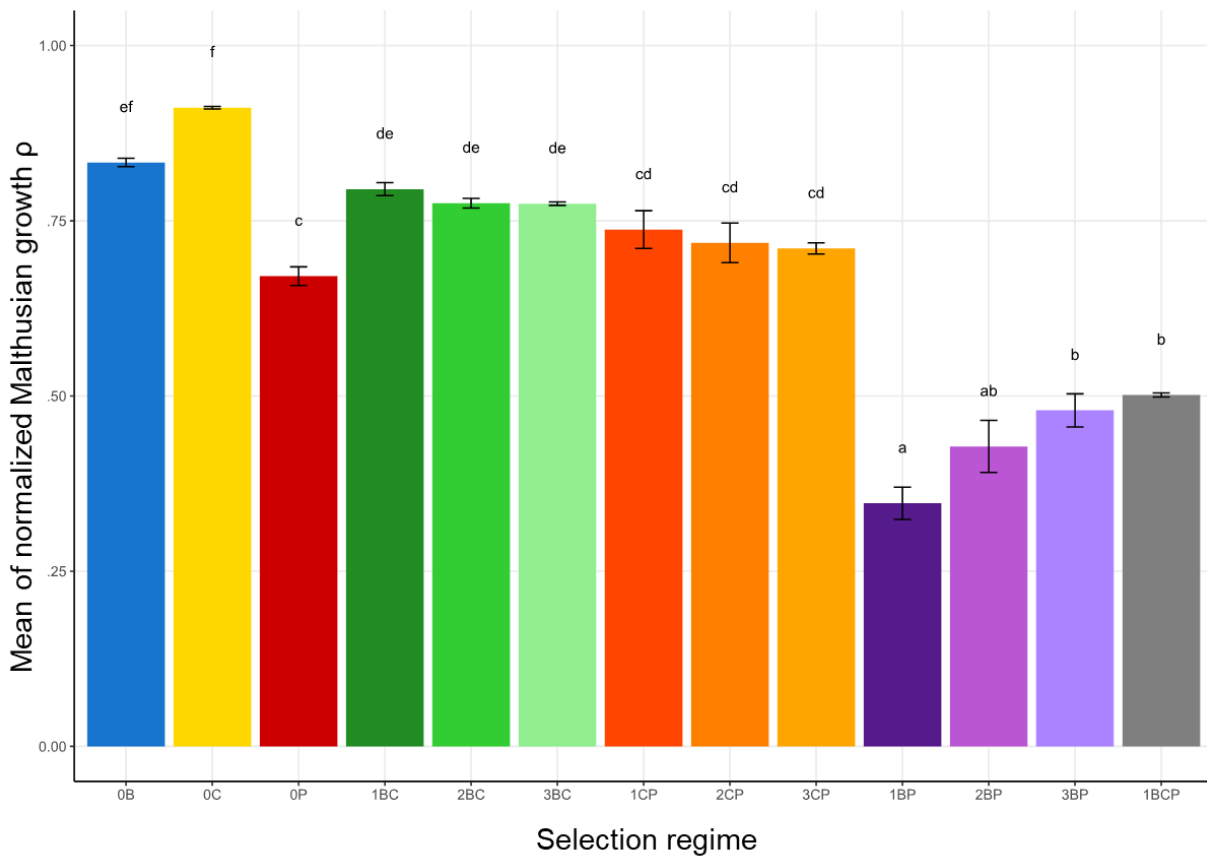
## 3. Regular PCR program

Initiation	95°C	3 min	35 cycles
Denaturation	95°C	30 s	
Annealing	T <sub>m</sub> °C	30 s	
Elongation	72°C	1min	
Final	72°C	5 min	
	10°C	To the end of the cycle	

## Supplementary Information 3:

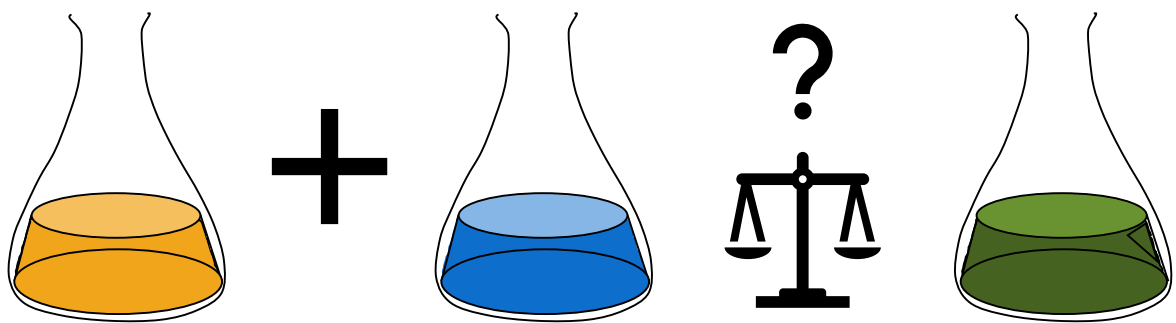
### Variation of resistance evolution in selection regimes

$\rho$ , 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.



## Part IV.

# Performance of mixture and dose modulation strategies and impact of their components



# Why is mixture efficient at delaying the evolution of resistance?

## An experimental evolutionary approach on fungicides

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## Abstract

Resistance to pesticides is a critical threat to agriculture, human health and biodiversity. Among resistance management strategies, mixtures of fungicides are the most recommended and widely used. However, the components of the efficiency of the mixtures remain unclear. Here, we carried out an experimental evolution study on the fungal pathogen *Z. tritici* to determine how mixture manage resistance. We compared the effect of the continuous use of single active ingredients to that of mixtures, at the minimal dose that fully controls the disease, that we called the efficient dose. We showed that the performance of efficient-dose mixtures applied against an initially susceptible population strongly depended on its mixture components. They were found either as durable as the best mixture component used alone, or worse than all components used alone. Moreover, regimes of efficient-dose mixture most likely selected generalist resistance profiles which might result from the combination of the selection exerted by each component and from their reduced dose. Our results warn that mixture is not a universal strategy. Therefore, experimentally evaluating the specificities of the targeted pathogens, their interactions with fungicides and the interactions between fungicides are crucial requirements for designing sustainable resistance management strategies.

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

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## Introduction

The widespread use of pesticides and drugs has led to the rapid evolution of resistance that reduces or even negates their efficacy [1]. Resistance management is therefore critical to avoid the overuse of pesticides that would be deleterious to human health and biodiversity, and to maintain sufficient qualitative agricultural production, in a context where new modes of action (MoA) dwindle and where agricultural practices favour the emergence and spread of resistance [2]. Management strategies aim at slowing down the rate of resistance build-up by maximising the heterogeneity of the selection pressure. They might involve dose reduction and/or the combination of different MoAs in space and time [3].

Among those strategies, mixtures of fungicides (*i.e.* the combination of two or more fungicides within the same treatment) are most particularly used, studied and recommended to control plant pathogens (FRAC recommendations for fungicide mixtures 2010; REX Consortium 2013). The efficacy of this strategy at delaying resistance and maintaining disease control has been proven in empirical as well as in modelling studies [4–6]. This plebiscite is also due to its operability, as many manufacturers offer ready-to-use commercial mixtures, as well as some possibility to design tank mixtures with the same active ingredients (AIs) [7]. At last, a side benefit of mixtures is that they enable the control of multiple pathogens (*i.e.* broadening of the activity spectrum) in a single spray.

The efficacy of mixtures is explained by several non-exclusive processes. First, mixtures expose pathogens simultaneously to several fungicides (*i.e.* multiple intragenerational killing (REX Consortium 2013)) and evolving specific resistance to each of the mixture components (*i.e.* multiple resistance) is less probable than evolving resistance to a single fungicide [8]. Secondly, as described in formerly established governing principles of resistance management, the growth rate of simple resistant individuals (*i.e.* resistant to one AI) is reduced when fungicides are used in mixture [4,9]. As mixing partners control both resistant and susceptible strains, both growth rates are diminished and so is the selection coefficient, defined as the

difference between these respective growth rates.

Dose reduction is another strategy to control resistance, acting particularly by reducing the growth rate of resistant individuals [4]. A majority of empirical and theoretical evidence supports that once resistance has emerged, high doses increase selection, although there are counter-examples that could be explained by the convergence of dose-response curves of resistant and susceptible strains at high doses [10]. During the emergence phase, the effect of the dose is very specific to the interaction between the fungicide and the pathogen, since high doses may have either beneficial or deleterious influence on resistance. Maintaining a low pathogen population size with high dose limits the mutation load but accelerates the selection of a mutation once it has emerged [11]. Theoretical studies demonstrated that for an overwhelming majority of realistic parameters of fungicide-pathogen combination, low-dose strategy limits the emergence of resistance of qualitative resistance [11,12].

Therefore, combining mixture and dose reduction in “efficient-dose mixtures” (*i.e.* mixture with reduced doses of its components but still allowing similar disease control to that provided by these components when used alone at their full authorized rate) may decrease the selection rate of resistant individuals and thus enhance fungicide durability [4]. While the socio-environmental benefits of reducing rates in mixtures are obvious, in practice, commercial mixtures yet include fungicide components close to or at their full-rate, *i.e.* the rate at which they are marketed alone (*e.g.* commercial products used on wheat to control *Septoria* leaf blotch; Table S1). Nonetheless, efficient-dose mixtures remain rarely adopted, which may result from the complexity to evaluate their potential advantages. First, such mixture might not benefit from the effects of high-dose strategies, long advocated to reduce the occurrence of mutations and especially the selection of partially resistant mutants, putative mutational stepping stones to high-level resistance. Second, the efficacy of efficient-dose mixtures might be equivocal because it may depend on the biology



of the pathogen (*e.g.* its ploidy and the reproduction mode; [3,13]), on fungicides performance [14], on the interaction between mixture components (antagonism or synergism; [13,15,16]) and on resistance costs [17]. Third, most studies on mixture durability consider the evolution of a specific resistance to the at-risk fungicide rather than the durability of the mixture itself. And finally, the assessment of mixture strategies usually focuses on their performance during the selection phase rather than during the emergence phase of resistance dynamics [3,12]. Here, we carried out an experimental evolution study to determine how an efficient-dose mixture may manage resistance, in a view to improve the comparison with strategies based on single AIs. In particular, we dissected how mixture components drive the quantitative and qualitative performance of this strategy. We used *Zyoseptoria tritici*, an ascomycete responsible for Septoria leaf blotch (STB) on winter wheat, a major wheat disease [18]. STB is responsible for up to 70% of fungicide use in Western Europe [19]. Resistance to all authorised unisite inhibitors (*i.e.* exhibiting a single biochemical mode of action), namely inhibitors of the polymerization of  $\beta$ -tubulin or benzimidazoles, inhibitors of cytochrome b within the complex III of respiration or Qols, inhibitors of succinate dehydrogenase within the complex II of respiration or SDHIs, and inhibitors

of sterol 14 $\alpha$ -demethylase or DMIs, has been observed for *Z. tritici* in France, at various extents [20]. Resistance results from target-site mutations for this four MoAs. Target overexpression is also demonstrated for DMIs. Enhanced efflux is caused by the overexpression of the MFS1 transporter [21]. It is a generalist mechanism causing multidrug resistance (MDR) affecting all MoAs but with limited impact on the susceptibility of the isolates when alone.

Using an approach previously developed for the study of resistance selection in alternation strategies (Ballu et al., submitted), we observed the evolution of resistance in the haploid yeast-like easily-cultivable form of a fully susceptible strain of *Z. tritici*. We first compared the rate of evolution of resistance under single or mixed fungicides treatments for three AIs representing distinct modes of action, set at the same efficacy (*i.e.* EC<sub>90</sub>). Next, we determined the cross-resistance profiles of the evolved lines, examining whether the efficacy of fungicide mixtures is counterbalanced by an increase in the occurrence of generalist profiles. Finally, we investigated how the heterogeneity in selection pressure associated to dose-efficient mixtures determines the patterns of cross-resistance profiles in evolved strains, by contrast to strains exposed to single fungicide at similarly-efficient or reduced doses.

## Material and methods

### General design

The protocol of the experimental evolution was adapted from (Ballu et al., submitted). We refer the interested reader to this article for details and justification.

The ancestral *Z. tritici* isolate was IPO323, susceptible to all fungicides. YPD plates (20 g.L<sup>-1</sup> dextrose, 20 g.L<sup>-1</sup> peptone, 10 g.L<sup>-1</sup> yeast extract, 20 g.L<sup>-1</sup> agar; USBiological) of a culture inoculated at 18°C in the dark for 7 days were used to prepare a founding culture in 25 mL liquid YPD (same as previously but without agar) in a 50 mL Erlenmeyer flask capped with cotton wool. This primary culture was incubated in similar

conditions for 7 days with 50 rpm stirring and used to initiate all lines.

Lines were conducted similarly in 25 mL liquid YPD medium, contained in 50 mL Erlenmeyer flasks, incubated as previously. Each fungicide treatment was repeated as four independent populations (*i.e.* lines). Each Erlenmeyer flask was inoculated with 10<sup>7</sup> spores (500  $\mu$ L of the primary culture). Control lines were not treated with fungicides and contained the same amount of solvent as introduced in treated lines. Experimental evolution was conducted over 7-day cycles (*i.e.* roughly six to seven generations per cycle). This cycle duration allows to maintain cultures in their exponential growth phase (before the stationary phase). At each end of cycle, 2% of the evolved culture was transferred

to a new Erlenmeyer flask containing fresh medium. To assure equal population size at the beginning of each cycle, we mimicked immigration from outside population by adding the complementary amount of spores from the untreated line to reach  $10^7$  spores in each line.  $OD_{405}$  was measured at the end of each cycle and used to compute population size (see (Ballu et al., submitted) for details). The Malthusian growth was calculated for each line as in [22] :

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

The spore concentration and the Malthusian growth were given after normalization by the concentration and the Malthusian growth of the control lines, respectively.

#### Selection regimes and selection doses

Selection regimes were designed to study the influence of three different factors on resistance evolution. First, selection regimes differed in the number of AIs used (from 1 = direct use to 2-3=mixtures). Secondly, AIs varied according to the modes of action they represented: prothioconazole-desthio (*i.e.* P; representative of DMIs), benzovindiflupyr (*i.e.* B; representative of SDHIs) and carbendazim (*i.e.* C; representative of benzimidazoles). Finally, each particular combination of AIs was applied at several concentrations: an efficient dose and reduced ones (noted with the subscript r in line names). All single fungicide and all combinations of AIs were applied at the full efficient dose and at reduced doses, continuously over the course of the experiment. A total of  $16 \times 4$ , *i.e.* 64 independent lines was observed (Table 1). The experiment lasted ten cycles except for six lines (BP, BCP, Br2, Cr1, Cr2, Pr2) for which it lasted only nine cycles (lag of the first cycle due to a calibration problem).

Table 1: Doses of fungicides B, C and P and of their mixtures used to select resistance in the various regimes of experimental evolution. The proportion of the reference dose applied referred to the efficient dose of the mixture. For example, the selection dose of the CP mixture was  $EC_{90}(CP)=0.082 \text{ mg.L}^{-1}$  of C +  $0.00205 \text{ mg.L}^{-1}$  of P, *i.e.*  $0.41 \times (EC_{90}(C) + EC_{90}(P))$ . The interaction between AIs was calculated with the Wadley formula (Wadley, 1945).

Lines name	Proportion of the reference dose applied per cycle	Between AI interaction	Efficacy observed at the 1 <sup>st</sup> cycle	Benzovindiflupyr $\text{mg.L}^{-1}$	Carbendazim $\text{mg.L}^{-1}$	Prothioconazole-desthio $\text{mg.L}^{-1}$
<b>B</b>	1.00		0.92	0.5		
<b>Br1</b>	0.53		0.62	0.263		
<b>Br2</b>	0.50		0.73	0.25		
<b>C</b>	1.00		0.90		0.2	
<b>Cr1</b>	0.45		0.92		0.09	
<b>Cr2</b>	0.40		0.95		0.08	
<b>P</b>	1.00		0.89			0.005
<b>Pr1</b>	0.80		0.86			0.004
<b>Pr2</b>	0.60		0.54			0.003
<b>BC</b>	0.68	0.74	0.90	0.34	0.136	
<b>CP</b>	0.41	1.22	0.90		0.082	0.00205
<b>BP</b>	0.60	0.83	0.92	0.3		0.003
<b>BCP</b>	0.42	0.79	0.89	0.21	0.084	0.0021

Efficient doses were chosen so that each treatment, whether a mixture or a fungicide alone, exerts a selection pressure of similar intensity on a naive population. Dose response-curves were established for the three AIs B, C and P. Their  $EC_{90}$ s were established at 7 days as the fungicide concentration inhibiting 90% of growth compared to untreated lines. We considered  $EC_{90}$  as our reference dose since the MIC (*i.e.* the minimal inhibitory concentration) could not be obtained experimentally. Fungicide mixtures were composed with the same proportion of  $EC_{90}$  from each AI, to ensure similar contribution of each fungicide to the global efficacy. Dose-response curves of each of the three possible pairs of AIs were established using a range of 1:1 fraction of the  $EC_{90}$ s of each fungicide (*i.e.* roughly from 0.41 to 0.68 times the  $EC_{90}$  of each AI; Table 1). Table 1 details the final doses used in the different selection regimes. We computed their level of interaction R, as  $R = EC_{90}^{theo}/EC_{90}^{obs}$ , using the Wadley formula,

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

with  $M$ , the AIs of the mixture,  $f_i$ , the fraction of AI  $i$  in the mixture (computed from AIs concentrations) and  $EC_{90}^i$  the  $EC_{90}$  of the AI  $i$ .  $EC_{90}^{obs}$  was the sum of the AIs concentrations in the mixture [23]. By definition, additive interactions were positive resulting in synergism if R was higher than one and were negative resulting in antagonism if R was lower than 1.

#### Establishment of resistance phenotype profiles at the end of the experiment

At the end of the evolution experiment, we realized droplet tests on each line having undergone 9 cycles (*i.e.* the last cycle common to all lines) of selection to characterize their resistance profile.

For each line, four droplets adjusted to  $10^7$ ,  $10^6$ ,  $10^5$  or  $10^4$  spores.mL<sup>-1</sup> were deposited on YPD solid medium amended with a discriminatory dose of fungicide in a square Petri dish. Discriminatory doses were validated in preliminary experiments to prevent the growth of the susceptible ancestral IPO323 isolate but to allow the growth of reference resistant isolates

available in our collections. The ancestral isolate IPO323 and a negative control were included in each test. Lines evolved under efficient doses were submitted to a set of eight different modalities: the efficient doses of each of the single AIs, the efficient doses of each of the four AI combinations and tolnaftate at 2 mg.L<sup>-1</sup>. We used tolnaftate as a marker of generalist resistance. Lines exposed to reduced doses were submitted to the previous set of discriminatory doses but also to nine additional discriminatory doses, corresponding to the selection dose of each AI in mixtures (Table 1).

Each test was scored according to the rank of the droplet with the lowest concentration of spores that enabled growth (*e.g.* a score of 2 was given if growth was observed on both the first and the second but not on the third or fourth spore dilution).

#### Statistical analysis

The mean growths of lines over the course of the experiment were compared by one-way ANOVA with lines as factor. Four ANOVAs were conducted, one per mixture. Pairwise comparisons between lines were obtained using Tukey's *post-hoc* correction. The dynamics of resistance were analyzed by a non-parametric permutation test analysis ( $10^4$  permutations) for repeated measures with spore concentration as dependent variable, selection regime and cycle as explanatory variables and lines as repeated unit of observation. The multiple pairwise  $P$  values were obtained after Bonferroni correction. The number of selection regimes against which a line was resistant, and its mean resistance score, were computed as the number and the mean of scores strictly higher than zero in its resistance profile, respectively. They were analyzed in linear models, the number of resistances being modeled with a quasi-Poisson distribution and the mean resistance score with a logGaussian distribution, with the type of selection regime (one-single-AI or two-or-three-AIs mixture) and the selection regime nested in the type were the explanatory variables.

The structuration of the resistance profiles of lines exposed to single- or mixture-efficient

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

The effects of AI number, alternation partner (C or P) and their interaction with reduced dose

## Results

Mixture durability strongly depends on mixture components

In this experiment, each selection regime, whether a mixture or a single AI, was designed to have the same efficacy, equal to 90%, compared to the untreated control, *i.e.* the selection doses were fixed as the  $EC_{90}$  (hereafter “efficient dose”) after the establishment of dose-response curves for each AI and their four possible mixtures. The level of interaction of CP was  $R=1.22$ , being higher than one and suggesting some synergy as  $R>1$ . Otherwise, antagonism was suggested for the other mixtures as  $R<1$  (BC: 0.74, BP: 0.83 and BCP: 0.79) (Table 1). These interactions (synergy or antagonism) were considered non-significant as  $R<1.5$  and  $R>0.5$ , following the criteria proposed by [23].

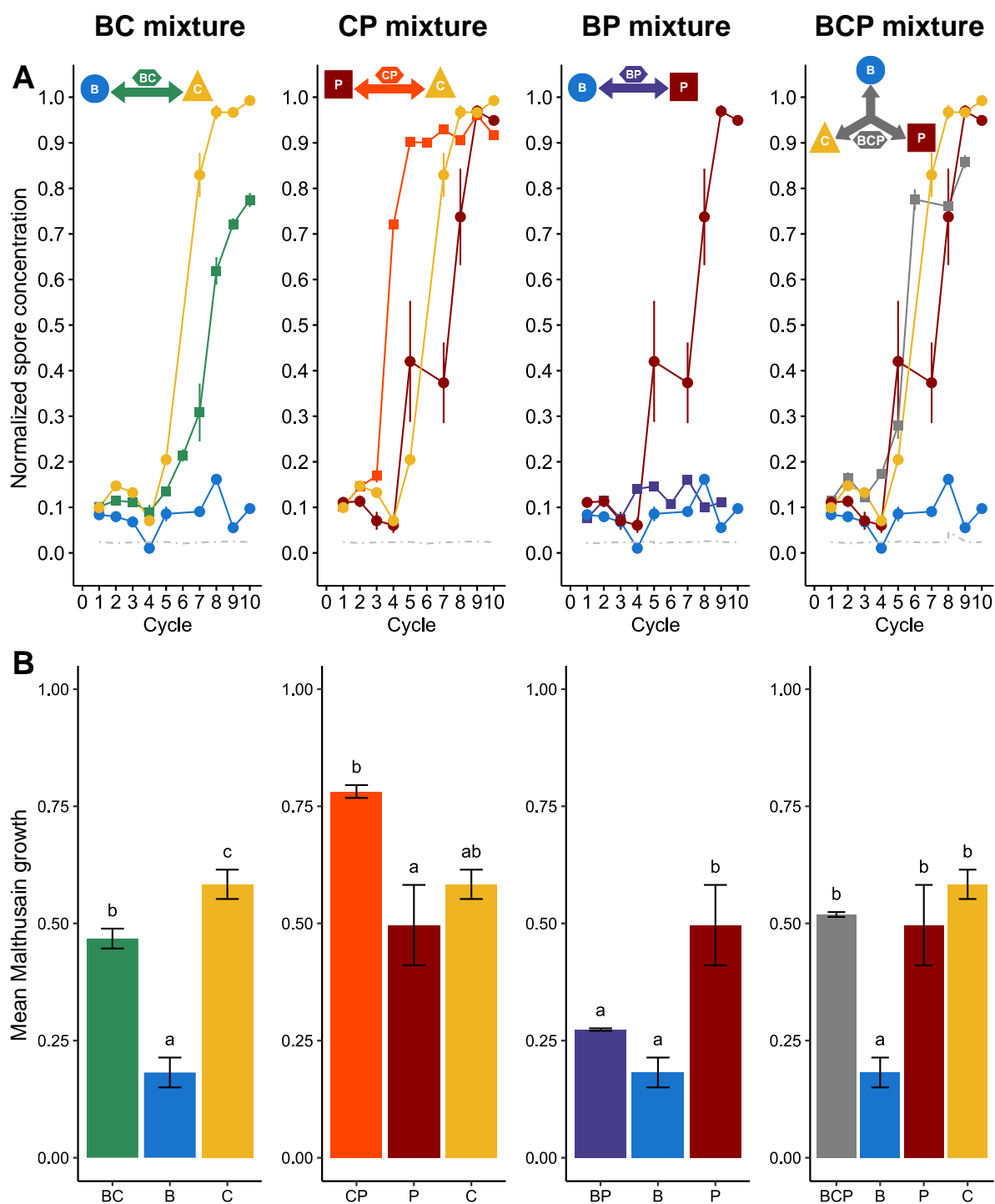
We observed the dynamics of *Z. tritici* after experimental evolution in independent lines under single- or mixed fungicides treatments set to induce the same 90% efficacy, for three fungicides representing distinct modes of action: benzovindiflupyr (B), carbendazim (C) and prothioconazole-desthio (P) (Figure 1A). The variability among the four repeated lines exposed to the same treatment was most often very low. For lines under continuous exposure of a single AI at its efficient dose, resistance first emerged in lines exposed to C and P: the normalized spore concentration (hereafter simply referred to as concentration) of C and P lines was higher than 20% (double the initial one)

exposure (in single-fungicide or in mixture) on the score of resistance to tolnaftate were investigated with a linear model (quasi-Poisson GLM model determined from variable selection by stepwise method on a Poisson GLM), where the lines where no resistance has emerged, *i.e.* the control lines and B and BP lines, were excluded.

All analyses and figures were produced with R 4.0.4 and the packages CAR, EMMEANS, FACTOEXTRA, EZ, GGLOT2, GGPUBR, COWPLOT, GRIDEXTRA, MULTCOMP and FACTOMINER.

after five cycles and resistance was generalized (spore concentration higher than 90%) after eight and nine cycles for C and P, respectively. For lines exposed to B, no resistance clearly emerged, since the spore concentration remained lower than 20% after ten cycles.

The evolution of lines under efficient-dose mixtures exposure was extremely contrasted. The BP mixture fully delayed resistance as no resistance emerged in these lines after ten cycles, as in the B lines. BP dynamics was very significantly different from P ( $P<1e^{-3}$ ) but slightly from B ( $P=0.56$ ). The BC mixture exhibited intermediate performance, significantly different from B and C ( $P<1e^{-3}$  for both), with resistance emerging after six cycles (*i.e.* one cycle later than in the case of direct exposure with C but before exposure with B) and a normalized spore concentration reaching 80% at the 10<sup>th</sup> cycle, *i.e.* after resistance was generalized in C lines. The CP mixture displayed poor sustainability, as the emergence and generalisation of resistance respectively at the 3<sup>rd</sup> and 5<sup>th</sup> cycles were faster than in lines exposed to C or P alone (resistance emergence at the 5<sup>th</sup> cycle and generalisation at the 8<sup>th</sup> and 9<sup>th</sup> cycles, respectively) and was significantly different from P and C dynamics ( $P<1e^{-3}$  for both). The three-way mixture BCP exhibited intermediate results, with resistance emerging and generalising more slowly than in lines exposed to the least durable CP mixture (but not in a significantly way  $P=0.20$ ) but allowing resistance to emerge contrarily to the BP mixture ( $P<1e^{-3}$ ).



**Figure 1: Dynamics of resistance evolution in the lines selected at the same 90% treatment efficacy.** Each column represents results for a pair of fungicides used alone or in mixture, at their efficient dose, as explicated in the pictograms on the top. B: benzovindiflupyr (SDHI), C: carbendazim (benzimidazole) and P: prothioconazole-desthio (DMI). **(A)** The normalized spore concentration corresponded to the spore concentration observed at the end of a cycle compared to the spore concentration in the control line, *i.e.* a susceptible population not exposed to fungicides. **(B)** Mean of the Malthusian growths. Results are normalized with the Malthusian growth of the control (histogram bars) and presented with their standard deviations (upper and lower lines). Different letters indicate significant differences between groups ( $P < 0.05$ ).

Comparing the global resistance growth while using the cycle-averaged Malthusian growth rates produced similar ranking of these strategies (Figure 1B). Resistance growth in BC lines was intermediate, *i.e.* significantly higher than in lines B but lower than in lines C ( $P < 0.05$ ). Resistance growth in CP lines was significantly equal to or higher than in the corresponding single-fungicide lines. BP lines did not perform significantly differently from the B lines, exhibiting the best durability. The results of BCP lines were intermediate, *i.e.* not significantly different from the two least durable AI treatments.

It is worth noting that CP, the least “durable” mixture, was the only mixture which exhibited (non-significant) synergism and was applied with an efficient dose lower than the sum of half the efficient doses of its components.

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

We determined the phenotypic resistance profile of each population at the 9<sup>th</sup> cycle (Figure 2) using droplet tests. As expected, control lines did not exhibit resistance to any of the testing fungicide modalities. The lines exposed to single fungicides showed contrasted patterns of resistance. Those exposed to C exhibited a unique and narrow resistance profile characterized by a strong resistance to C (mean resistance score of 4, *i.e.* the maximal score) and medium resistance to the mixture BCP (mean resistance score of 2). By contrast, lines exposed to P exhibited specific profiles in each of the four repeats, all broader than for C (in average, P lines resistant to 3.25 out 8 discriminatory doses, whereas C lines were resistant to 2) and including various degrees of resistance to P and to CP but also to tolnaftate (for 3 out 4 lines). Tolnaftate is known to reveal multidrug resistance due to enhanced efflux in *Z. tritici* [21,24]. Such patterns are consistent with multiple and/or generalist resistance mechanism. Lines exposed to B, where no resistance had emerged, displayed no resistance on any modalities of this droplet test.

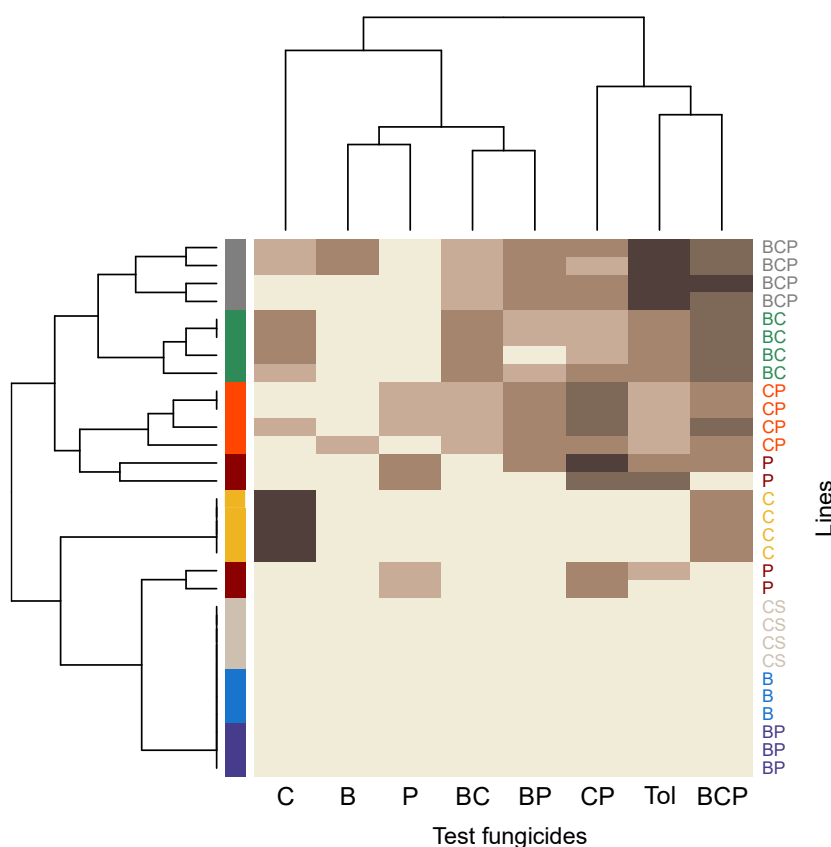
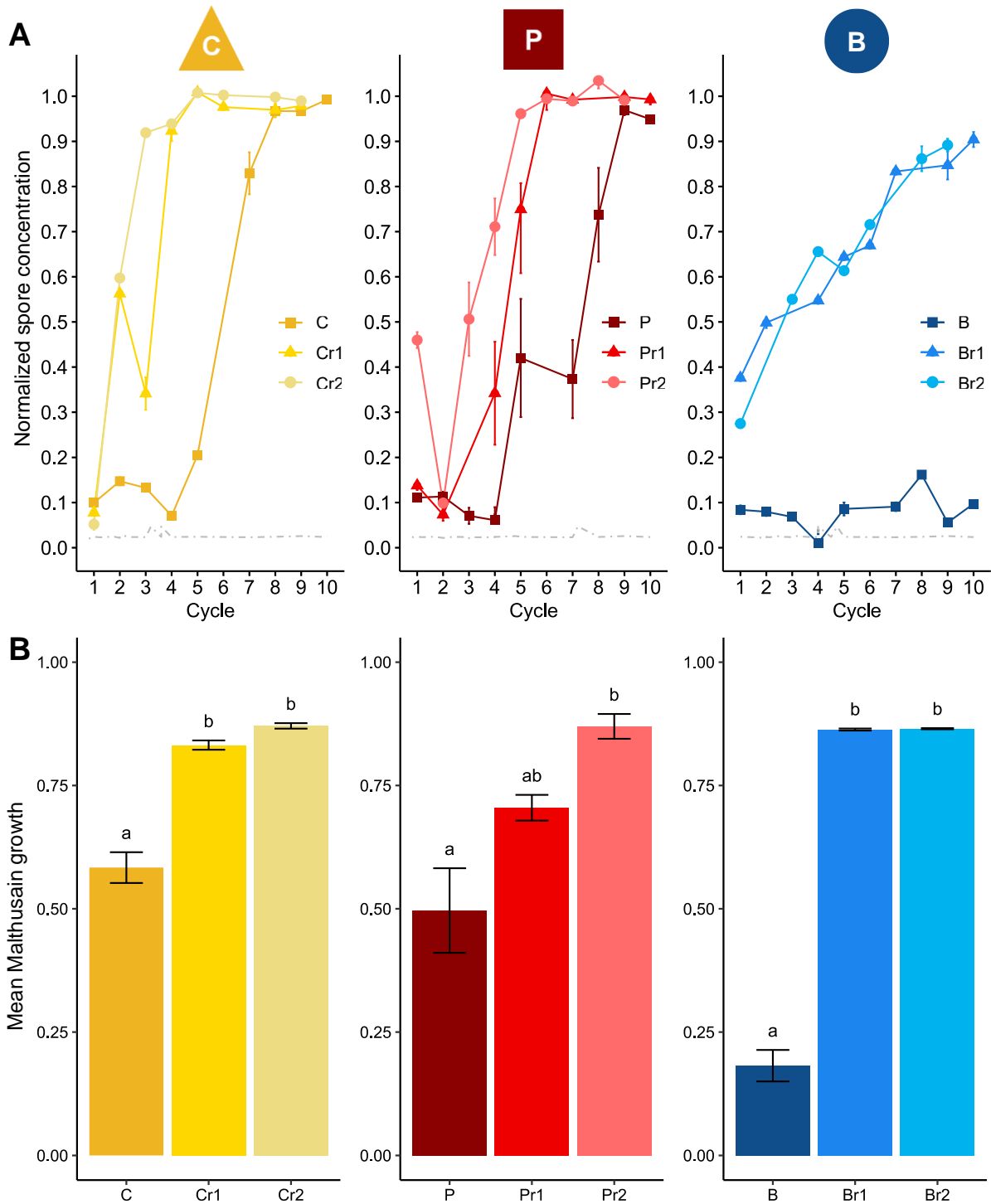


Figure 2: Heatmap of the phenotype resistance profiles at the 9<sup>th</sup> cycle. The resistance scores (0-4; represented by the brown scale) are shown for each of the twelve populations evolved under one of the eight selection regimes (four or three replicate lines per regime; regimes represented by the rainbow scale, as described in Figure 1) and for each fungicide or mixture tested. Heatmaps established using the pairwise Euclidean distance.



**Figure 3: Dynamics of resistance evolution in the lines exposed to a single fungicide at full- or reduced efficient doses.** Each column represents results for an AI used at its EC<sub>90</sub> selection dose or at two reduced doses, representing a fraction this EC<sub>90</sub> (Table 1). B: benzovindiflupyr (SDHI), C: carbendazim (benzimidazole) and P: prothioconazole-desthio (DMI). **(A)** The normalized spore concentration corresponded to the spore concentration observed at the end of a cycle compared to the spore concentration in the control line, i.e. a susceptible population not exposed to fungicides. **(B)** Mean of the Malthusian growths. Results are normalized with the Malthusian growth of the control (histogram bars) and presented with their standard deviations (upper and lower lines). Different letters indicate significant differences between groups (P<0.05).

The lines exposed to efficient-dose fungicide mixtures where resistance had emerged (BC, CP and BCP) exhibited wider resistance profiles than the ones exposed to a single AI, even to P. Indeed, in average, they were resistant to 2.3 times more testing modalities than those exposed to a single AI ( $P < 1e^{-4}$ ) but at a lower degree, their score being 0.8 times lower for selection regime against which they were resistant. These lines were resistant to their selection mixture, at various degrees, but also to the other three mixtures and to tolnaftate, especially for BCP lines that exhibited the highest possible resistance score to tolnaftate. This suggested again multiple and/or generalist resistance evolving in these lines. However, these lines were not necessarily resistant to the efficient dose of their components used alone: BC lines were resistant to C but not B; CP lines were mostly resistant to P but stayed susceptible to C; half of the BCP population displayed resistance to B and C while the other half did not reveal resistance to any single AI. The lines exposed to BP, where no resistance had emerged, showed no resistance in the droplet tests.

#### Reduced dose of single AIs still selects for resistance

As expected, over the course of the experiment, the control of *Z. tritici* was weaker in the lines exposed to reduced doses than in those exposed to the efficient dose of the same fungicide (Figure 3). In particular, resistance to B emerged in populations undergoing reduced doses of this fungicide whereas it was prevented at its efficient dose. For each AI, the mean Malthusian growths were significantly greater in reduced-dose lines than in efficient-doses ones ( $P = 0.04$  and  $P = 0.003$ , for Pr1 and Pr2 compared to P respectively, and  $P < 1e^{-4}$ , for all pairwise comparisons between efficient and reduced doses of B and C). Surprisingly, Cr lines undergoing exposure under reduced efficient dose of C (*i.e.* set in preliminary data at 0.4 and 0.45 of the efficient dose), initially exhibited similar control to that of the line undergoing full exposure (Table 1). Nevertheless, the control of these lines was reduced as expected from the second cycle (Figure 3). The higher continuous

increase in spore concentration over time cycles signalled that reduced-dose regimes selected for resistance, at the same time as they displayed lower control over the populations. However, we could not test the effect of dose reduction on resistance selection since lines exposed either to full or reduced-doses were not submitted to the same treatment intensity, preventing the dissociation of the resistance selection from the growth control.

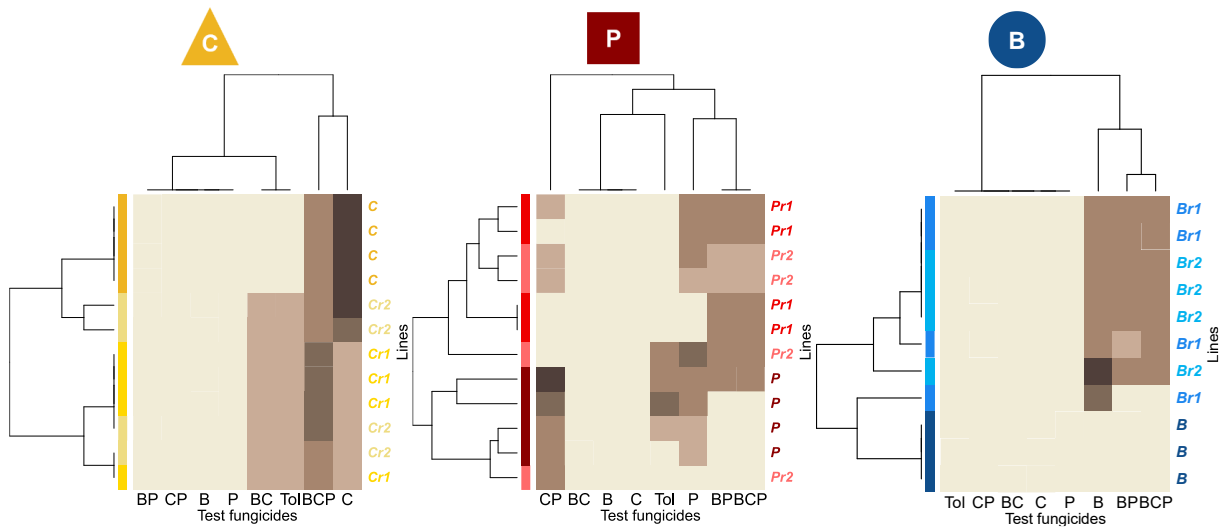
#### Reduced dose of fungicides also selects for generalist phenotypes

Heatmaps of the phenotype resistance profiles confirmed that the reduced doses of B, C or P selected for resistance (Figure 4). Lines undergoing selection with reduced doses of B or C and almost all exposed to reduced doses of P (five out of eight) were resistant to their selection fungicide at its efficient dose. Resistant profiles selected at reduced dose were wider than or different from those selected at efficient dose by the same fungicide. For fungicide C, the efficient-dose regime selected a unique resistance profile with high resistance to C and medium resistance to BCP, whereas the reduced-dose regime selected for a generally weaker resistance but also for an extra resistance to tolnaftate. For fungicide P, the efficient-dose regime selected for resistance to P and CP and also to tolnaftate in three out of four lines. Whereas the reduced-dose P regime selected for BP and BCP resistance (except for one line), only half of the lines exhibited resistance to CP or P and all lines were susceptible to tolnaftate. For fungicide B, the reduced-dose regime mostly selected for resistances to B, BP and BCP that could not be compared to the efficient-dose regime since no resistance emerged from it.

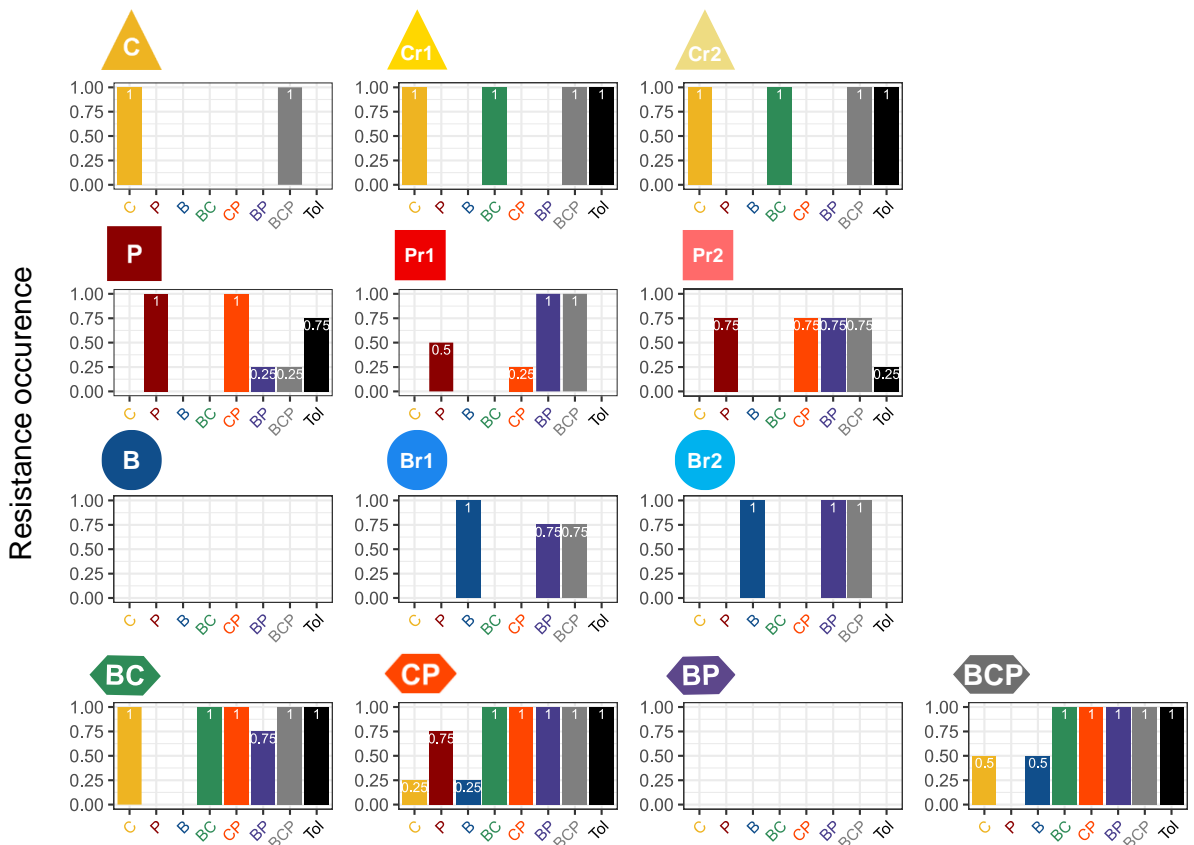
#### The determination of resistance profiles results from the balance between selection heterogeneity and dose reduction of single AIs in efficient-dose mixtures

Resistance spectra differed for the number of fungicides for which resistance was displayed, and also for the occurrence of these resistance among the replicates of the different





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



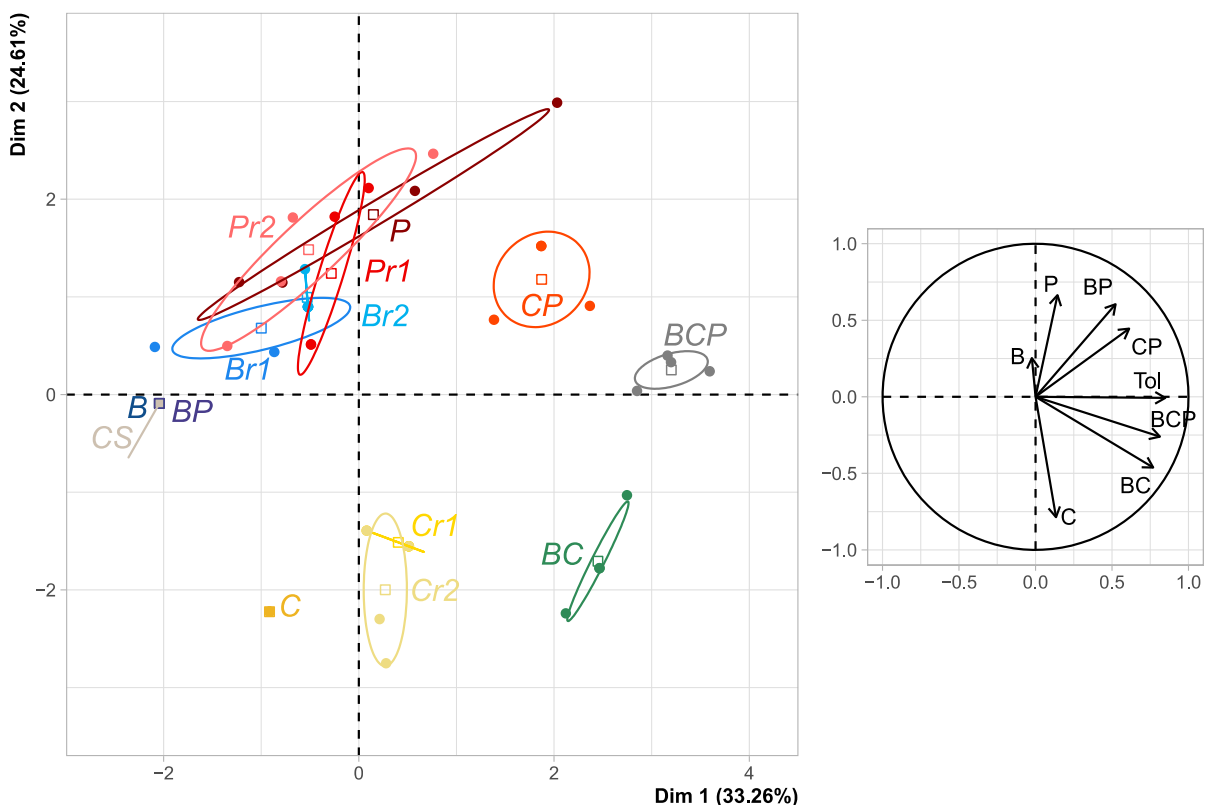
**Figure 5: Occurrence of resistance evolved in each selection regime.** Histograms show the occurrence of resistance within a line for each testing modality examined in a droplet test. For example, a score of 0.25 means that one of the four replicated lines of this selection regime had a resistance note higher than zero).

selection regime (Figure 5). The resistance spectrum of BC including six resistances almost corresponded to the union of the resistance spectra of Br and Cr (with an extra resistance to CP and a missing one to B). By contrast, the cumulative resistance spectra of B and C included only 2 resistances. The CP lines showed the same pattern since the CP resistance spectrum included a common resistance to BC and BP observed only for reduced-dose regimes of C and P and not in their efficient-dose spectra. The spectrum of BCP was also better explained by the spectra of the reduced-dose B, C and P regimes where resistances to BC, BP and B are more present than in efficient-dose ones.

The PCA of the resistance profiles established for each line showed a first axis explained mainly by resistance to tolnaftate and BCP and secondarily by resistance to the two-way mixtures (Figure 6). This first axis showed that efficient-dose mixtures often selected higher intensity generalist resistance. Indeed, to the left of this axis were lines with the narrower resistance spectra (*i.e.* selected with efficient-dose single-AI

regimes). In the relative centre of the PCA were lines with low resistance to tolnaftate and BCP (*e.g.* Cr1, Cr2) and to the right were lines with greater occurrence of resistance to tolnaftate and BCP (all from lines under effective-dose mixtures). The analysis of the occurrence of tolnaftate resistance showed a significant effect of mixture when selecting resistance to this fungicide, with significantly higher scores for two- and three-way mixtures compared to the use of the same AIs alone ( $P=0.19$  and  $P=0.002$ , respectively). Such analysis also revealed a positive significant effect when selecting generalist resistance, for lines exposed to reduced dose of C ( $P=0.0059$ ). No negative or highly positive cross-resistance was observed between the different MoAs (*i.e.* correlations scores between testing fungicide modalities ranged between 0.14 and 0.66; SI Figure 1).

Generalist resistance profiles selected in efficient-dose mixtures would then result both from the multiplicity of selection pressures exerted by the mixtures and the reduction in the dose of each of their components.



**Figure 6: Phenotype resistance profiles established in all lines at the end of the experiment.** PCA was structured by generalist resistance, as detected by resistance to tolnaftate and the mixture BCP.

## Discussion

To investigate the effect of efficient-dose mixtures on the emergence and selection of fungicide resistance, we submitted multiple lines of a susceptible isolate of *Z. tritici* to fungicides representative of three modes of action, applied either singly at efficient dose or with a fraction of this dose targeting  $EC_{50}$ , or as two- or three-component mixtures. Efficient-dose of single AI or mixture strategies displayed the same treatment efficacy ( $EC_{90}$ ). The effect of efficient-dose mixtures on resistance dynamics appeared highly contrasted depending on mixture components: such mixtures were either as durable as the best mixture component used alone, or worse than all AIs used alone. Moreover, efficient-dose mixtures favoured generalist resistance phenotype profiles, with all lines undergoing such regime being resistant to all mixtures but also to tolnaftate, an indicator of multidrug resistance (MDR), a generalist resistance mechanism already described in field strains of *Z. tritici*. The resistance profiles characterized in efficient-dose mixtures resulted from the combination of selection exerted by each component of the mixture at its reduced dose. Indeed, these profiles were similar to the union of profiles obtained after exposure to reduced-doses of the corresponding single AIs, but with higher scores recorded for modalities associated to generalist resistance (*i.e.* resistance to tolnaftate and mixtures).

This experiment followed a similar experimental design from (Ballu et al., submitted), notably using the same AIs but to address the sustainability of alternation strategies. In the present study, the ranking of times to resistance emergence did not reflect the assumed hierarchy of the intrinsic risks of resistance associated with benzimidazoles (high; C), SDHs (medium to high; B) and DMIs (medium; P) [25]. Indeed, if resistance emerged first in C lines and later in P lines, resistance was never selected in B lines. This could result from changes in temperature, in humidity between the two evolution experiments or from changes in treatment efficacy determination (especially  $EC_{90}$  instead  $EC_{95}$ , leading to a substantial difference in the selection dose for B and C). Therefore, we considered that the lines of the present

experiment, which have evolved in the same environment, could be compared but we focused our conclusions on the effects of the C and P AIs and did not interpret our results invoking intrinsic risks.

Durability of mixture could not outperform solo-fungicide used when applied at efficient dose.

We observed highly contrasted resistance dynamics, despite the same initial disease control, depending on the strategy (single or two- or three-way mixtures) and on the mixture components. We thus demonstrated that the mixture strategy does not always systematically improve resistance control compared to single fungicide treatments. This result contradicts the main knowledge and recommendations about mixtures [3,4,26]. Indeed, previous studies demonstrated that the mixture strategy enables to delay the emergence [11] and selection [6] of resistance to a high-resistance risk fungicide, increasing the effective life of this fungicide. However, significant differences between our study and previous ones could explain our divergent conclusions.

First, we studied efficient-dose mixtures as suggested by [27], arguing that, to decrease resistance selection, mixtures could be used at lower doses, and in particular at the minimal dose still giving an effective control. Though, half-dose mixtures have yet received few attention [13] and almost all of the studies on mixtures consider full-dose mixtures (but see [14] for an exception). To study the acclaimed “redundant-killing” effect of mixtures and disentangle it from any AI additivity or synergist effect, we exposed each line to treatments exhibiting the same efficacy. The resulting tested mixtures then varied for the fraction of efficient dose of each of their component. The CP selection regime included a 1:1 ratio of 0.4 times the  $EC_{90}$  of each fungicide whereas the doses of the other mixtures were higher than half the  $EC_{90}$  of their component (or one third of the dose for BCP). Considering half-dose for instance could have changed the ranking of mixture strategies, *e.g.* the CP selection regime that was the least sustainable at efficient dose mixture (at  $0.4 \times EC_{90}$ - dose mixture) would have higher dose and

could gain in durability whereas the other mixtures would have lower dose and could lost in durability. Second, we used a naive ancestral population, susceptible to all fungicides, while most of the studies focus on the selection phase of resistance dynamics, *i.e.* after resistance to at least one of the components has already emerged. Third, a majority of studies focus on the evolution of resistance to one of the mixture components only, and it is often the high-risk fungicide. Resistance to the other partners of the mixture is often assumed to be insignificant, although it may contribute to the gradual growth of the population, and generalist mechanisms are neglected. [3] identified only four papers that considered resistance to both mixing partners in their review. Therefore, our findings are interpretable as the overall durability of the mixture, instead of as the delaying selection effect of mixture on a specific resistance phenotype. Finally, we carried out an experiment which enables to study the resistance dynamics without requiring *a priori* assumptions about resistance phenotypes or mechanisms that are likely to be selected [28–30], whereas previous theoretical studies were constrained to consider single or few resistance phenotypes. Our results support the conclusions of the empirical study from Mavroei and Shaw (2006) that demonstrated that the benefit of mixtures strongly depended on the specific combination of its components and required to be demonstrated experimentally.

#### Mixture favoured generalist resistance of a phytopathogenic fungus

We found that mixtures favoured the selection of broad resistance phenotype profiles, consistent with multiple resistance and/or generalist mechanisms. Indeed, lines evolved under mixture regimes often exhibited wider spectra of resistance than those exposed to the use of only one AI, with lower resistance intensity and growth on tolnaftate. As this fungicide is an indicator for MDR [24], we assume that generalist resistance was more likely to occur than multiple specific resistances, without excluding the presence of specialist resistance. Indeed, both can coexist within an individual or within a population, as described by [32] in the

“bet-hedging” hypothesis where in an isogenic population, differently-specialized phenotypes, more or less fit depending on conditions, may co-exist in a dynamic equilibrium in a heterogeneous environment. Genetic analysis (*e.g.* of the promoter of the *mfs1* gene, associated to MDR in field isolates of *Z. tritici*; [33]) could precise the resistance structure of evolved populations, although non-target-site resistance could also be acquired by epigenetic mechanisms [34].

Our result that mixture favours generalist resistance supports the studies from [35] and [15] for herbicides mixtures and [36] for antibiotics combination. As MDR is a global increasing issue [37], greater attention should be paid to this trade-off while designing resistance management strategies, *e.g.* by including consideration about management of non-target site resistance, as suggested by [38] and [39] for SDHI fungicides.

Resistance profiles are shaped by dose variation and should therefore be considered in management strategies

While dealing with resistance management in fungi, the question of the dose rate was so far focused on variation in resistance dynamics, *i.e.* the time to resistance emergence or the selection rate [3,6,11,12]. Our experiment did not enable to settle this debate, since the growth of susceptible and resistant variants were confounded in observations of the fungal growth and since the reduced doses considered here were too low to realistically describe resistance management strategies with sufficient disease control. But it enabled to tackle the question of the dose rate from a new standpoint by considering the qualitative outcome of selection rather than just the dynamics of resistance.

We observed that strains resistant to the efficient dose of B, C or P could be selected with reduced doses of the same fungicides, even for the lines exposed to benzovindiflupyr (B) for which resistance never emerged at full dose. This is consistent with a previous observation on antibiotics [30,40,41]; and herbicides [42]. Indeed, low dose treatment allows the survival of a higher frequency of small-effect resistance

mutations, which combined lead to high-level resistance [42].

Some specific resistances in lines submitted to reduced-dose regimes could indicate that dose mitigation also favours the selection for generalist mechanisms. Indeed, resistances to tolnaftate and to the BCP mixture were found in lines exposed to reduced doses of B and P, respectively, but not in the lines undergoing efficient-doses of the same fungicides. These results are consistent with multiple previous studies, although not in plant pathogens, which demonstrated that low doses could select for off-target mutations [43–45] and for polygenic resistance mechanisms [43,46] that are more prone to result in multiple or generalist resistance (see Raymond (2019) for a review).

The selection exerted by reduced-doses could also shape the resistance profiles of lines exposed to efficient-dose mixtures as these are more similar to the union of resistance profiles of lines exposed to reduced doses of their components than to the union of those selected at efficient doses. One should point out in particular that resistance to tolnaftate was observed in lines undergoing reduced-dose of C (but not in efficient-dose ones) and in all lines exposed to efficient-dose mixtures including C. As emphasized by [16] for antibiotics, low doses in resistance strategy management should be considered with caution as they do not prevent resistance even at low doses and could lead to generalist mechanisms, possibly in mixtures also.

#### Experimental evolution a useful tool to compare strategies

The experimental evolution framework enabled us to submit naive populations to resistance management strategies exhibiting various degrees of selection heterogeneity and to compare their performance in standardized conditions. In such controlled environment, we could disentangle and assess the performance of several drivers of mixture and dose reduction strategies, which could have been hardly achieved in field experiments. We also benefited from the observation of selected resistance profiles, contrary to model studies. Despite these multiple advantages, the experiment remained delicate to handle, and a limited number of

strategies have been studied here. To consolidate our conclusions, especially concerning the effect of the dose on mixtures, future work testing other AIs, different dose-ranges of fungicides alone or in mixtures, including double-efficient-doses, are still required. From an application point of view, a better understanding of the predictive capacities of such experiment (*e.g.* by relating growth dynamics and resistance profiles to disease control and in-field resistance frequency), appears a key step to design resistance strategies tailored by the intrinsic properties of pathogens and fungicides. Finally, we tested our strategies on naive populations, susceptible to all fungicides. Applying this approach to populations where resistance is initially present could allow us to propose useful complementary advice to farmers, as contrasting resistances status were described in monitoring studies [20].

#### Conclusion

Our results warn that mixture is not a universal strategy. At the minimal dose to control the disease, a mixture applied against a naive population could shorten durability and more generalist resistance, compared to single fungicides exhibiting comparable efficacy. However, efficient-dose mixtures, provided adequate components, could provide as effective disease and resistance control as single-fungicide treatments, at a lower environmental and economic cost. Therefore, taking into account the specificities of the targeted pathogens, their interactions with fungicides and the interactions between fungicides, as demonstrated here, as well as the frequency and the type of resistance already present in population, are crucial requirements for designing sustainable resistance management strategies. Resistance management remains a key challenge for the development of a more sustainable agriculture. To meet this challenge, experimental evolution appears to be a very promising tool that usefully complements theoretical studies and field monitoring.

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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. AB and FC performed the statistical analysis, with contributions from ASW and AD. The paper was written by AB and FC, with significant contributions from ASW and AD.

## Competing interests

AB contributed as part of a PhD studentship partly funded by Syngenta, supervised by ASW, FC and AD.

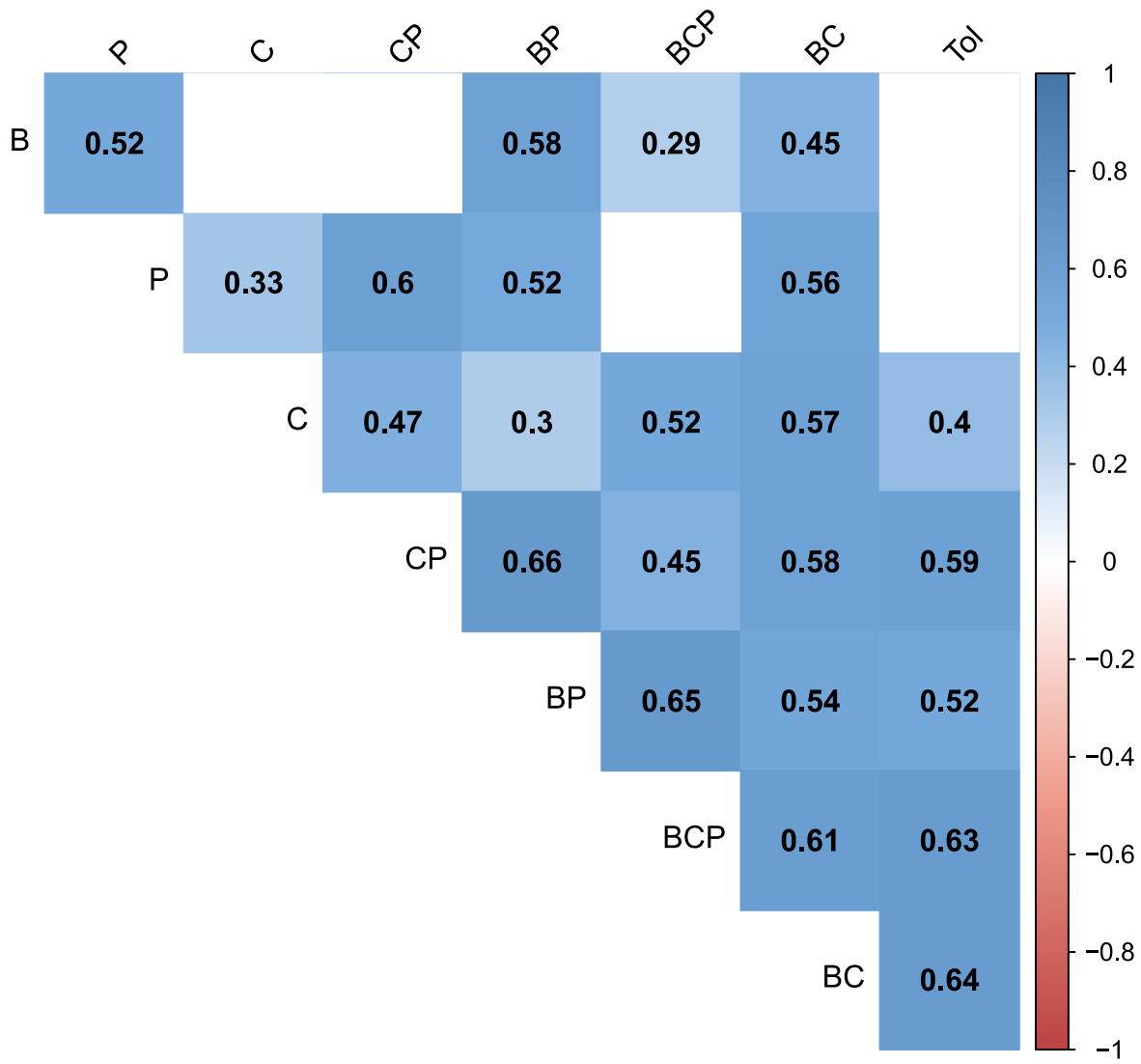
**Table S1: Commercial mixtures and solo formulations used on wheat to control Septoria leaf blotch in France.** For each commercial product, the composition, recommended rate and use are detailed. Percentages indicate the fraction of each AI used in the mixture, compared to the solo commercial product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

Use in mixture						Commercial Product	Aviator Xpro Oceor Xpro SDH2 Pro	Karosse Xpro Skyway Xpro	Cavando Korema Korema Star Osiris Star Osiris Win Epomet	Adexar Tenax XM SDH1	Librax SDH-CO	Ceratavo era Elatus era Velogy era	Kestrel Onnel Piano Prosaro Prosafort Prosatop	Ampera Diams Epopee Galactica Nebraska
						Composition g.L <sup>-1</sup>	75+150	75+100+100	56.25+41.25 (or 37.5+27.5)	62.5+62.5	62.5+45	150+75	160+80 (or 125+125)	132.5+267.1
Use as solo compounds						Recommended rate l.ha <sup>-1</sup>	1.25	1	2 (or 3)	2	2	1	1	1.5
						Recommended use g.ha <sup>-1</sup>	93.75+156.25	75+100+100	112.5+82.5	125+125	125+90	150+75	160+80 (or 125+125)	198.75+400.65
						Fungicides	Bixafen + Prothioconazole	Bixafen + Prothioconazole + Tebuconazole	Epoxiconazo+ Metconazole	Fluxapyroxad + Epoxiconazole	Fluxapyroxad + Metconazole	Prothioconazole + Benzovindiflupyr	Prothioconazole + Tebuconazole	Tebuconazole + Prochloraz
Fungicide	Chemical class	Commercial product	Composition g.L <sup>-1</sup>	Recommended rate l.ha <sup>-1</sup>	Recommended use g.ha <sup>-1</sup>									
Benzovindiflupyr	SDHI	Elatus plus	100	0.75	75							100%		
Bixafen		Thore	125	1	125		75%	60%						
Epoxiconazole		Rubric	83	1.5	124.5				90%	100%				
Fluxapyroxad		Imtrex Syrex Fluxatop	62.5	2	125					100%	100%			
Metconazole	DMI	Sirena	90	1	90				92%		100%			
Prochloraz		Eyetak Proca Prochlorflash Pro Plex 450 Faxer	450	1	450									89%



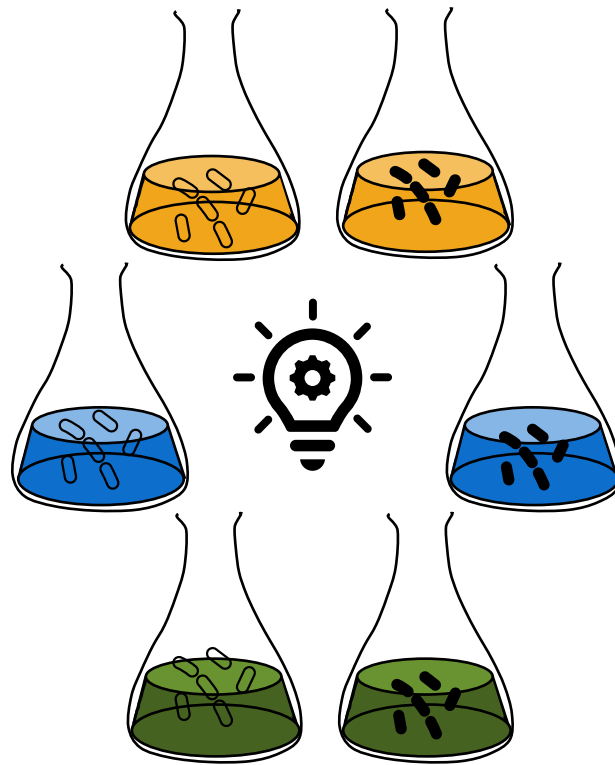
		Fujara Saranta Sporaz Septoraz												
Prothioconazole		Joao Protioline	250	0.8	200		94%	50%				75%	80% (or 62.5%)	
Tebuconazole		Illide Mystic Ew Fezan Colnago Rivazon Erasmus Spekfree Curzol Ulysses	430 (or 250)	0.6 (or 1)	258 (or 250)			39-40%					31-32% (or 48-50%)	77-79.5%
						<b>Total</b>	<b>169%</b>	<b>149-150%</b>	<b>182%</b>	<b>200%</b>	<b>200%</b>	<b>175%</b>	<b>111-112% (or 110.5-112.5)</b>	<b>166-168.5%</b>

Figure S2: Correlation between the susceptibility towards test fungicides observed in droplet tests.



## Part V.

# Maximising the heterogeneity of selection as a proof of concept for sustainable anti-resistance strategy



# **Skill and style to maximise heterogeneity in selection for fungicide adaptation: resistance status does matter. A proof of concept in a plant pathogen**

## **1 Introduction**

Exposure to pesticides or drugs creates an environment favourable to the emergence and the selection of resistant individuals. Anti-resistance strategies aim at delaying the evolution of resistance by subjecting populations to heterogeneous selection pressures or by reducing their exposure time to selection (van den Bosch et al., 2014a; REX Consortium, 2013). Multiple sources of heterogeneity can be considered to mitigate selection, such as the number of active ingredients (AIs) or of modes of action (MoAs) displayed, their intrinsic resistance risk, their dose, their pattern of application over time (either concomitantly *i.e.* in mixture, or successively *i.e.* in alternation) or over space (*i.e.* mosaic). Exemplifying the adaptation of the plant pathogenic fungus *Zymoseptoria tritici*, several empirical or modelling studies concluded that these strategies are globally efficient to limit the evolution of resistance (van den Bosch et al., 2014a; Dooley et al., 2016c; Heick et al., 2017). Yet, the identification of the most sustainable strategy is still a matter of debate, as the ranking of strategies was not proved unequivocal in many agricultural or clinical situations (van den Bosch et al., 2014a; REX Consortium, 2013). Several reasons may explain this finding. First, the performance of a strategy may differ according to the criteria used for its assessment. The evolution of the frequency of resistance is among the most frequently used criterion, and other ones, such as disease control or the effective life of fungicides, may conclude to different rankings. Second, drivers of the heterogeneity of the selection pressure may impact resistance management differently, depending on how and when they are implemented in the course of resistance dynamics. Indeed, strategies efficient at delaying the emergence of resistance may differ from those relevant to slow down selection once resistance is already present in populations (Hobbelen et al., 2014). At last, the initial frequency of resistant mutants and their nature (*e.g.* inducing resistance to one or multiple MoAs) may also influence the effective life of AIs (Hobbelen et al., 2013). Indeed, the performance of a strategy also relies on the respective efficacy of its components at controlling susceptible and resistant individuals over time and space and its overall efficacy might be compromised according to the status of resistance in population (FRAC, 2010).

Septoria tritici blotch (STB) is the main disease affecting the yield of wheat worldwide (Fones and Gurr, 2015; Torriani et al., 2015). It is caused by the ascomycete *Zymoseptoria tritici*, an hemibiotroph plant pathogenic fungus, mostly controlled by fungicide applications. However, this pathogen has adapted, at various degrees, to all the authorized unisite MoAs (*i.e.* benzimidazoles, preventing the polymerization of  $\beta$ -tubulin; QoIs, inhibitors of the complex III of the mitochondrial respiration; DMIs, inhibitors of sterol biosynthesis and SDHIs, inhibitors of the complex II of the mitochondrial respiration), at least in Western Europe. Resistance is caused by one or multiple mutations in genes encoding the target protein of these fungicides or a generalist mechanism of enhanced efflux causing multidrug resistance (MDR) (Garnault et al., 2019; Heick et al., 2017; Hellin et al., 2020; Huf et al., 2018; Jørgensen et al., 2018a; Omrane et al., 2015; Rehfus et al., 2018; Samils et al., 2021; Torriani et al., 2015). In this context, *Z. tritici* reveals a relevant model to identify the drivers determining the performance of anti-resistance strategies in contrasted field situations. To our knowledge, the relative efficacy of the sources of selection heterogeneity is poorly understood for this pathogen of high agronomic relevance.

In this context, this work aims at understanding the interplay of strategy components that maximizes the heterogeneity of the selection pressure in relation to resistance status. It relies on several assumptions, based on literature and on previous work from Ballu et al. (2021a, 2021b). First, the various sources of selection heterogeneity display a contrasting efficacy in delaying resistance evolution. Second, combining these sources of heterogeneity is likely to increase the overall sustainability of strategies (REX Consortium, 2013). Third, the efficacy of anti-resistance strategies is highly dependent on population structure as regarding resistance (Hobbelen et al., 2013). This work is then a proof of concept of these hypotheses that we tested by an approach of experimental evolution. By using strains carrying field resistances, we combined some characteristics of natural populations with accelerated experimental evolution in controlled and miniaturized conditions (Bailey and Bataillon, 2016). The evolution of resistance mutations was followed under several selection regimes and helped characterizing their impact on the efficiency of anti-resistance strategy factors. Several sources of heterogeneity usually promoted for the durable control of STB were tested. Those were the number of AIs used in the strategy (*i.e.* 1-3), their diversity (*i.e.* intra- *vs.* inter-MoA diversity), their intrinsic resistance risk (*i.e.* from very low to high) and the temporal heterogeneity of fungicide exposure (*i.e.* intra- *vs.* inter-cycle). Several combinations of one or several of these drivers (*i.e.* several selection regimes) were compared for their sustainability while facing artificial populations differing for their resistance structure (*i.e.* fully susceptible *vs.* encompassing low frequencies of single resistance to either SDHIs or DMIs *vs.* composed of low frequencies of single resistance to either SDHIs

or DMIs and also to their combination) (Bank et al., 2014). Indeed, as sexual reproduction of *Z. tritici* was not possible *in vitro*, the introduction of multiple (*i.e.* SDHI+DMI) resistance in our population mimicked the recombination occurring naturally in field populations of *Z. tritici*, notably *via* sexual reproduction, and helped to establish the potential effect of such events (Fisher and Lang, 2016). Ancestral strains were first tested for their fitness and resistance pattern. Then, we analysed the quantitative performance of the various selection regimes while quantifying the growth dynamics of our populations as well as the variation in frequency of the introduced resistance alleles throughout the course of the experimental evolution. We also considered the qualitative performance of strategies by performing droplets tests describing the resistance phenotype profiles of evolved populations. This design allowed multi-criteria assessment of anti-resistance strategies.

It should be noted that at the time of writing this manuscript, and because of delay due to the Covid19 situation, some experiments were still running, preventing the analysis of the impact of some drivers. In this preliminary version, only the impact of the number of AIs (from 0 to 2) and two AIs (benzovindiflupyr and prothioconazole-desthio, respectively of SDHI and DMI MoAs) could be considered and presented. Nevertheless, the full experimental design is described in the M&M section, to allow assessing the relevance of this study. A full report of results should be available by the end of September 2021 and this article will be adapted for its submission to an international peer-reviewed journal in the wake.

On the basis of these preliminary results, we nevertheless validated the previous findings stating that heterogeneous selection pressure does limit emergence and selection of resistance, either in a naive population or a population with low frequency of single resistance. However, we also assessed that population structure does matter as the presence of a multiple mutant encompassed the sustainability of heterogeneous selection regimes.

## 2 Materials and methods

### 2.1 Biological material and its characterization

#### 2.1.1 Isolates and artificial ancestral populations

To study the durability of anti-resistances strategies on the selection phase (*i.e.* once resistance has already emerged), we artificially created different field populations by introducing at low frequency field mutations that determine resistance to DMIs or SDHIs. These populations were prepared while mixing the susceptible reference strain IPO323 strains (Goodwin et al., 2011) with two or three resistant. Those were semi-isogenic because isolated from the progeny between IPO323 and a

field resistant strain (G. Gazeau, unpublished; codes and phenotypes in Table 1). This choice minimized the bias of genotype background, when assessing fitness or resistance. The SDHI resistant isolate was characterized by the histidine (H) substitution with an arginine (R) at the codon 152 of the *sdhC* gene (H152R change). SdhC is one of the four subunits constituting the succinate dehydrogenase, the target of SDHIs. This amino-acid substitution was associated with high levels of SDHI resistance (Rehfus et al., 2018). The DMI resistant isolate exhibited a combination of five changes (V136A, A379G, I381V, Y461S and S524T) in the sterol 14 $\alpha$ -demethylase protein, encoded by *cyp51*. This genotype was named G31 in Garnault et al. (2019) and was associated with moderate-high levels of resistance to DMIs (Huf et al., 2018). The double mutant isolate displayed the same mutations in *sdhC* and in *cyp51* and was resistant to SDHIs and DMIs. These three resistant strains also inherited additional resistances from their field ancestor. They were resistant to benzimidazoles (inhibitors of the polymerization of  $\beta$ -tubulin) and the R\_SDHI\_DMI mutant was resistant to strobilurins (QoIs; QoIs inhibit the complex III of mitochondrial respiration at the Qo site of cytochrome b). We assumed here that these additional resistances would not affect the dynamics of resistance to SDHIs and DMIs, as no benzimidazoles and QoIs were included in selection regimes and as there is no cross-resistance between these four MoAs when target-site mutations occur. At last, the fitness of all isolates was measured (see details below), to detect any penalty putatively related to these additional resistances.

Table 1: Semi-isogenic isolates and derived ancestral populations used in the experimental evolution. Genotypes on *sdhC* and *cyp51* loci (respectively encoding the targets of SDHIs and DMIs) simplified as measured by pyrosequencing and qPCR. In addition, the three R isolates were also resistant to benzimidazoles. The R\_SDHI\_DMI isolate was resistant to QoIs.

Isolates	Genotypes	Phenotypes		Population composition		
		SDHI	DMI	PopS	PopR	PopRR
S	<i>sdhC</i> <sup>wt</sup> ; <i>cyp51</i> <sup>wt</sup>	S	S	100%	95%	95%
R_SDHI	<i>sdhC</i> <sup>H152R</sup> ; <i>cyp51</i> <sup>wt</sup>	R	S	-	2.50%	2%
R_DMI	<i>sdhC</i> <sup>wt</sup> ; <i>cyp51</i> <sup>I381V</sup>	S	R	-	2.50%	2%
R_SDHI_DMI	<i>sdhC</i> <sup>H152R</sup> ; <i>cyp51</i> <sup>I381V</sup>	R	R	-	-	1%

These semi-isogenic isolates were used to found three ancestral populations : i) a naive population named PopS, composed only of the susceptible strain S (IPO323), ii) a population, PopR, mostly composed of strain S but also of 5% simple mutants resistant to SDHIs (called R\_SDHI) or to DMIs (referred as R\_DMI) and lastly iii) a population, PopRR, similar to the previous one but with the addition of a multiple mutant (named R\_SDHI\_DMI) resistant to both SDHIs and DMIs, and with also final

proportion of 5% of mutants. This double mutant carrying both the SDHI and DMI resistance alleles was used to study the effect of recombination in our populations, as there was no sexual reproduction in the culture conditions of this experimental evolution.

To mimic the early phase of resistance selection, we aimed to set the initial resistance frequencies at the lowest detectable frequency that ensured acceptable repeatability of its measure. To this end, in a preliminary experiment, we prepared artificial populations of  $10^7$  spores in 50 mL Erlenmeyer flasks containing a range of resistant spores (0.1%, 0.5%, 1%, 2% and 5%) from the R\_SDHI\_DMI isolate, in addition to spores from the susceptible strain (S). For each ratio, three artificial populations were prepared. The experiment had also been repeated three times. The frequency of the resistant alleles was estimated as described below, by qPCR or pyrosequencing, depending on the alleles. We then calculated the experimental error, as

$$Error = \frac{Targeted\ ratio - Observed\ ratio}{Targeted\ ratio},$$

where the targeted ratio was the resistance frequency prepared experimentally for a given population, and the observed ratio, the resistance frequency measured by genetic analysis. The 1% ratio was found to be the smallest ratio exhibiting one of the lowest mean and standard deviation of the experimental error and constituted our detection limit. The initial frequency of resistance in PopR and PopRR artificial populations was similar and set at 5% total. As the R\_SDHI\_DMI mutant was expected to appear more scarcely in natural populations, we arbitrarily set its initial frequency at half that of the simple mutants. The initial structure of artificial populations is described in Table 1.

## 2.1.2 Fitness and resistance profiles of ancestral strains

We measured the fitness of the resistant strains and compared them to the fitness of the susceptible isolate in order to detect putative fitness penalties. Fitness was inferred for each strain from its growth in the culture conditions of the experimental evolution without fungicides. Population size was measured daily from OD<sub>405</sub> data for 7 days. This experiment was repeated five times. The growth curve of each strain was modelled using a mixed non-linear logistic model, described by

$$\text{Spore concentration} = \text{Asym} / (1 + \exp((xmid - t) / sca)),$$

where  $t$  is the day,  $Asym$  the asymptote, *i.e.* the maximum growth,  $xmid$  the time needed to halve the value of the asymptote and  $sca$  the inverse of the slope. The strains were considered as a fixed effect and the repetitions as a random effect. We compared the logistic parameters between strains using the Tukey method for pairwise comparisons. Estimations were performed in the R version 4.1.0 using the packages NLME, CAR and EMMEANS.



We established the resistance profile of each strain by estimating their  $EC_{50}$ s and resistance factors (RFs) for a wide range of fungicides.  $EC_{50}$ s were computed from dose-response curves in microtiter plates established for pure cultures of each isolate, as described in Part II. RFs were estimated as the ratio between the  $EC_{50}$  the resistant isolate and of the susceptible one. RFs were calculated for three SDHIs (benzovindiflupyr B, boscalid Bo and fluxapyroxad F), four DMIs (prothioconazole-desthio P, metconazole Me, tebuconazole Te and mefentrifluconazole M), a QoI (azoxystrobin A), a benzimidazole (carbendazim Ca), a multisite inhibitor (chlorothalonil C) and tolnaftate T (inhibitor of squalene epoxidase within sterol biosynthesis, used in medicine and marker of multidrug resistance). Patterns of positive cross-resistance within SDHIs or DMIs are recognized in field isolates of *Z. tritici*.

## 2.2 Selection regimes

We studied how multiple selection regimes differed at delaying the selection of resistance, depending on their components.

### 2.2.1 Sources of selection heterogeneity

Starting from the three possible ancestral populations, we tested 14 different selection regimes that differed in four components (31 lines in total; Table 2):

- **Number of AIs.** We hypothesized that selection regimes including more AIs will be more efficient to delay resistance selection. The number of AIs varied between one (straight selection) and two or three. In some regimes, the third AI was a multisite inhibitor, added to two unisite ones.  
**Resistance risk of AIs.** We expected selection regimes displaying the AIs with the lowest risks of resistance to be more efficient to delay resistance selection. We selected AIs representative of their MoAs among those currently used to control STB in the field. MoAs, and then their representative AIs, differed in their contrasting risk of resistance. They were described either at medium to high risk (SDHIs, represented by benzovindiflupyr (B) and fluxapyroxad (F)), at low to medium risk (DMIs, represented by prothioconazole-desthio (P) and mefentrifluconazole (M)) or at very low risk of resistance (multisite inhibitor, represented by chlorothalonil (C)).

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Table 2: Selection regimes and their respective components and codes.

Population tested	Number of AIs		Intrinsic resistance risk of AI					Temporal heterogeneity		AI diversity		Name of the selection regime	Experimental batch
	Number of fungicides	Includes a multisite inhibitor	High/Medium (SDHI)		Medium/Low (DMI)		Very low (multisite)	Intra-cycle (mixture)	Inter-cycle (alternation)	Intra-MoA	Inter-MoA		
			Benzovindiflupyr (B)	Fluxapyroxad (F)	Prothioconazole-desthio (P)	Mefentrifluconazole (M)							
PopS	0											PopS_Ethanol	A
PopS	2		X		X			X			X	PopS_Mixt_PB	A
PopS	2		X		X				X		X	PopS_Alt_PB	A
PopR	0											PopR_Ethanol	A/B
PopR	0											PopR_DMSO	B
PopR	1		X									PopR_B	A
PopR	1				X							PopR_P	A
PopR	2		X	X				X		X		PopR_Mixt_BF	B
PopR	2		X	X					X	X		PopR_Alt_BF	B
PopR	2				X	X		X		X		PopR_Mixt_MP	B
PopR	2				X	X			X	X		PopR_Alt_MP	B
PopR	2		X		X			X			X	PopR_Mixt_PB	A
PopR	2		X		X				X		X	PopR_Alt_PB	A
PopR	2			X		X		X			X	PopR_Mixt_MF	B
PopR	2			X		X			X		X	PopR_Alt_MF	B
PopR	3	X	X		X		X	X			X	PopR_Mixt_BPC	B
PopR	3	X	X		X		X	X	X		X	PopR_Alt_BPC	B
PopRR	0											PopRR_Ethanol	A/B
PopRR	0											PopRR_DMSO	B
PopRR	1		X									PopRR_B	A
PopRR	1				X							PopRR_P	A
PopRR	2		X	X				X		X		PopRR_Mixt_BF	B
PopRR	2		X	X					X	X		PopRR_Alt_BF	B
PopRR	2				X	X		X		X		PopRR_Mixt_MP	B
PopRR	2				X	X			X	X		PopRR_Alt_MP	B
PopRR	2		X		X			X			X	PopRR_Mixt_PB	A
PopRR	2		X		X				X		X	PopRR_Alt_PB	A
PopRR	2			X		X		X			X	PopRR_Mixt_MF	B
PopRR	2			X		X			X		X	PopRR_Alt_MF	B
PopRR	3	X	X		X		X	X			X	PopRR_Mixt_BPC	B
PopRR	3	X	X		X		X	X	X		X	PopRR_Alt_BPC	B

- **AIs diversity.** We assumed that increasing the number of AIs might be of limited interest if those exhibit positive cross-resistance (*e.g.* if they share a similar MoA). We then compared intra-MoA and inter-MoA diversity of the AIs, assuming that inter-MoA diversity would be more efficient to delay resistance than intra-MoA diversity.
- **Temporal heterogeneity.** We studied whether the timing of application of AIs over the successive pathogen generations affected the performance of the strategy. We then compared simultaneous application of AIs (intra-cycle heterogeneity, *i.e.* mixture) with sequential application of AIs (inter-cycle heterogeneity, *i.e.* alternation). For this last option, AI changed every cycle, as short alternation rhythm was found efficient in a previous work (Ballu et al, 2021a). Alternation regimes all started with the AI with the lowest resistance risk (or highest intrinsic activity for intra-MoA regimes), since it was demonstrated more efficient (Elderfield et al., 2018; van den Bosch and Gilligan, 2008). We assumed that the efficacy of regimes undergoing inter-cycle temporal heterogeneity would be enhanced if a resistance cost applies to any of the isolates.

Because of the important size of this experimental design, all lines could not be conducted in a single experiment. Therefore, selection regimes were distributed into two batches (A and B; Table 2) that were carried out successively. In particular, regimes whose direct comparison was of special interest were carried out in the same batch. Control lines were repeated in the two batches if necessary.

Fungicides were used as technical products and purchased at Sigma Aldrich (US). They were dissolved into ethanol 80%, except chlorothalonil, which was dissolved in DMSO because of its lower solubility. Ethanol 80% and DMSO solvent control lines were added to the experiment. Stock solutions of fungicides were prepared at the beginning of the experiment and kept at 4°C throughout. Fungicides and solvents added to culture medium never exceeded 0.5% of the final volume to avoid any toxicity on the growth of *Z. tritici*.

## 2.2.2 Doses of selection

The selection doses of fungicides used alone (in sequence or alternation lines) were defined as their  $EC_{97}$  for the susceptible strain S, grown in conditions of the experimental evolution (described below).  $EC_{97}$ s were established from dose-response curves for each fungicide considering a logistical regression model (see Part II), with the growth of the populations inferred from  $OD_{450}$  measurement after 7 days normalized by that of the control line grown without fungicide (Table 3). Validation of this dose of selection included the cultivation of the S isolate in the conditions of the experimental evolution at this theoretical  $EC_{97}$  and at close doses. The lowest dose providing at least 97% inhibition was finally defined as the selection dose.

The selection doses of two-way fungicide mixtures were set as half the selection dose of each AI when used alone, in order to compare alternation and mixture regimes receiving the same global fungicide quantity. The only three-way mixture contained  $\frac{1}{2}$  EC<sub>97</sub> of chlorothalonil,  $\frac{1}{4}$  EC<sub>97</sub> of benzovindiflupyr and  $\frac{1}{4}$  EC<sub>97</sub> of prothioconazole-desthio.

Table 3: Selection doses used in experimental evolution.

mg.L <sup>-1</sup>	Benzovindiflupyr	Fluxapyroxad	Prothioconazole-desthio	Mefentrifluconazole	Chlorothalonil
Used alone	0.7	0.7	0.005	0.002	5.5
Used in 2-way mixtures	0.35	0.35	0.0025	0.001	-
Used in three-way mixtures	0.175	-	0.00125	-	2.75

## 2.3 Design of experimental evolution

### 2.3.1 Ancestral populations

$10^7$  spores of each strain (S, R\_SDHI, R\_DMI, R\_SDHI\_DMI) were inoculated into 25 mL of YPD (Yeast extract Peptone Dextrose, Broth Traditional Formulation with Peptone powder from USBiological, composed of 20 g/L of dextrose, 20 g/L of peptone and 10g/L of Yeast Extract) medium amended with 0.25% of antibiotics (100 mg.L<sup>-1</sup> streptomycin and penicillin) in 50 mL Erlenmeyer flasks. Pre-cultures were incubated for seven days in the dark at 18°C, 70% RH and stirred at 150 rpm. Then we artificially created the population PopR and PopRR from the pre-cultures of each strain. To limit variability due to small pipetting, the populations were prepared in high volumes (15 mL) before the inoculation of  $10^7$  spores into the Erlenmeyers flasks. The 26 different selection situations (14 selection regimes \* 1-3 populations) and the 7 controls (in A and B batches) were repeated as three independent lines each. Control solvent lines received 0.5% of solvent (either ethanol 80% or DMSO), whereas treated lines received the fungicide treatment as 0.5% of their final volume, according to the selection regimes described in Table 2. 99 independent lines were finally carried out (26 selection situations and 7 controls = 33\*3).

## 2.3.2 Serial transfer experimental evolution

Absorbance at 405 nm (OD<sub>450</sub>) was measured from two wells filled with 200 µL of each fungal culture in a 96-well microtiter plate, at the end of the first and subsequent cycles. OD<sub>450</sub> was used to determine the spore concentration of each line (expressed in sp.mL<sup>-1</sup>; see Part II for more details).

To start a new cycle, 500 µL of an evolving line were transferred to a new Erlenmeyer flask filled with fresh medium. If this 500 µL transfer was not providing 10<sup>7</sup> spores, the new medium was complemented until reaching the threshold with spores from the control (PopS) or with mixtures of spores reconstituting PopR or PopRR from the pure cultures of the different isolates. Every cycle then started with the same population size of 10<sup>7</sup> spores and was kept as previously described.

The experimental evolution was conducted on a basis of cycles of seven days each. If the normalized Malthusian growth (defined below) of a line reached at least 95% during three consecutive cycles, then the line could be ended. In that case, to ensure fair comparison between alternation and mixture lines, the lines were stopped only after an even number of cycles (*i.e.* at the soonest, at the 4<sup>th</sup> cycle). It is worth noting that the experiment had originally been carried out on 9 cycles. A power failure occurring at the 7<sup>th</sup> cycle led us to focus our analysis of population dynamics on the six first cycles. We considered that our qualitative analyse of evolution, based on phenotyping tests performed at the 9<sup>th</sup> cycle (see § 2.4.3) may still provide relevant insights in the evaluation of anti-resistances strategies until we repeat this test with fossil samples from the 6<sup>th</sup> cycle.

Finally, a 2 mL aliquot of each line was sampled at the end of each cycle and kept at -80°C with glycerol 25% as cryoprotectant, for further analyses, or in case of a technical problem. Two samples of about 200 µL were also kept in microtiter plates for further DNA extraction.

## 2.4 Measurement of the performance of strategies

### 2.4.1 Growth rate of evolved lines

From the measured OD<sub>405</sub> and the corresponding spore concentration, we calculated  $M_i^n$ , the mean normalized Malthusian growth for each line  $i$ . This was the mean over all cycles of the Malthusian growth normalized by the Malthusian growth of the solvent control line:  $M_{it}^n$ , computed as  $M_{it}^n = \frac{M_{it}}{M_{Ct}}$ , with  $C$  the solvent control line. The Malthusian growth was calculated as follows:

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

We used a beta regression (package BETAREG for R) to investigate the effects of the founding populations and the selection regimes, included as fixed factors, on the  $M_i^n$  of each line. We compared

regimes and ancestral populations using the Tukey adjustment method for pairwise comparisons (package EMMEANS).

## 2.4.2 Molecular quantification of resistance alleles in evolved lines

We evaluated the selection regimes with respect to the evolution of the frequencies of resistance alleles initially introduced. The DNA of samples stored in microtiter plates were extracted with the DNeasy® Plant Kit (Qiagen), using the manufacturer procedure.

The *sdhC*<sup>H152R</sup> allele was quantified by Syngenta in samples using a qPCR procedure. Allele-specific primers were designed with the goal to amplify only this allele by discriminating all other alleles recorded in the field. The allele-specific forward primer CGT TGA ATG GAG TGA GGC A and the reverse primer TGT ACC ATC TCT CTT CAT CCT C amplified the product of the wild-type allele H152, while the allele-specific forward primer GTT GAA TGG AGT GAG GCG used with the same reverse primer amplified the product of the mutant allele R152. The unspecific forward primer TCG TTG AAT GGA GTG AGG C was used to estimate the concentrations of the reference plasmids (wildtype, mutant). The reactions were performed with FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) on a Bio-Rad CFX-384 machine. The PCR was run under the following conditions: initial denaturation at 95°C for 10 min, 40 cycles at 95°C for 15 sec, 58°C for 30 sec and 75°C for 30 sec (measurement of fluorescence). After the PCR, a melt curve was recorded from 65°C to 95°C. The detection threshold was estimated at 1%.

The *cyp5*<sup>1381V</sup> allele, included in the G31 genotype of the DMI-resistant isolate, was quantified by Syngenta using a pyrosequencing procedure. Primers for the pyrosequencing assays were designed with the PyroMark Assay Design 2.0 software (Qiagen). A 216 bp fragment containing the 381 codon was amplified with the primer pair CCC GAC ATC CAA GAC GAA C and Biotin-TGG AAT GAC GTA TGC CGT ACC. The PCR ran under the following conditions: initial denaturation at 95°C for 2 min, 50 cycles at 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec and final elongation at 72°C for 5 min (GoTaq® Hot Start Polymerase, Promega, Madison, WI). 20 µL of these PCR products were prepared for pyrosequencing reactions using the PyroMark Q96 Vacuum Workstation (Qiagen) and Streptavidin Sepharose High Performance Beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as described in the instructions of the workstation and in the PyroMark Q96 ID User Manual. The single-stranded DNA templates were transferred to 40 µL annealing buffer (Qiagen) containing the sequencing primer. The primer used in the reaction was ACC CTT CGT ATT CAC G [0.4 µM] for sequencing the codon 381 mutation site. The sequencing run was set up by PyroMark-Software v.1.0 (Qiagen). After incubation of samples at 80° C for 2 min and equilibration to room temperature, the

sequencing reaction was performed with PyroMark Gold Q96 reagents on a PyroMark Q96 ID machine (both from Qiagen). Dispensation order of the nucleotides was AGC GTC AGA TCA. The detection threshold was estimated at 5% for the I381V change in preliminary experiments.

From qPCR and pyrosequencing data, we calculated the normalized concentration of resistant mutants R\_SDHI and R\_DMI in the population as the proportion of the resistant alleles *sdhC*<sup>H152R</sup> and *cyp51*<sup>I381V</sup>, respectively, multiplied by the size of the population (in spores.mL<sup>-1</sup>). We divided it by the concentration of spores of the control lines to obtain the normalized frequencies of resistance (hereafter normalized alleles frequency). qPCR and pyrosequencing did not allow to distinguish combinations of *sdhC*<sup>H152R</sup> and *cyp51*<sup>I381V</sup> within the same individual (*i.e.* as in the R\_SDHI\_DMI isolate) from the co-occurrence of the same alleles in several individuals (*i.e.* as in the R\_SDHI and R\_DMI isolate). Therefore, we calculated the maximum possible concentration of this double mutant as the common proportion of both resistant alleles. This calculation may overestimate the concentration of the R\_SDHI\_DMI isolate in PopRR and was then interpreted cautiously.

A quasi-binomial generalized model was fitted to determine how the normalized allele frequency of *sdhC*<sup>H152R</sup> and *cyp51*<sup>I381V</sup>, as well as the maximum potential frequency of the multiple mutant R\_SDHI\_DMI, observed from the global concentrations of each line, varied according to funding populations and selection regimes set as fixed factors. Pairwise comparisons were adjusted using the Tukey method implemented in the EMMEANS R package.

As previously mentioned, some lines were stopped after three consecutive cycles exhibiting population size at least 95% of the one of the control. To provide proper comparisons between the selection regimes, we extrapolated population size and alleles frequencies from the “generalized” phase to complete the missing cycles and carried out the analyses previously described. It was the case for the regimes of sequence of benzovindiflupyr alone and prothioconazole-desthio alone for PopR and PopRR (*i.e.* lines PopR\_B, PopR\_P, PopRR\_B, PopRR\_P for the 5<sup>th</sup> and 6<sup>th</sup> cycles)

### 2.4.3 Phenotype of evolved lines

At the end of the experiment, we conducted droplet tests to describe the phenotype resistance profile of each line. Lines were tested for their susceptibility to the selecting fungicides used alone at their selection dose (EC<sub>97</sub> of the susceptible strain), to mixtures at the dose used in the experiment (*e.g.* half of selection doses of their component) and to mixtures composed of components at their full selection doses. Some fungicides not used in selection regimes were also tested, *e.g.* boscalid (Bo), carbendazim (Ca) and azoxystrobin (A). Finally, tolnaftate (T) was tested to detect

multidrug resistance caused by enhanced fungicide efflux (Table 4). We systematically added the ancestral strains (S, R\_SDHI, R\_DMI and R\_SDHI\_DMI) as controls.

Table 4: Doses of the fungicide used to perform droplet tests. Modalities used during the experimental evolution are noticed with an asterisk.

Test fungicide modality	Fungicide class	Dose mg.L <sup>-1</sup>
B*	SDHI	0.7
P*	DMI	0.005
½(BP)*	SDHI+DMI	0.35+0.0025
BP	SDHI+DMI	0.7+0.005
Bo	SDHI	2
Az	QoI	0.5
To	SBI class IV	1.5

Drops of different concentrations of spores ( $10^7$ ,  $10^6$ ,  $10^5$  and  $10^4$  spores.mL<sup>-1</sup>) of each line were deposited on solid YPD medium in square 9 cm side Petri dishes amended with 0.5% of test fungicides (or solvent for controls). The resistance of populations was scored from 0 (no growth, *i.e.* susceptible population) to 4 (*i.e.* resistant population, with score assigned according to the number of droplets at which growth was observed).

The distribution of the phenotype resistance profiles between and within selection regimes were represented as heatmaps. The Euclidean pairwise distance was used for the hierarchical clustering of these profiles, with dendrograms for the rows and columns, performed in the R version 4.1.0.

## 3 Results

### 3.1 Phenotypic characterisation of ancestral isolates

#### 3.1.1 Resistance patterns of the ancestral isolates are in agreement with their respective genotypes

The S isolate was susceptible to all fungicides, as expressed by the low EC<sub>50</sub>s observed for each MoA. These results also reveal the within- and between-MoA differences in intrinsic activity (Table 5). Boscalid was the weakest SDHI to control *Z. tritici*, while mefentrifluconazole was the most efficient DMI. A comparatively low intrinsic activity can be noticed for chlorothalonil, which is expected for contact multisite fungicides measured *in vitro*.



The three resistant isolates exhibited a high RF towards the benzimidazole carbendim, as expected from their genetic background. The isolate R\_SDHI carrying the *sdhC*<sup>H152R</sup> allele was associated with high RFs to all tested SDHIs, suggesting positive cross-resistance, as expected from literature (Dooley et al., 2016b; Yamashita and Fraaije, 2018). R\_SDHI was susceptible to the four DMIs, the QoI azoxystrobin, the multisite chlorothalonil and also to tolnaftate (all RFs < 1.2), which likely implies the absence of multidrug resistance. By contrast, the R\_DMI isolate carrying the *cyp51*<sup>I381V</sup> allele (within the G31 genotype) displayed resistance to all tested DMIs (RFs > 10) but not to SDHIs (low RFs) (Huf et al., 2018). DMI cross resistances was established between the four DMIs but affected AIs differently, a lower RF being noticed for mefentrifluconazole as previously observed (Ishii et al., 2021). This strain was also susceptible to all the other AIs tested except carbendazim. Finally, the R\_SDHI\_DMI isolate-carrying both *sdhC*<sup>H152R</sup> and *cyp51*<sup>I381V</sup> resistance alleles presented medium to high RFs to the SDHIs, DMIs, benzimidazoles and QoIs, once again consistent with its expected recombinant phenotype and original background. As the other resistant isolates, R\_SDHI\_DMI was also susceptible to the multisite chlorothalonil and to tolnaftate. Even though the three resistant isolates were semi-isogenic and carried the same mutations associated to SDHI and DMI resistances, we noticed some variation between RFs of the simple and the double mutants. This may be attributed to the measurement method and will be repeated. Overall, this experiment validated the expected phenotypes of our strains.

Table 5: Resistance patterns to multiple fungicides of ancestral isolates. EC<sub>50</sub>s and RFs calculated after dose-response curves established in microtiter plates. 4 repeats per isolate and AI. Dose-response curves were repeated between 2 to 4 times depending on the AI. ND: not determined due to experimental issues.

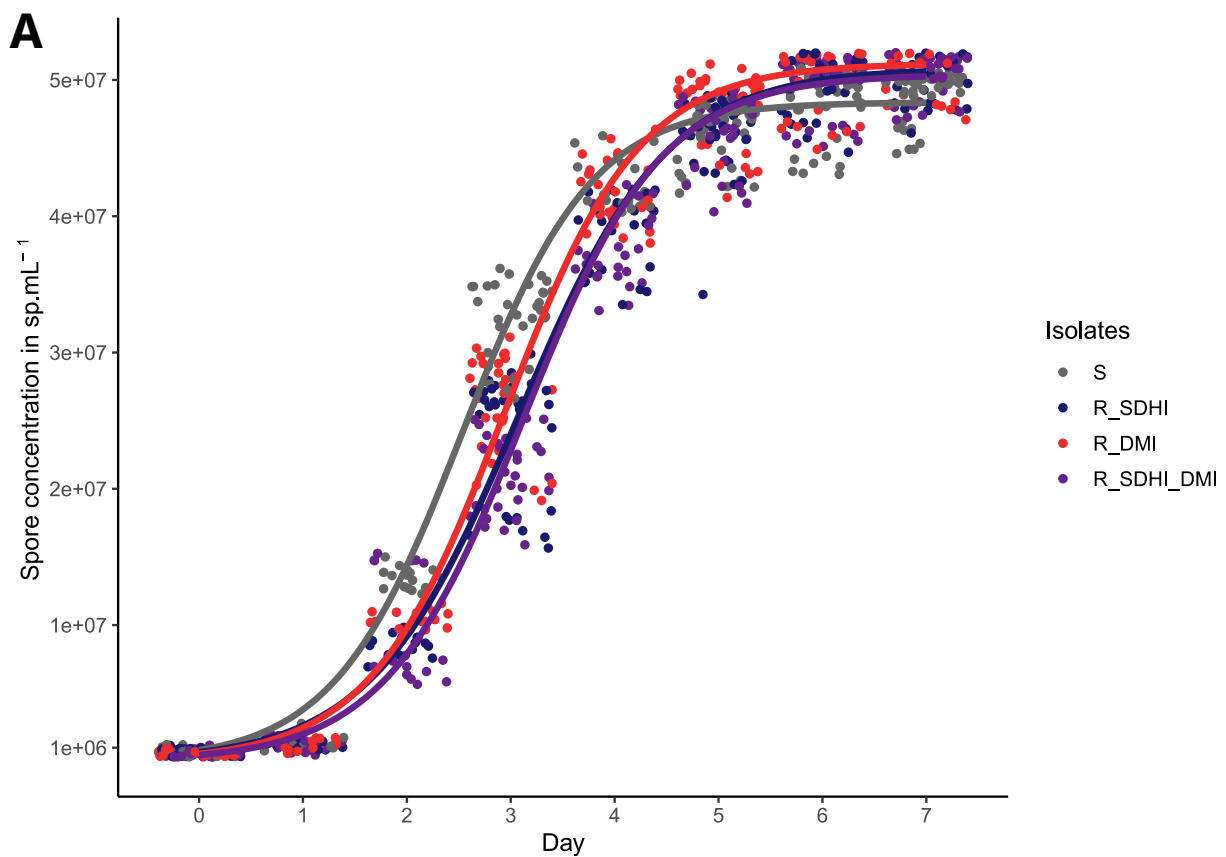
Chemical class	Fungicide	EC <sub>50</sub> (mg.L <sup>-1</sup> )		Resistance factor (RF)	
		S	R_SDHI	R_DMI	R_SDHI_DMI
SDHIs	Benzovindiflupyr	0.044	58.4	2.4	156
	Boscalid	0.262	169	1.5	ND
	Fluxapyroxad	0.051	> 1000	3.1	> 1000
DMIs (SBI class I)	Mefentrifluconazole	0.0008	1.2	10.9	ND
	Metconazole	0.0038	1.1	22.8	8.4
	Prothioconazole-desthio	0.0016	0.7	25.6	41.1
	Tebuconazole	0.0076	1.0	40.2	17.2
Benzimidazoles	Carbendazim	0.111	>450	>450	>450
QoIs	Azoxystrobin	0.061	0.6	0.5	353
Multisites	Chlorothalonil	1.18	0.4	0.7	0.8
SBI class IV	Tolnaftate	0.353	ND	0.7	0.3

### 3.1.2 A weak and early penalty entails the fitness of the resistant isolates in our experimental conditions

The growth curves of strains cultivated in the absence of fungicide selection are shown in Figure , as modelled with a logistic regression.

The four isolates were compared for three parameters of the logistic model (Figure 1). The time necessary to reach half of the maximum spore concentration,  $xmid$ , was significantly lower for S (2.5 days) than for the resistant mutants, either simple or multiple (between 2.94 and 3.08 days), and especially for the double mutant R\_SDHI\_DMI whose  $xmid$  was significantly higher than the one of R\_DMI. The increase in growth rate during the exponential phase was higher for S strains, since the inverse slope,  $scal$  of S was the lowest and was significantly lower than R\_SDHI (equal to 88% of its value). The maximum growth represented by  $Asym$ , the asymptote, was significantly higher for the resistant mutants than for the susceptible isolate ( $Asym$  of S about equal to 95% of the mutant ones). Consequently, our experiments suggested low fitness cost of the resistant mutants in our experimental conditions, preferentially expressed during the first days of the cycle. Nevertheless, the growth of the resistant isolates caught up with that of the susceptible isolate and significantly exceeded it by the end of the experiment. It is then difficult to predict if such penalty might affect the evolution of resistant isolates when competing with the reference isolate, in the absence of selection pressure.

The weak and early resistance cost observed here for the resistant isolates is not fully consistent with previous observations. The  $sdhC^{H152R}$  allele carried by the R\_SDHI and R\_SDHI\_DMI isolates has previously been associated to a strong fitness cost expressed in *in vitro* cultures and competition experiments. This change is supposed to lower the activity of the SDH enzyme (Gutiérrez-Alonso et al., 2017; Scalliet et al., 2012). We confirmed this penalty only for the early stages of the growth dynamics. Similarly, the  $cyp51^{I381V}$  allele, carried by the R\_DMI and R\_SDHI\_DMI isolates, has been associated with a reduced enzyme activity but restored by the change Y461H, and then potentially by Y461S, carried by our DMI resistant strains (Hawkins and Fraaije, 2018). This may explain why we observed only a weak and early penalty on the growth of these isolates. We assumed that resistance to benzimidazoles (*i.e.* the E198A change affecting  $\beta$ -tubulin), exhibited by all resistant mutants, did not biased fitness measurement, as fitness penalty was never observed in *Z. tritici*, nor in other species (*e.g.* in *M. fructicola*) (Chen et al., 2014; Hawkins and Fraaije, 2018). Resistance to QoIs (*i.e.* the G143A change affecting cytochrome b) was found to reduce the virulence of *Z. tritici* and the growth reduction of some other pathogens (Hawkins and Fraaije, 2018), but we extrapolate that this cost might not express under artificial culture conditions.



**B**

Isolates	xmid			scal			Asym		
	mean	SE	group	mean	SE	group	mean	SE	group
S	2.54	0.05	a	0.62	0.02	a	4.84E+07	4.37E+05	a
R_SDHI	3.08	0.05	bc	0.71	0.02	b	5.08E+07	4.43E+05	b
R_DMI	2.94	0.05	b	0.65	0.02	ab	5.12E+07	4.40E+05	b
R_SDHI_DMI	3.13	0.05	c	0.67	0.02	ab	5.04E+07	4.42E+05	b

Figure 1: Fitness of ancestral isolates in the conditions of the experimental evolution; A. Growth curves of the four isolates used in the experimental evolution. Logistic regression. Experiment repeated 5 times. S (susceptible) in grey; R\_SDHI (resistant to SDHI) in blue; R\_DMI (resistant to DMI) in red and R\_SDHI\_DMI (resistant to both SDHI and DMI) in purple. Growth was measured as a spore concentration ( $\text{sp.mL}^{-1}$ ) increase over time (7 days), without fungicide, at 18°C, 150 rpm, in the dark. B. Estimation of parameters of logistic non-linear model of growth curves for ancestral isolates. Pairwise comparisons were adjusted with the Tukey method for multiple comparisons. Statistical differences ( $P < 0.05$ ) were indicated by different group letters.

### 3.2 Impact of the heterogeneity of the selection on the performance of strategies.

This section includes results on selection regimes used in batch A only. Lines evolving under selection regimes of batch B were still running or just finished at the time when this manuscript was written.

### 3.2.1 Increasing the number of AIs delays the evolution of resistance differently in ancestral populations

The growth in control lines (without selection pressure) reached  $4.9 \times 10^7$  sp.mL<sup>-1</sup> (about 125 times the initial concentration) for each cycle, independently from the composition of the ancestral population. The quantification of resistance alleles remained below the detection limit for all cycles and populations (Figure 2).

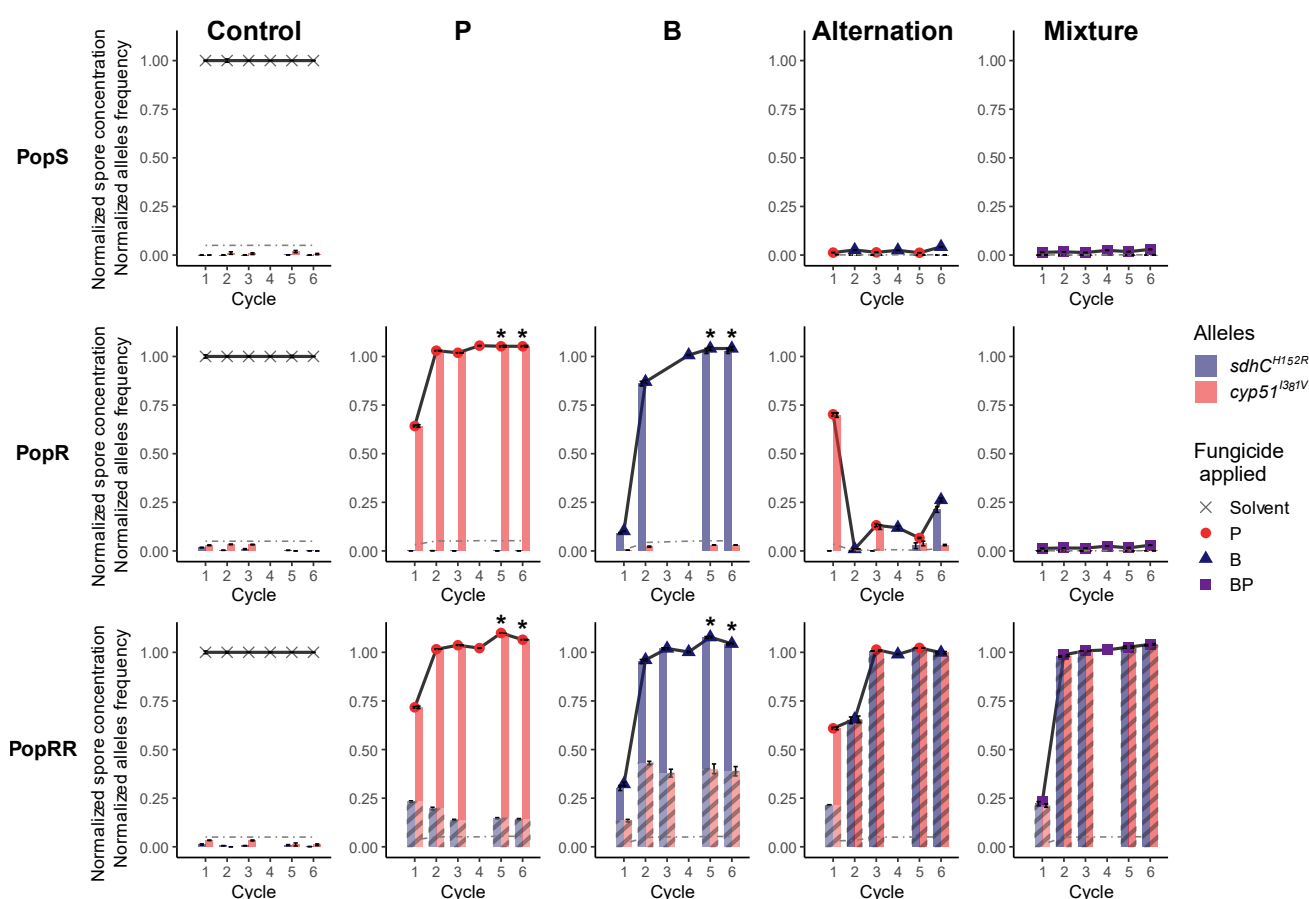


Figure 2: Evolution of normalized growth and resistance allele frequency in experimental evolution. The initial populations, as detailed in Table 1, are represented in rows: PopS susceptible population, PopR: includes initially 5% of single resistant isolates; PopRR: includes initially 5% of single or multiple resistant isolates. The selection regimes are represented in columns, as detailed in Table 2. Black lines represent the mean normalized spore concentration at the end of each cycle calculated from 4 repeated lines evolving under the same selection regime. Normalization was achieved according to growth measured in control lines containing only solvent. Symbols details which fungicides were used for each cycle (cross: solvent only; dot: prothioconazole-desthio; triangle: benzovindiflupyr; square: mixture of both). Histograms represent the proportion of resistant alleles normalized according to frequencies of the same alleles measured in control lines: blue and red histograms represent alleles providing resistance to SDHIs (*sdhC*<sup>H152R</sup>) and to DMIs (*cyp51*<sup>I381V</sup>), respectively. Grey stripes on histograms from lines evolving from the ancestral population PopRR represent the maximal possible frequency of the combined genotype *sdhC*<sup>H152R</sup> + *cyp51*<sup>I381V</sup>, providing resistance to SDHIs and DMIs. Asterisks indicate that the spore concentration and alleles frequencies were extrapolated from previous cycles. Absence of some histogram bars (e.g. in the 4th cycle) indicate missing data in allele frequency. B: benzovindiflupyr (SDHI); P: prothioconazole-desthio (DMI).

Unidirectional selection regimes (*i.e.* one AI, B or P, used continuously) were tested only in presence of resistant isolates in the ancestral population (PopR and PopRR). The mean Malthusian growth was hardly decreased by B or P alone and ranged between 90% to 99% of the one of the control, depending on the ancestral population (Figure 3). The selection of both resistant alleles was very strong, since the frequency of *cyp51*<sup>H152R</sup> and of *sdhC*<sup>H152R</sup> rose up to 100% and 86-95% after only two cycles of exposure to P and B, respectively (Figure 2).

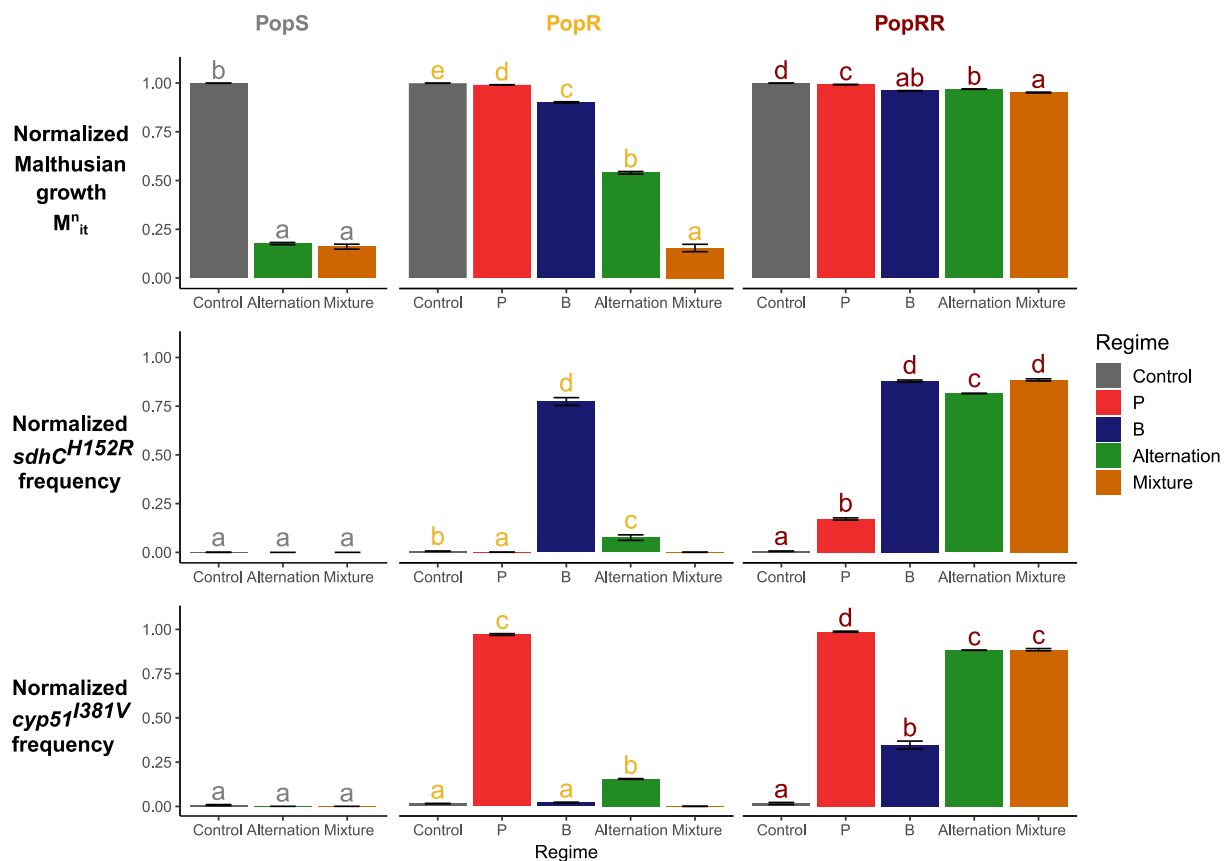


Figure 3: Normalized Malthusian growth  $M_{it}^n$  (top) and normalized frequencies of SDHI (middle) and DMI (bottom) resistance alleles averaged over 6 cycles of experimental evolution. The ancestral populations are presented in columns with from left to right, PopS susceptible population, PopR: includes initially 2.5% of each single resistant isolate; PopRR: includes initially 2% of single or 1% of multiple resistant isolates. Statistical differences between regimes within a given population are indicated with coloured letters.

Yet, in the PopRR ancestral population, P and B preferentially selected their associated single resistant genotypes rather than the double resistant genotype, as the maximal frequency of the combined genotype always stayed below the one of the single genotypes (*i.e.* 12-26% under P direct selection and 20-45% under B direct selection) (Figures 2 and 4). The lower frequencies of the double resistant genotype comparatively to the single resistant genotypes could be explained by its half-lower initial proportion, but also by a possible fitness penalty, as already suggested in Figure 1. This later as-

sumption is also suggested by the decrease of the frequency of the double resistant genotype observed over the 6 cycles and will be tested with the whole dataset when the experiment will be finished while quantifying the *cytb* G143A change (uniquely associated to R\_SDHI\_DMI).

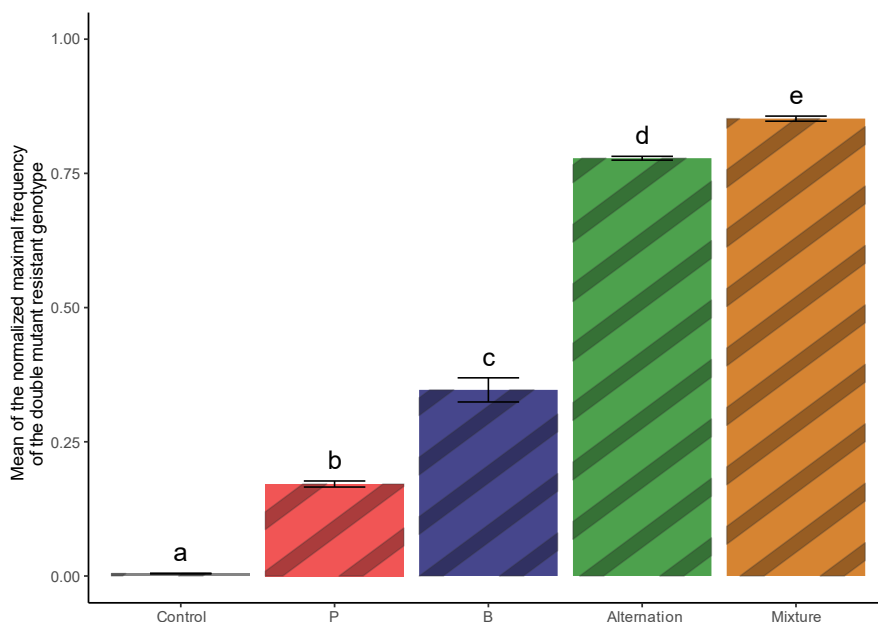


Figure 4: Mean of the normalized maximal frequency of the double resistant genotype in PopRR lines. For each selection regime, the mean over 6 cycles of the maximal common frequency of both *sdhC*<sup>H152R</sup> and *cyp51*<sup>I381V</sup> resistance alleles. Statistical differences between regimes are indicated with letters.

Heterogenous selection regimes (*i.e.* including two AIs, in alternation or mixture) were more efficient at delaying the emergence of resistance in the naive PopS lines and the selection of the resistance alleles in PopR lines, comparatively to unidirectional selection regimes (Figures 2 and 3). The Malthusian growths of S populations exposed to BP alternation and mixture reached 16% and 18 % of those of control ones, respectively. Reduced growth suggests that resistance did not emerge, and in particular, the two quantified resistant alleles *cyp51*<sup>I381V</sup> and of *sdhC*<sup>H152R</sup> were never detected in these lines. In PopR lines, the mean Malthusian population growth reached 54% and 15% of the ones of control lines, in alternation and mixture regimes, respectively, which was still significantly lower than growth in regimes of continuous selection. The two single resistant genotypes initially introduced were poorly selected and represented in average 16% and 4% of the population, for *cyp51*<sup>I381V</sup> and of *sdhC*<sup>H152R</sup>, respectively. Nevertheless, more variability was observed between cycles under alternation compared to under mixture. By contrast, the Malthusian growth was only very slightly decreased, reaching 95%-97% of that of the control lines, in PopRR populations. It was significantly lower than the ones obtained under continuous exposure to P but not to B. Both *cyp51*<sup>I381V</sup> and *sdhC*<sup>H152R</sup> alleles were quickly generalized, suggesting the rapid selection of the double mutant only (Figure 4). The

mean concentrations of the resistance alleles were often lower for heterogeneous than for homogeneous selection pressure which suggest a lower fitness of the R\_SDHI\_DMI compared with R\_DMI and to lesser extent to R\_SDHI. The comparison between alternation and mixture will be discussed later.

### 3.2.2 Modulating the intrinsic risk of AI is of limited interest when resistance has emerged in populations

AIs used in a strategy may drive the evolution of resistance, depending on their relative intrinsic resistance risk and diversity (Part III). The relative fitness of the selected isolates may also influence the outcome of the selection and therefore balance the importance of AIs. On this first batch of experimental evolution, we could only study the effect of AI diversity, using benzovindiflupyr or prothioconazole-desthio representing two MoAs, *i.e.* SDHIs and DMIs, respectively.

The AI little affected the dynamics of resistance. In PopR and PopRR lines, the *sdhC*<sup>H152R</sup> and *cyp51*<sup>I381V</sup> alleles were rapidly and similarly generalized in response to the continuous exposure of B or P, reaching at least a normalized proportion of 95% after two cycles. However, the mean Malthusian growth was significantly lower in response to the sequence of B rather P in both populations but were still quite high, *i.e.* 90-95% *vs.* 99%, which anyway do not imply a strong benefit due to the AI (Figure 3). This slight difference in population dynamics might confirm a lower fitness of R\_SDHI relatively to R\_DMI, which was consistent with the dose-response curves measured without selection (Figure 1), but which was not sufficient to imply a strong discrepancy in resistance growth rate. Besides, in PopRR, the maximum frequency of R\_SDHI\_DMI was lower in the P sequence regime than in the B sequence regime (17% *vs.* 34%, respectively). In the first cycles we noticed a decrease of *sdhC*<sup>H152R</sup> normalized frequency during the sequence of P (Figure 2). As R\_SDHI wouldn't be fit in an environment with prothioconazole-desthio, it suggests a decrease of R\_SDHI\_DMI in favour of R\_DMI. A fitness disadvantage of R\_SDHI\_DMI compared to R\_DMI when P was applied, would explain this evolution of frequency and is consistent with previous statements without fungicide pressure. *cyp51*<sup>I381V</sup> did not decrease as much in presence of B, which advocated a comparable fitness of R\_SDHI\_DMI and R\_SDHI in such environment.

To conclude, although the frequency variation of resistant alleles is consistent with their relative fitness as described previously, these fitness differences observed in our conditions were not sufficient to strongly influence the course of resistance selection under continuous exposure of fungicides. When resistance was initially present in a population, its selection occurred rapidly, and similarly for

the two tested AIs, possibly because they were used at equivalent efficient doses ( $EC_{97}$ ). After emergence, the performance of sequence strategies may then highly rely on the dose of the selecting fungicide, pending some modulation due to the fitness of the selected resistant individuals.

### 3.2.3 Mixture and alternation display similar poor efficacy on multiple resistant genotypes

The temporality of the selection pressure was compared in alternation and mixture selection regimes. In PopS, where no resistance was introduced, both alternation and mixtures of B and P were similarly and strongly efficient at controlling fungal growth (Figure 2). We assumed that resistance did not emerge, at least after the selection of the *cyp5*<sup>T381V</sup> and *sdhC*<sup>H152R</sup> alleles, as their frequencies both remained under the detection threshold. However, when resistances were originally present in the population, some distinction could be made between intra- and inter-cycle temporal heterogeneity. When only simple mutants were present (PopR), both strategies significantly reduced fungal growth but at different extents. Indeed, the Malthusian growth and resistance allele frequencies were higher in alternation ( $M_{it}^n = 54\%$  of that of the control) compared to mixture regimes ( $M_{it}^n = 15\%$  of that of the control) (Figure 3). In alternation, a given genotype is selected when its associated AI is applied and is counter-selected at the following cycle and conversely for the other mutant. On the contrary, in mixture, the co-occurrence of both AIs established a non-advantageous environment for the two mutants. Similarly, a significant but very weak advantage was observed for mixtures by contrast to alternation regimes (95% and 97% of the normalized Malthusian growth, respectively; Figure 3). This limited interest is consistent with the more intense selection of the double resistant genotype in mixture compared to alternation (*e.g.* mean frequency of *sdhC*<sup>H152R</sup> at 81% and 88%, respectively Figure 3).

### 3.2.4 Final population structure is mainly driven by its ancestral composition and secondly by selection regimes

To understand whether additional resistance mechanisms, different from the introduced *cyp5*<sup>T381V</sup> and *sdhC*<sup>H152R</sup> alleles, were selected in the experimental evolution, and to assess whether the final phenotypes of evolved lines differed according to the selection regimes, we systematically established the phenotype of all lines at the end of the experiment. The growth of lines was observed in droplet tests in medium amended with the fungicides used for the selection, their mixture but also with fungicides representing modes of action unused for the selection.



## Resistance was counter-selected in control lines

PopS lines, which evolved under mixture or alternation of B and P, stayed susceptible at the end of the experiment (as identical to the S isolate), *i.e.* no resistance was selected whatever the selection regime (Figure 5A). This is consistent with previous studies (Parts III and IV) that identified mixture and alternation with benzovindiflupyr and prothioconazole-desthio as efficient anti-resistance strategies for a naive population.

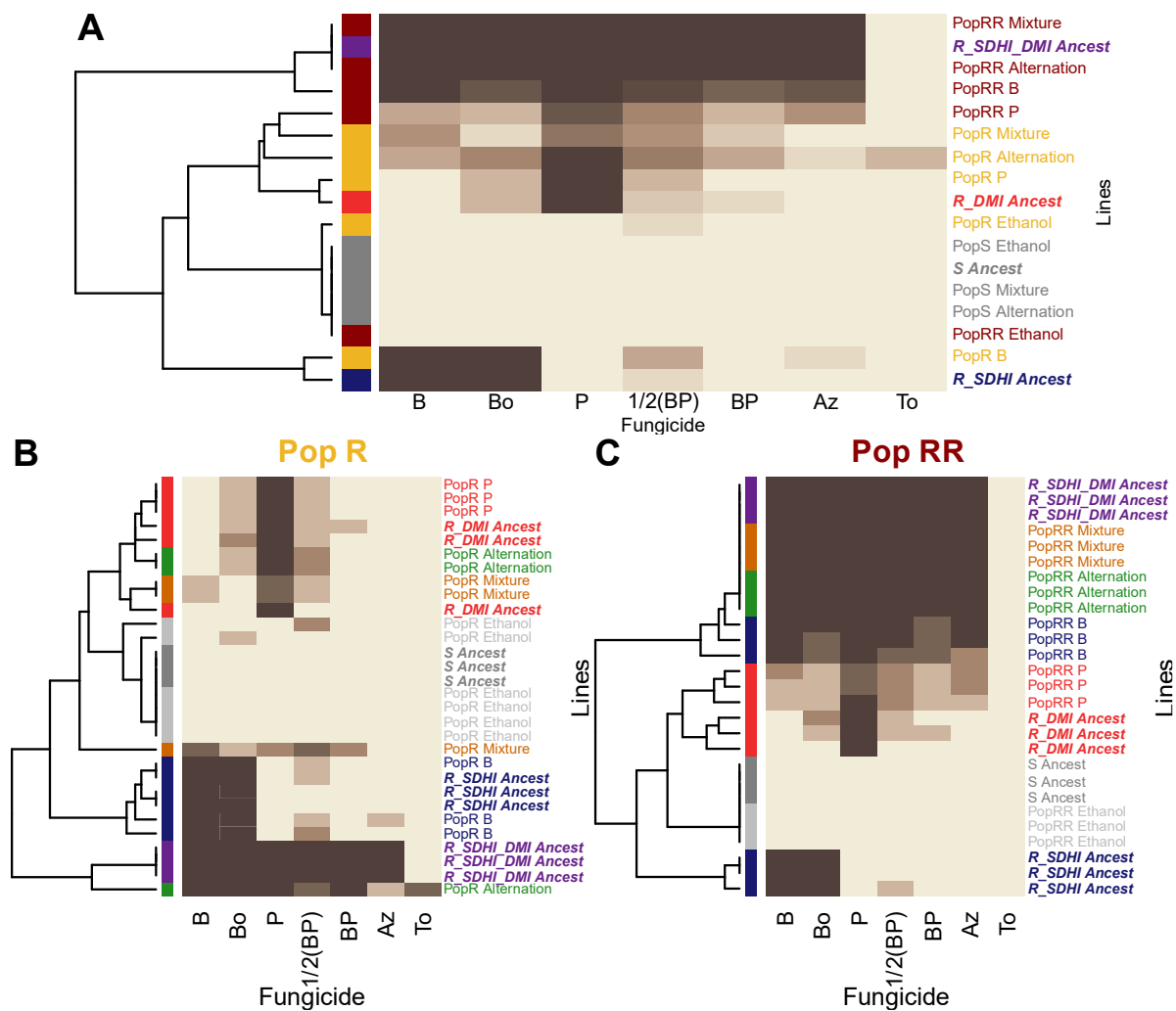


Figure 5: Phenotypes of the evolved population established in droplet tests. Ancestral strains used to found the initial populations were added in bold in the heatmaps. Populations evolved after nine cycles of selection were tested for resistance towards benzovindiflupyr (B), boscalid (Bo), prothioconazole-desthio (P), azoxystrobin (Az) and tolnaftate (To) at their respective  $EC_{97.5}$ , a mixture of half the  $EC_{97}$  of benzovindiflupyr and prothioconazole-desthio (1/2(BP)), a mixture of benzovindiflupyr and prothioconazole-desthio at their  $EC_{95}$  each (BP). The discriminatory doses were established for the susceptible strain (IPO323), otherwise stated. A. Mean of the scores for each line (population x regime) tested. B. Scores for PopR lines. C. Scores for PopRR lines.

Control lines derived from PopR and PopRR artificial populations, in which resistances were initially introduced, exhibited fully susceptible phenotype, *i.e.* similar to that of the S ancestral isolate but not of the R\_SDHI, R\_DMI or R\_SDHI\_DMI isolates (Figure 5A). We assumed that resistance was at too weak frequency to be detectable in this test or that it was lost during the experiment. This last assumption is supported by the undetectable frequency of the resistance alleles at the end of the experiment (Figure 2). As the initial frequency of resistance alleles was initially low (1% to 2.5%), and in the absence of selection, their loss might result from their slightly altered fitness compared to strain S and/or by genetic drift, acting after the regular bottlenecks occurring between each cycle (*i.e.* a maximum of 2% of the population is transferred to the next cycle).

*The homogeneous use of fungicides exclusively drove the selection of introduced resistance alleles.*

The phenotypes of PopR lines exposed to the continuous selection of B or P were similar to those of the ancestral introduced isolates R\_SDHI or R\_DMI, respectively (Figure 5). This was consistent with allele quantification (Figure 2). Indeed, lines exposed to P only (PopR\_P) homogeneously exhibited a strong resistance against P, and weak resistance against boscalid (not expected as boscalid is a SDHI) and the mixture BP. Those resistances were already described for the ancestral R\_DMI isolate (*i.e.* the only one resistant to DMIs and introduced in PopR) bearing the *cyp51<sup>I381V</sup>* change, although more irregularly. Its RFs for P and boscalid were 25 and 1.5, respectively (Figure ). Symmetrically, the final phenotype of PopR lines continuously exposed to B (PopR\_B) were similar to the one of the R\_SDHI ancestral isolate, *i.e.* high resistance towards the two SDHIs B and boscalid (RF  $\approx$  58 and 169) and a low resistance to the BP mixture (Figure ; Figure 5). These findings are consistent with the intense selection of the *sdhC<sup>H152R</sup>* allele only. The weak resistance to azoxystrobin observed in only one PopR\_B line is either an artifact or a hint of the additional selection of a generalist resistance mechanism, as it was not observed from the ancestral strains.

The PopRR lines exposed to either B or P selection regimes were finally resistant towards B and P, their mixtures at half and full doses, boscalid, as well as on azoxystrobin. This was consistent with the co-selection of the three resistant alleles and/or the preferential selection of the double resistant allele (as carried by R\_SDHI\_DMI). This last assumption is supported by resistance frequencies (Figure 2) at the end of the experiment and by the maintenance of the azoxystrobin resistance, exclusively carried out by the double resistant strain. The quantification of the G143A change in lines might have

contributed to conclude about the scenario of selection. No hint for the selection of additional resistance selection (*i.e.* growth on tolnaftate) was recorded.

### *The heterogeneous use of fungicides diversified the evolutionary trajectories toward resistance*

Previous work from parts III and IV demonstrated that mixture and alternation may favour the emergence and selection of generalist isolates. Here we tested whether this phenomenon would also occur when specialist and multiple resistance were already present in the population.

At the end of the experiment, all PopR lines exposed to BP mixture selection regime exhibited moderate resistance against the half-dose mixture but also against the single mixture components, B and P, at their full efficient dose (average resistance score of 2; Figure 5B). This is consistent with the maintenance of both resistance alleles, even at low frequency (Figure 2), the final phenotype of the population depending on the relative frequencies of each allele as regards to the frequency of the susceptible alleles. This might explain why resistance to boscalid (introduced with the *sdhC*<sup>H152R</sup> genotype) and to the full-dose BP mixture was detectable in only one out the three lines exposed to the BP mixture selection regime. Similar allele competition could be hypothesized in PopR lines exposed to alternation of B and P, with the frequency of a given allele increasing at the end of the cycle exposed to the associated AI (Figure 2). Indeed, two out the three PopR lines exposed to the alternation regime of B and P exhibited a resistance phenotype similar to that of the R\_DMI ancestral strain, probably because P was used at the last cycle. However, the last line exhibited an original and generalist resistance pattern, associated to medium to high resistance for all tested fungicides, and especially for azoxystrobin, unused in these selection regimes, and whose resistance was neither introduced in PopR, and for tolnaftate, indicating multidrug resistance. This suggested the selection of at least one non-target site resistance mechanism such as enhanced efflux, on any or both the R\_SDHI or R\_DMI backgrounds.

When single resistances were introduced, increasing the heterogeneity of the selection might drive the selection of the present genotypes, but also of new generalist ones, as they might be more adapted to multi-directional selection. Final populations may then encompass a greater diversity of genotypes than in lines exposed to direct selection, including specialist and generalist genotypes. By contrast, when a genotype enabling to survive to this multiple selection pressure, it is massively selected, as shown in PopRR lines from alternation and mixture regimes, poorly diversified, and not exhibiting hints of generalist resistance.

## 4 Discussion and perspectives

In this work, we aimed at validating that providing smart interplay of strategy components would maximize the heterogeneity of the selection pressure and then improve fungicide durability. We also questioned the capacity of the initial resistance status to mitigate this general principle. Then, using a still undergoing approach of experimental evolution, we preliminary compared selection regimes differing for the MoA of the two used AIs B and P, and for their pattern of application over time. We compared the qualitative and quantitative performance of these selection regimes after the evolution of a naive ancestral population, but also of populations encompassing either low frequencies of single resistances to B or P, or of single and multiple resistances to B and P. We demonstrated that the most heterogeneous strategies (*i.e.* mixture and alternation) were the most durable, but their efficiency is jeopardized as soon as multiple resistance is present in populations, even at low frequency. Finally, we confirmed that heterogeneous selection regimes may select additional generalist resistance even if specialist resistance is already present in populations.

### *The heterogeneous use of fungicides is sustainable in some situations.*

In this final experiment, we concluded that heterogeneous selection pressure provided by the mixture or the alternation of two AIs of different MoAs prevented the emergence of resistance in a naive population of *Z. tritici*, at least during the 6 cycles tested (*i.e.* roughly  $\approx 45$  generations in our conditions). This finding is in agreement with previous experiments conducted in parts III and IV of this PhD but also with modelling studies carried out for the same biological model. For example, target-site resistance to a high-risk fungicide took longer to emerge when this latter was mixed with a low-risk fungicide, compared to when it was used alone (Hobbelen et al., 2014).

To a certain extent, this conclusion also applies in some cases where resistance is already present. Indeed, populations of *Z. tritici* fully susceptible to all MoAs cannot be found any more in France, as well as in Western Europe. We therefore considered an ancestral artificial population were independent single target-site resistances to B (SDHI) or P (DMI) where introduced at low frequency and explored the relevance of our previous findings. In particular, we aimed at validating that heterogeneous selection pressure was still more sustainable than homogeneous selection in this situation. We found that the sequential use of a given fungicide massively selected the associated resistance allele, but that selection was still highly delayed in mixture and in alternation. Our results are consistent

with literature where empirical and modelling studies conclude that the sequential use of DMIs fungicides quickly selected the corresponding resistant isolates initially present in the population, resulting in a rapid loss of control which was delayed when DMIs were mixed with SDHIs or QoIs for example (Heick et al., 2017; Hobbelen et al., 2013; Mavroeidi and Shaw, 2006). Yet, we could not extend these results to situations where multiple resistance was present in the populations. This general finding was also described for several pathogens and can be supported by the trade-off constraint imposed by the first resistance to one MoA and the acquisition of resistance to a second MoA resistance (Kim et al., 2014; REX Consortium, 2013).

### *Does mixture really outperform alternation?*

Although we verified that mixture and alternation similarly prevented and delayed the evolution of resistance in a naïve population, we found that the B+P mixture was more efficient than the alternation of the same AIs in a population where both single resistances were present. The performance of alternation was also more variable from one cycle to another, displaying partial growth control. The discrepancy between alternation and mixture, or their respective efficacies, might also grow with the increase in initial frequency of resistance.

Mixtures are often (but not systematically) described as better anti-resistance strategy than alternation, in field as well as in modelling studies (van den Bosch et al., 2014a; REX Consortium, 2013). However, it is important to recall that the total dose of each AI may greatly vary among mixtures, and efficacy accordingly (Barzman et al., 2015). This may lead to inconsistent dose comparison with alternation. To study only the impact of temporal variation in selection, we used comparable total doses of fungicides in each selection strategy while using a full dose (*i.e.* equivalent to EC<sub>97</sub>) of each AI in alternation, and half-doses of each AI in mixture. Still, in these conditions, the relative efficacy of mixture might still be greater than that of sequences with the same AIs because of synergy. Then, if the efficacy of mixture relies on the synergy between AIs, it might vary with pairs and ratios of AIs and also be jeopardized by the increase of resistance frequency. In particular, multiple resistance might decrease synergy while decreasing the relative efficacies of each AI (Kosman and Cohen, 1996). This may explain why mixture might not be neither a satisfactory strategy in the long term once resistance has emerged.

### *Resistance status is critical to determine the performance of strategies*

A major finding in this preliminary work is that the efficacy of selection regimes depended first on the structure of the population, well before the effect of the strategy components that we

were able to test at this stage (*i.e.* the number of AIs, their diversity and the temporality of the selection pressure). Indeed, heterogeneous regimes of selection were as inefficient at decreasing resistance than those displaying continuous selection pressure when multiple resistance was present in the population, even at low initial frequency (1%). In particular, the double resistant genotype was probably massively selected in mixture as well as in alternation regimes, whereas the direct use of the same AIs selected preferentially the single resistant genotypes. This difference possibly operated because of the initial lower proportion but also of the slight fitness differences measured for the various resistant strains but might not exist in situations of similar fitness for all genotypes. Modelling also suggested that the time before loss of control of a strategy was decreased with the increase of the frequency of double resistance and that it was similar for mixture and alternation (Hobbelen et al., 2013). Consequently, our work suggests that the most important factor to consider for resistance evolution is the initial composition of the population, then the degree of heterogeneity in selection, as mostly determined in our experiment by the number of AIs temporality of the selection pressure. The AI diversity had finally relatively low importance as the two AIs induced similar growth rates of resistance in our conditions.

We also assumed in this experimental evolution that the weak and early fitness penalty of resistant isolates compared to the susceptible isolate might improve the efficacy of alternation strategies (*i.e.* exhibiting inter-cycle heterogeneity), while allowing this resistance cost to express between two applications with the selecting AI. Growth curves in absence of selection suggested the following ranking of the fitness  $w$  of tested isolates:  $w_S > w_{R\_DMI} > w_{R\_SDHI} \geq w_{R\_SDHI\_DMI}$ . The impact of fitness variation might have operated only in lines containing the single resistant genotypes, as the double resistant genotype could be selected indirectly (*i.e.* because of genetic hitchhiking) by any of the AIs. We observed strong efficacy of the alternation strategy but weaker than that of mixture. This first suggests that the measured fitness was not relevant (*e.g.* because it is too weak or because it was not expressed in the conditions of this competition experiment). Indeed, fitness and selection coefficients are often more properly assessed *in natura* (Giraud et al., 2017). Here, the S isolate was laboratory-adapted, while resistant isolates derived from a cross with a field strain and were only semi-isogenic, which may have led to differences among strains independently from resistance, even if all cultures have been conducted similarly (Garland and Rose, 2009). Second, the rhythm of alternation (*i.e.* the temporal variation) might have been insufficient to allow the counter-selection of the resistance alleles. Third, fitness penalty might have contributed to the efficacy of alternation, but mixture was anyway more efficient because of the unfair comparison between the strategies, as mentioned be-

fore. To explore this question further, it might be useful to use fully isogenic isolates and allow unbiased comparison between strategies. We could also use isolates more contrasted for their fitness in our conditions or genotypes widely distributed in French populations (*e.g.* the *sdhC*<sup>T79N</sup> or *sdhC*<sup>W80S</sup> (Hawkins et al., 2018; Rehfus et al., 2018). Similarly, complexifying the ancestral artificial populations while introducing conjointly diverse strains responsible for a given resistance in a population may be interesting as well to confirm whether clonal interference slow down resistance fixation due to competition between the advantaged strains (McDonald, 2019).

#### *Towards a better consideration of generalism in resistance management*

The dynamics of resistance to fungicides is quite often the only criterion used to compare anti-resistances strategies and case studies generally consider the evolution of resistance to a high-risk fungicide only, determined by TSR. However, resistance dynamics might be much more complex, as recalled Heick et al. (2017) for DMIs. This underlines the fact that selection regimes might also influence the diversity and the relative frequencies of selected genotypes and pleads for the qualitative assessment of their performance.

The systematic analysis of the phenotype resistance profiles of evolved strains at the end of the experimental evolution, *via* droplets tests, confirmed that selection of the introduced genotypes mostly occurred. Growth variation between the drops exhibiting serial spore dilutions potentially highlighted the variation in frequency of these selected genotypes, depending on the selection regimes. However, in PopR lines undergoing selection of B+P, we noticed growth on the mixture of these two fungicides. As only single resistant genotypes were introduced and as recombination is unlikely in our artificial system, this observation suggested the co-selection of the two genotypes. Yet, we cannot fully exclude the selection of a resistance on background resistant to the other MoA, and conversely. We can neither exclude the selection of tolerance to one or the two MoA. Tolerance is well known in the medical field. It is a transient state of lower susceptibility, usually associated to low to high RFs but not genetically supported (Berman and Krysan, 2020). Additionally, we observed resistance to toltaftate, suggesting the selection of the generalist mechanism of enhanced efflux, in one line exposed to the alternation of B and P. This additional mechanism of resistance can either have been selected in the S background independently from pre-existing TSR or could have been combined with introduced TSR, as in field populations of *Z. tritici* (Cools and Fraaije, 2013; INRAE, Anses, Arvalis - Institut du Végétal, 2021; Leroux and Walker, 2011).

Heterogeneous environments have been described to enhance the genetic diversity of populations (Bell and Reboud, 1996; Kassen, 2002) and to favour the evolution of more generalist resistance (Parts

III and IV). Indeed, while preventing the evolution of specialist resistance, alternation and mixture may also favour the selection of generalist phenotypes by shaping original mutational landscapes (Comont et al., 2020; Kim et al., 2014; Lagator et al., 2013b, 2013a; Vogwill et al., 2012). This trade-off was confirmed again in this experiment, despite the initial introduction of resistance. This trade-off is also poorly considered in the field. However, high frequency of generalist individuals in population might narrow the number efficient AIs even more than TSR. In weeds or insects, NTSR is generally combined with TSR and associated to high RFs as well as unexpected resistance patterns (Gaines et al., 2020; Mitchell et al., 2014).

### *Perspectives*

Even if experimental evolution simplifies environmental conditions and therefore consciously desert some biotic and abiotic factors, it proved a very useful conceptual tool for comparative studies, testing many hypotheses concerning the evolutionary dynamics of resistance and disentangling its main drivers (Bank et al., 2014; Cowen, 2001). Experimental evolution including field isolate-based populations informed directly on the potential evolution of current genotypes under multiple anti-resistance strategies, either actual or innovative.

At the time of writing this manuscript, not all data were available, and several drivers of selection could not be analysed. In the near future, we expect to draw final conclusion about their respective impact. In particular, we expect intra-MoA diversity to be of limited interest on TSR, because of cross-resistance (Dooley et al., 2016c; Hagerty et al., 2020; Heick et al., 2017). Therefore, we assume that neither the alternation and mixture of fluxapyroxad and benzovindiflupyr nor of mefentrifluconazole and prothioconazole-desthio would limit the selection of their associated genotypes or would sufficiently control populations. We will also further prospect the impact of AIs intrinsic resistance risk and activity while introducing fluxapyroxad instead of benzovindiflupyr and mefentrifluconazole instead of prothioconazole-desthio. To increase heterogeneity as much as possible in our design, we also introduced a third MoA, as represented by the multisite inhibitor chlorothalonil, which is not concerned by resistance in any of the strains introduced in this experiment. Chlorothalonil is of lower a low intrinsic activity compared to the unisite inhibitors used in this study, but we expect that at its  $EC_{97}$ , it will control both population growth and resistance selection, jointly with its unisite partners. When multiple resistance is introduced, the efficacy of a strategy should rely on the third partner, and then on its intrinsic risk of resistance and on its relative efficacy. In such scenario, we assume that the efficacy of alternation would fluctuate from one cycle to another, depending on chlorothalonil use, while mixture should provide smaller but more regular control of fungal growth, because



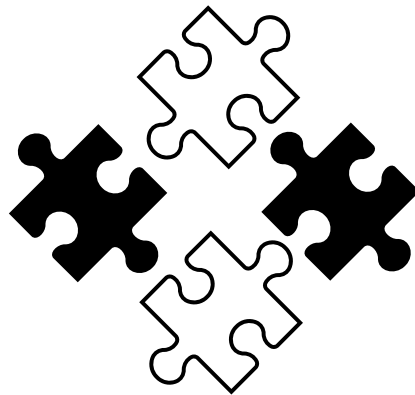
of the dose reduction of each AI. From an educational point of view, introducing a chlorothalonil illustrates the benefit of AIs not concerned by resistance to improve the efficacy of anti-resistance strategies. This selected example could be extrapolated to soon-to-be-registered (*e.g.* QiIs, metyltetraprole), to explore their inner risk in relation to their current partners already concerned by resistance.

### *Concluding remarks*

Our experimental evolution confirmed the importance of maximizing the heterogeneity of the selection pressure to limit the emergence and selection of resistance evolution, while confronting our previous findings to more realistic situations, *i.e.* to populations containing field-representative resistant isolates. It also highlighted that focussing strategies on fitness penalty might be hazardous, not to mention compensatory mutations that may ultimately reduce the interest of resistance costs (Lenormand et al., 2018). Finally, this work stressed the importance of considering the trade-off between the quantitative and qualitative components of the performance of strategies, as well as the status of resistance in populations, recombination being an aggravating factor of resistance evolution. In this sense, this study is a step forward sound tailoring management of resistance. In field situations where MoAs are used sequentially because of their successive discoveries and withdrawals, as a result of resistance or regulation, new AIs are often combined with more ancient ones, *i.e.* they are never confronted to the ideal situation where all their partners are still fully efficient. Considering the whole of our findings, and because fungal recombination cannot be prevented, this situation argues in favour of preventing resistance emergence rather than selection, *e.g.* while using new AIs as solo treatments but at maximal dose made possible by toxicity and registration, and while preferring non-chemical control measures.

## **Part VI.**

# **General discussion and conclusion**



# General discussion and conclusion

## Main findings

This PhD work aimed at enhancing the performance of anti-resistance strategies by characterizing the drivers of their optimization and at determining the effect of the initial state of resistance on that performance. This was achieved while using experimental evolution (EE), as a promising tool to bring new insights to the debate on the relative performance of anti-resistance strategies, and while using the economically important plant pathogen *Zymoseptoria tritici*. From three comprehensive experiments, we concluded that:

### → Fungicide alternation on a naive population (part III):

- was not a risky strategy when considering the dynamics of resistance: even in the worst case, resistance did not evolve faster under the alternation regime than under the continuous exposure to any of its components. This beneficial or neutral effect of alternation on resistance evolution was not only related to the spreading of selection pressure over longer periods.
- enhanced the selection of generalist phenotypes.
- revealed the choice of AIs, characterized by their intrinsic risk of resistance, as the main driver affecting resistance evolution. The effect of the number of AIs was not consistent as it finally depended on the AIs involved, while a short alternation rhythm had a very light positive effect.
- resulted in a trade-off between the rate of resistance evolution and the level of generalism of evolved isolates, that depended mostly on AIs choice. This trade-off was dynamic and varied over time.

### → Mixtures on a naive population (part IV):

- When designed at efficient-dose, mixtures revealed either a beneficial, a neutral or even a deleterious effect on resistance evolution compared with the sequential use of single AIs. Mixture at efficient-dose is not always a safe anti-resistance strategy.
- Performance of mixtures was principally dependent on the AIs combined, *i.e.* on their intrinsic risk of resistance. Incorporating a high resistance risk AI into a mixture may shorten the durability of its mixture partner(s).
- Reducing the dose of single AIs and mixtures may favour generalist or multiple resistant phenotypes. This pattern observed with efficient-dose mixtures is partly

explained by the reduced doses of each mixture ingredient.

→ Increasing the degree of heterogeneity in selection pressure on naive and resistant populations (part V):

- Heterogeneous selection limited the emergence and selection of resistance, even if resistances to all MoAs applied are initially present as independent genotypes, at a low frequency. By contrast, homogeneous selection intensively selected introduced resistances.
- Heterogeneous selection regimes selected additional generalist resistance mechanisms.
- Both homogeneous and heterogeneous selection regimes performed poorly when a multiple resistant genotype is initially present. Population resistance status does mitigate the efficiency of strategies.
- To avoid recombination and the potential apparition of multiple mutants, it is important to tailor strategies according to local populations and implement them as soon as possible.

## **Experimental evolution as a configurable and relevant tool to study anti-resistance strategies.**

Experimental evolution was found a convenient tool to study evolutionary trajectories in microorganisms (Remigi et al., 2019). In particular, it allows for the rigorous comparison of contrasting selection pressures by simplifying natural environments, and thus the interaction with background factors, and by allowing evolutionary parameters to be easily modified. Indeed, by adapting a serial transfer protocol to our scientific questions, our EE studies provided both quantitative and qualitative insights on the adaptation to fungicides on multiple and independent lineages of a model fungal species, *Z. tritici*, and on a relative short-time scale. Specifically, our experimental design made it possible to easily vary the selection pressures induced by fungicide applications and the initial structure of the ancestral population, which was explored in our EE. I will further discuss the associated limitations. This approach could also be used to study the impact of immigration on adaptation. This option was not studied here as it was fixed after preliminary experiments, which will also be justified below.

## Modulating the selection pressure in experimental evolution

Several components of the selection pressure were studied in our EE and their respective performances for delaying resistance were compared. Selection pressure differed in selection regimes for several reasons.

In the field, the registered dose of a fungicide is the one providing an acceptable and regular control of the disease (*i.e.* generally the MIC validated in field situations), with some security anticipating AI degradation and losses due to the conditions of application. This “field dose” is then a compromise between its efficacy against the disease and its safety for the users and the environment. To mimic this “field dose”, and assuming that low fungicide degradation and loss occurred in our system, we set similar intensity of the selection pressure in our EEs while establishing the minimum dose providing a good control of the population (*i.e.* ranging between the EC<sub>90S</sub> and EC<sub>97S</sub> of each AI, depending on the EE). The intensity of the selection was thus comparable between all the selection regimes within the experiments, allowing the effect of other sources of heterogeneity to be studied in an unbiased manner. Nonetheless, we also studied the effect of modulating the intensity of selection by reducing the doses applied. For example, we used mixtures at their efficient dose but also at half-efficient doses of mixture components alone, both assessed qualitatively and quantitatively, which remains original in literature on agricultural fungicides and extrapolates field practices aiming at decreasing pesticide load. As already studied in literature, we confirmed biologically that dose reduction impacted the resistance dynamics of *Z. tritici* (van den Bosch et al., 2011; Mikaberidze et al., 2017). We also noted that none of the resistant phenotypes selected with a full dose of a single AI was similar to those selected with reduced doses of the same AI, which could imply different resistance mechanisms. Dose splitting (*i.e.* the partitioning of the dose in several applications) has not been explored because of a lack of time but is another current practice in the field. Dose splitting is similar to dose reduction but with an increased application frequency over the cycles. We assume that because of dose reduction and by spreading selection pressure over longer period without the interruption from other AIs, dose splitting is likely to increase the rate of selection (van den Bosch et al., 2014a).

Selection pressure also varied qualitatively in our experiments, as induced by using several MoAs and several AIs within the same MoA. MoAs were chosen to represent contrasted intrinsic risks of resistance and our observations roughly ranked MoAs as predicted by the FRAC. Partial discrepancies observed between some parts of this PhD probably result from the use of different doses for the same AI. The intrinsic risk of resistance was repeatedly found a major driver, if not THE major driver

of resistance evolution throughout our experiments. Indeed, this so-called risk in literature is inherent to the fungicide MoA. It can hardly be manipulated and therefore is mostly a hazard (Brent and Hollomon, 2007b; Grimmer et al., 2014b). We observed at multiple occasions that the risk of using hazardous fungicides could be mitigated while decreasing their exposure, by comparison to their direct use, for example while mixing or alternating them with other AIs or while reducing their dose (van den Bosch et al., 2011; Heick et al., 2017; Hobbelen et al., 2013). The relative contribution of AIs to the overall selection pressure is one of the keys to direct evolution, towards enhanced or tainted performance of a strategy, whether one considers mixture, alternation or dose reduction. In that respect, we found that complex strategies including hazardous fungicides were less efficient than the repeated use of less hazardous AIs. With an educational perspective, we also investigated if increasing AI diversity while using AIs of the same MoA would be sufficient to delay the build-up of resistance. These results are still in progress, but preliminary observations validate that strategies based on AIs displaying cross-resistance (because they share the same MoA or a generalist resistance mechanism) are not sustainable, as already mentioned in literature (Hagerty et al., 2020; Heick et al., 2017).

Selection pressure may also vary according to time in our EE, *i.e.* exposure to a single AIs was either concomitant (*e.g.* in mixture strategies) or independent (*e.g.* in alternation or dose reduction strategies) from those of the other AIs. We tested whether this driver interplayed with some other factors (*e.g.* frequency of exposure change; number of AIs) and have not noticed strong interaction. We could neither close the debate on mixture systematically outperforming alternation since other factors have to be considered (see the next section) but again we noticed the strong impact of the relative contribution of hazardous AIs when directing selection, whether in a mixture or in an alternation. We nevertheless noticed that both alternation and mixture implied the selection of generalist phenotypes, probably not because of the variation in the exposure to each AI, but rather because of the *de facto* multiplicity of the AIs that are used to design these strategies.

At last, in addition to the multiple variations of mixture, alternation or dose modulation strategies that could have been explored in our experiments (difficult choices had to be decided!), the selection pressure may also vary for reasons that have not been explored in our three EEs. For example, the impact of population migration, as underlying the mosaic strategy, could be tested while immigrating spores between lineages undergoing different selection regimes. This original approach might be relevant for further experiments, as mosaic is hardly considered to manage resistance in arable crops but is a key strategy elsewhere (REX Consortium, 2013).

Then, while many environments and selection pressures can be tested and compared, the findings of EE might be cautiously examined, as they might not be directly transposable to field situations. Indeed, several differences may question the relevance of EE to mirror resistance evolution *in natura*. Despite the comparable intensity of the selection pressure set up in each EE and applied in the field, selection pressure was homogeneous in dose and over time (or slightly heterogeneous if we consider some fungicide decay over the 7-day exposure of a population) in our Erlenmeyer flasks. All the individuals of 6-7 generations were equivalently exposed to the same selection pressure within a cycle. On the contrary, in the field, population size is much bigger, submitted to emigration and immigration. Fungicide decay is increased by environmental conditions, inducing dose variation. Some individuals may escape treatments (*via* untreated reservoirs or plant organs) and only a few generations within *Z. tritici* life cycle are finally exposed to fungicide sprays, assuming 1-2 treatments per season in France. The selection is thus heterogeneous in time, space and dose over the generations, even in the case of the sequential use of the same AI. In addition, we tested simplified population structures (*e.g.* fully susceptible in parts III and IV), whereas the structure of field populations reflects historical and multiple selection pressures, is much more complex in terms of resistance, not considering neutral variation, and hence should have contrasted effects on resistance evolution. In addition, more different adaptive trajectories might be found where populations are spatially structured (Ally et al., 2014; Zhang et al., 2011), which is the case for *Z. tritici* to some extent (Garnault et al., 2019; Hellin et al., 2020; Huf et al., 2018; Jørgensen et al., 2020; Rehfus et al., 2018). This additional diversity associated to the spatial structure of resistance is supposed to slow down the fixation of beneficial alleles but would be advantageous in fluctuating environments. The additive variance or the initial number of alleles potentially selected displays contrasting effect on short- or long-term, depending on if the population is spatially homogeneous or subdivided and if the selection pressure is continuous or not (Caballero and García-Dorado, 2013). Finally, it has been suggested that local adaptation might promote the co-existence of generalists and specialists in a population, then influencing not only resistance dynamics but also the phenotypes selected (Kröner et al., 2017). Altogether, the "local adaptation" potentially occurring in the field and in EE may differ because the patterns of heterogeneous selection pressure we studied might just differ over the life cycle of the pathogen, despite our effort to mimic field phenomena. We also omitted in the simplified laboratory environment of our EEs the potential interaction with the other selection pressures, biotic or abiotic, present *in natura*, which may bias the evolutionary trajectory of the selected alleles in EE, depending on their fitness in both environments. Consequently, non-synonymous mutations may be overrepresented in EE as natural selection exerts differently, but purifying selection should still be occurring

following the selection pressures applied in EE (Remigi et al., 2019). Another discrepancy between EE and field evolution lies in the timescale transfer. The analysis of samples collected throughout the course of EEs define snapshots of the result of selection pressure generally established at what is more or less objectively considered to be the “end” of the evolution. By contrast, selection in natural conditions is dynamic, the relative impact of the sources of heterogeneity varying with time, because of the variation of population structure. Here, we tempted to integrate the dynamic dimension of the selection pressure while considering critical time points in our first experiment, which is rarely provided in literature. In general, more dynamic comparisons between EE and field evolution might be necessary to adjust our understanding of selection and to enhance the predictive power of EE.

To conclude, at least until we can reproduce field evolution more faithfully in EE, the interest of this approach does not reside in its direct application to the field but in its theoretical perspectives. Indeed, with an appropriate experimental design, we were able to analyse accurately and without confounding effects the relative impact of multiple selection components as well as their interplay, which is crucial for those who want to tailor strategies in an informed way. This high number of comparisons could have never been achieved in field conditions and in such a short time. The produced dataset might be also of great interest for further exploration *via* mathematical modelling. In terms of predictability regarding field evolution, these experiments might be considered to mimic the “worst-case scenario” as in-field complexity might have mitigated the performance of strategies. This is anyway highly useful for stakeholders from an educational perspective and also raises awareness about prevention and early implementation of strategies.

## The influence of the structure of the ancestral population in experimental evolution

Controlling the initial structure of ancestral populations in EE offers at least two major interests. First, using ancestral naive populations makes it possible to explore the type of variants that emerge and is selected “spontaneously”, without trade-off occurring because of the combination with already present resistance alleles. Their evolutionary trajectories (*i.e.* time to emergence, fitness landscape, resistance growth rate...) can also be explored more easily. This is a major benefit because fully fungicide-susceptible populations cannot be found anymore in the field in France. Such studies are interesting to study the evolution of resistance to AIs exhibiting original MoAs. The comparison with the evolution of resistant strains allows predicting the emergence of new resistances when combined with current resistant alleles. Nevertheless, it is important to recall that population size is far



smaller in EE designs than *in natura*. Therefore, the probability that a *de novo* mutation responsible for resistance would emerge is higher in field populations, simply because the mutation load is greater (Kawecki et al., 2012). Therefore, emergence time cannot be directly transferred from EE to natural conditions. However, the emergence time can be compared between the different selection regimes of a same EE. In parts III and IV, we used a clonal ancestral population of the susceptible isolate IPO323, instead of using a variable population (*e.g.* a composite population or one made variable after UV or chemical exposure). The phenotype variability observed at the end of our experiments was therefore the result of selection regimes only and was not influenced by the initial genetic variability. Since adaptation values variability, evolution towards resistance might have been quicker if some genetic variation had been initially present. However, it might have been difficult to disentangle the initial variability from the selected one, especially since genetic variation is generally higher in populations undergoing heterogeneous selection pressures, whereas variation is more efficiently eliminated by homogeneous directional selection (Bell and Rebound, 1996).

Secondly, while introducing resistant alleles at low frequency, the emergence phase is skipped. The impact of selection drivers on the selection phase only (*i.e.* when resistance alleles are introduced) or on the whole dynamics (*i.e.* when starting with a fully susceptible population) can then be compared. In part V, we introduced field-resistant genotypes to study the impact of strategies on the selection phase only. This choice was relevant because the implementation of anti-resistance strategies has not always been proactive in the past and is still not anticipated in some regions of the world. We introduced two alleles in semi-isogenic backgrounds currently found in French *Z. tritici* populations and of agronomic interest, as they are associated with moderate to high resistance factors to either SDHIs or DMIs. However, this choice did not represent the current allelic diversity described in field populations (Dooley et al., 2016b; Huf et al., 2018; Leroux and Walker, 2011; Rehfus et al., 2018). Considering that several alleles in the field are associated with resistance of lower intensity and that the interaction with multiple backgrounds could hardly be assessed, this experiment also evaluated a "worst-case scenario". Indeed, some clonal interference (*i.e.* the competition between several mutant clones) is in place in natural environments and interferes with the competition with ancestral strains (van den Bergh et al., 2018; McDonald, 2019). The competition between mutants in favourable environments should result in the fixation of the genotype with the highest fitness; however, adaptation might be slower in field conditions. Such phenomenon might be studied in EE by using natural populations collected in the field and fully sequenced to establish their initial diversity. Recombination is an important phenomenon for adaptation, as it increases its rate while combining adaptive mutations and separating beneficial mutations from deleterious ones, therefore potentially "solving"

clonal interference. This may ultimately contribute to a global increase of fitness under a selective environment, while adapting population structure (Felsenstein, 1974; Goddard et al., 2005; Gray and Goddard, 2012; McDonald, 2019). Here, recombination might have been useful to study the relation between genotype complexity (or phenotype generalism) and the fitness of recombinant mutants. Sexual reproduction might also affect the sustainability of MoAs differently. For example, cytochrome b, targeted by QoIs is encoded by a mitochondrial gene and is therefore inherited from the partner bearing the “female” mating-type, without any possible recombination during sexual reproduction. Combination of alleles giving rise to SDHI resistance is mostly observed as the rare co-occurrence of changes affecting two of the four sub-units forming the succinate dehydrogenase but not as the co-selection of mutations in the same sub-unit encoding gene. By contrast, recombination within the nuclear *cyp51* gene, encoding the target of DMIs, occurred more frequently in *Z. tritici* (Brunner et al., 2008). Recombination may also favour the build-up of multiple resistances through the recombination of several alleles or mechanisms of resistance, either specific or generalist. Such genotypes might be preferentially selected under heterogeneous selection pressures, by contrast to their parental strains. Yet, parasexuality is not described in *Z. tritici*. Sexual reproduction is hardly achieved in laboratory conditions, where it takes nearly a year to recover a proper progeny, and which requires a 4 to 6-month outdoor incubation on plant material (Suffert et al., 2019). It was then impossible to make recombination occur naturally in our EE. To overcome this limitation, we used a double mutant isolated from the same progeny than the single ones previously described. This double mutant carried the two independent resistant alleles and then exhibited multiple resistance to SDHIs and DMIs. This allowed us to mimic the impact of recombination on the performance of strategies (see discussion in §3).

## Role of immigration in experimental evolution

The last parameters that may have influenced resistance evolution in EE are the transfer and immigration rates. We define transfer rate as the size of the sample taken from the evolved population at the end of cycle  $n$  used to start cycle  $n+1$ . The immigration rate is the additional input of individuals retrieved from the untreated control used to adjust the size of the initial starting population of cycle  $n+1$  at  $10^7$  spores.

So far, two main approaches have been considered in literature to design EE. First, an EE approach without immigration from control lines has been carried out on *Z. tritici* to create SDHI resistant mutants by selection, while progressively increasing the dose of fungicide according to lineage

survival (*i.e.* ratchet selection) and up to the MIC and beyond. This design also included exposition to U.V. light and aimed at producing TSR mutant, *i.e.* not at assessing anti-resistance strategies (Gutiérrez-Alonso et al., 2017; Reboud et al., 2007). In this design, population structure is not artificially perturbed between the cycles. Alternatively, EE has also been used to study anti-resistance strategies. The selection pressure was set at the MIC (or close to it) from the start (Lagator et al., 2013b, 2013a; Perron et al., 2007; Vogwill et al., 2012). In these studies, immigration was systematically carried out to prevent line extinction, which might otherwise occur in populations of insufficient size and/or with limited number of replicates. In these situations, immigration may overcome this issue by providing new individuals and thus ensuring a critical population size. This minimal population is a renewed opportunity to create new genetic variation by mutation, while the mutation load and the maintenance of emerging alleles directly depend on this critical population size. This approach was shown to accelerate the adaptive evolution towards resistance (Perron et al., 2007, 2008). Thanks to immigration, we were able to work at doses close to the MIC throughout the course of the EE, and then to compare regimes displaying similar selection pressures, in terms of efficacy associated to each AI. It also ensured reasonable experimental conditions: manageable population size, powerful number of replicates and reasonable experimental timeframe. This also enabled us to start with the same exact concentration of spores at the beginning of each cycle. Indeed, as we set the maximum transfer volume at 2%, the number of spores to start a new cycle was completed with immigration only when necessary, *i.e.* mostly in the first cycles. Susceptible individuals added during immigration could logically dilute resistance in the first stages of resistance dynamics, when immigration was still necessary, leading to a biased measurement of population structure over time. In addition, the first phases of emergence and early selection might have been delayed, compared to a design without immigration. However, it is important to recall that agricultural systems are rarely perfectly closed either. Migration is intense in field populations of *Z. tritici* (Suffert et al., 2011; Zhan and McDonald, 2004). More than a bias, our EE design may rather mirror a cropping season, when considering the following assumptions. First, our system is settled with a given carrying capacity (defined by container size and medium volume). The transfer from the previous cycle would represent the endogenous inoculum (present on wheat debris or on volunteers) and immigration the exogenous inoculum invading a field until the carrying capacity is reached (Suffert et al., 2011). The exogenous inoculum may not necessarily have been submitted to the same selection pressures than the endogenous one. Secondly, only one treatment is now predominantly applied within a growing season in France. About five or six asexual cycles may occur during an average epidemic on current cultivars (Suffert et al., 2017), which is roughly consistent with the about

6 generations that unfold in one cycle of EE. Finally, population transfer might represent the bottleneck (*i.e.* strong reduction of population size) occurring during the inter-epidemic season. As bottleneck size and immigration rate may have direct impact on resistance dynamics, it would have been interesting to use similar parameters than those occurring in the field to enrich the parallel. Indeed, increasing immigration rate may also both increase resistance evolution and decrease a potential cost of resistance (Perron et al., 2007, 2008). When selection pressure is high, an important immigration may favour the acquisition of a rapid and high-level resistance. A significant bottleneck may favour genetic drift, which stochastically influences, either positively or negatively, the evolution of resistance (van den Bergh et al., 2018; Lanfear et al., 2014). Then, the impact of a bottleneck is dependent on population size. As we set its volume constant and independent from the spore concentration of the previous cycle, this may have affected differently the selection regimes tested. *In natura*, populations of *Z. tritici* are large and diverse enough at the field scale not to be affected by genetic drift during the inter-epidemic stage (Morais et al., 2019). However, mimicking the field situation would be actually complicated as the selective forces at play during the inter-epidemic season are not fully understood and as the importance of the different forms of inoculum depend upon the regional environmental conditions and the agricultural practices (Morais et al., 2016; Suffert et al., 2011).

## Can we close the debate about anti-resistance strategies?

The prevention and management of resistance to pesticides and drugs has been a matter of debate since early ages of chemical crop protection and healthcare, as evidenced by the prolific literature on the subject. This PhD did not aim to resolve this complex and multifaceted issue, but rather to provide new insights, while using an innovative approach. Some "principles", derived from this extensive work, are discussed below.

### Increasing the degree of heterogeneity in selection pressure generally delays emergence and selection of fungicide resistance but may favour more generalist phenotypes

Here, we varied the degree of heterogeneity in selection pressure when using alternation, mixture or dose variation, compared to sequence application of fungicides. For a naive population, the addition of a second MoA to the initial selection pressure, as may be the case with alternation,

delayed or had a neutral effect on the emergence and selection of resistance to both components. This effect is not only due to a reduction in time exposure, since at equivalent exposure some alternations were also still displaying lower resistance frequency than the sequential use of their components alone (part III). It could be explained by the selection of a mechanism inducing negative cross-resistance between the AIs used or determining a fitness cost leading to the counter-selection of this resistant allele when the environment is not favourable, *i.e.* when the corresponding AI is not used (Kim et al., 2014; Raymond, 2019; REX Consortium, 2013). Increasing the degree of heterogeneity by using concomitantly two or three MoAs in mixture may also delay resistance evolution compared to the use of a single AI at equivalent selection pressure, but our results showed that it is not systematically the case (see highlight 2). This theoretical “protective” effect of an AI on the emergence of resistance to other AIs is based on the assumption that it is unlikely that a mutant would simultaneously develop (target site-) resistance to all components of a mixture. The limitation of selection in mixtures relies then on the absence of positive cross-resistance allowing the control of the resistance to an AI by another AI (van den Bosch et al., 2014a), as corroborated by our study involving populations composed of two single resistances at low initial frequency. Alternation and mixture were efficient to delay resistance selection of both resistant alleles as well as controlling population size. However, generalist or multiple resistant phenotypes were more frequently associated with these heterogeneous selection regimes than with monotonous single AI sequences. Indeed, many samples taken from initially naive populations and that were exposed to more than one AI displayed reduced susceptibility to a high number of AIs, including to tolnaftate. More generally, environmental heterogeneity is associated with generalist phenotypes for many different traits as temperature, element enriched medium, nutrient medium or herbicide resistance (Boixel et al., 2019b; Comont et al., 2020; Sandberg et al., 2017; Wang et al., 2019). Yet, we did not expect this finding to apply to situations where selection was facilitated by the primary introduction of single resistant genotypes, suggesting the multidirectional nature of the selection is a major driver. In fungi, generalism might result from the selection of NTSR mechanisms, such as enhanced efflux, which is generally associated with lower resistance factors than TSR when considered as the only resistant mechanism. Detoxification is another option, which is found to induce intense cross-resistance between unrelated AIs in different organisms (Hu and Chen, 2021; Mitchell et al., 2014; Sang et al., 2018; Saves and Masson, 1998) but is still poorly explored in fungi. Generalism might also result from the accumulation of several independent TSR and/or NTSR mechanisms. Some pleiotropic effects might explain a reduction of susceptibility or resistance towards other AIs in heterogeneous environments (Wang et al., 2019). Our

results suggest that the potential fitness cost of being a generalist instead of a specialist in a beneficial environment should be lower than the cost of being a specialist in a maladaptive environment (Kassen, 2002). However, the co-occurrence of both specialist and generalist resistances in many of our lines is not excluded. Especially, the timescale of our experiment is still limited compared to *in natura* evolution. In part III, the observation of the evolution of the resistance patterns, of the lines under continuous exposure to a single AI, suggest that specialist strains are not generalized in all lines, *i.e.* that the genotypic equilibrium was certainly not reached at the time we stopped the experiment.

Generalist resistance due to NTSR mechanisms may not appear a major concern at first glance due to its apparently low impact on disease control. However when NTSR is associated with TSR, resistance levels may significantly increase and the accumulation of several NTSR mechanisms can also lead to high RFs consistent with possible efficacy loss in the field (Leroux and Walker, 2011; Nikaido, 2009). As generalist resistances are not specific to one MoA, their spread in field populations may compromise the efficacy of future AIs (synthetic fungicides as well as biologicals) even before they are launched. Therefore, resistance management should face the following trade-off: the most effective strategies against specialist resistances might also be the ones promoting the selection of generalist individuals, more hazardous for future AIs. The design of management strategies should then result from an informed choice by the stakeholders, depending on the local situation, their economic priorities and the term of their production.

## Strategies differ in efficacy, but population structure always matters when defining durability

Increasing the degree of heterogeneity in selection pressure did globally limit the evolution of resistance, at various extents, depending on strategy components (Parts III, IV and V). Such inconsistency prevents us from generalizing without caution our findings to other pathogens, other MoAs and maybe even to other AIs. Nonetheless, we highlighted that some drivers were more relevant than other and even that some combinations were irrelevant for resistance management and should therefore be avoided. As a proof of concept, we chose AIs with distinct MoAs and different associated resistance risks, from low, moderate to high, with no known cross-resistance, as it is a potential failure factor (Fardisi et al., 2019). This choice turned out to be the most important driver for all the anti-resistance strategies tested, when assessing performance starting from naive populations. In order to expand our findings, it could be interesting to see if similar patterns are found with other

AIs of the same MoA or with other MoAs but similar intrinsic resistance risk, or with other strategies. For alternation, the order in which AIs are applied should determine strategy performance and more regimes should have been conducted to check this effect (Elderfield et al., 2018).

The higher-risk fungicide is often considered as the one to be protected with the introduction of another MoA with a same or lower resistance risk, either simultaneously (mixture), staggered in time (alternation) or in space (mosaic) (Hobbelen et al., 2013). We noticed that the lower the partner risk, the better the strategy is (van den Bosch et al., 2014b). However, our results are not consistent with the cautious conclusion of a modelling study that the efficacy of mixtures, even made of two high-risk fungicides, is equivalent or superior to the use of single components (Hobbelen et al., 2013). Indeed, we found that the mixture of AIs with the highest resistance risks, at efficient-dose, developed resistance sooner than both of its AIs alone (part IV) and that the emergence and early phases of resistance of a low-resistance risk fungicide were shortened when it was alternated with a high-risk partner. In addition, the implementation of a third poorly efficient AI may decrease the efficacy of a strategy (mixture or alternation) already in place with two AIs of lower resistance risk. This highlights the fact that maximising the heterogeneity of pressure does not necessarily require increasing the number of AIs used but choosing a relevant combination. Lagator et al. (2013a) found similar results with three-way mixtures used at their equivalent dose. Therefore, we may need to shift the lens of resistance management away from protecting a high-risk fungicide at all costs if it also compromises the sustainability of its potentially lower-risk partner. Yet, these findings might be qualified with the consideration of the dose applied, particularly in mixtures. Lagator et al. (2013a) highlighted the importance of high doses in such cases.

AIs choice is also relevant to determine the pattern of resistance to be selected, as we noticed that the presence of prothioconazole-desthio was associated with an increased selection for generalist resistance, revealed by growth on tolnaftate. Prothioconazole-desthio is a DMI for which several resistant phenotypes have been observed that combined drug efflux with some TSR mechanism(s?) (Leroux and Walker, 2011). Generalist resistance may be associated with the intensive use of some specific MoA combinations (Garnault et al., unpublished). Other drivers of strategies such as alternation rhythm had a minor impact on resistance evolution. A short rhythm of alternation slightly decreased resistance evolution but was less frequently associated with multidrug resistance than long alternation.

In addition to AIs choice, the initial population structure highly mitigated the performance of heterogeneous strategies. We tested the best alternation and mixture defined from the previous experimentations on naive lines to test their suitability for the management of more complex resistances.

Unfortunately, neither population size nor resistance evolution were controlled by such strategies in presence of the multiple resistant genotype in the initial population, corroborating the conclusion of Hobbelen et al. (2013), that the durability of these heterogeneous anti-resistance strategies was quite similar to that of single fungicide sequences in this context. Double mutants are complex phenotypes that may result from recombination occurring potentially during sexual reproduction between two single mutants or from the sequential selection of a resistance on the genetic background already displaying resistance to another MoA. The rate of selection of those multiple resistant genotypes may depend on their initial frequency, on their potential fitness cost relatively to the other strains present in the population and on the presence of a synergy between the AIs used (Hobbelen et al., 2013; Shaw, 1993). If the absolute fitness of a multiple resistant genotype is lower than that of a simple variant in the absence of selection pressure or under homogeneous selection pressure, one may consider reducing the degree of heterogeneity of selection pressure to potentially decrease the frequency of that of the multiple mutants. However, fitness cost may be reduced over time through compensatory mutations for example, which does not make it a reliable ally in limiting selection. Therefore, it is all the more important to set up resistance management as early as possible in order to limit their emergence and then their selection as much as possible.

Increasing the degree of heterogeneity of selection pressure is not a magic formula always conducing to limit the emergence and selection of resistance. We emphasized how variable the response can be depending on the AIs combinations displayed and more importantly on the initial status of resistance in the population. To achieve a sound management of resistance, tailored strategies should then be implemented for local populations, which further reinforces the role of resistance monitoring and field diagnosis on a smaller scale than the national one.

## Mixture does not systematically outperform alternation depending on the comparison method chosen

Mixtures are often described as more effective than alternation in delaying resistance selection (van den Bosch et al., 2014a; REX Consortium, 2013). However, this comparison is rarely carried out at comparable doses (half-dose of each component in mixtures). The few studies that take into account this setting are more nuanced in their ranking of the two strategies but mixtures are still considered more advantageous than alternation in several cases (van den Bosch et al., 2014b; Hobbelen et al., 2013). Elderfield et al. (2018) highlighted the care that should be taken when strategies



are compared and the importance of the system, the model and the epidemiological and fungicide parameters influencing it.

In this PhD, we can first establish an indirect comparison of alternations (part III) and efficient-dose mixtures (part IV) with the combinations of three different MoAs by studying their relative relevance as anti-resistance strategies for naive populations. Alternation appeared always beneficial or neutral in comparison with the use of its components alone, while the worst mixtures could select resistance faster than the sequential use of either of their components. Both anti-resistance strategies favoured generalism in comparison with homogeneous selection pressures. At comparable intensity of selection pressure, alternation therefore appeared globally better than mixture, even if there were large variations in responses depending on the AI combinations. It is nonetheless important to note that the best alternations and mixtures were composed of the same AIs (*i.e.* of acceptable resistance risk) and were successfully preventing resistance selection in both cases, including generalist one in the study timeframe.

Secondly, we were also able to compare directly alternation and mixture of the same two AIs (the best strategies identified previously) but at equivalent dose (part V) and on several population differing for their structures. We confirmed that they were equally preventing resistance emergence by controlling the naive population. Compared with sequence, they both limited population growth and the selection of simple resistant mutants in the population. They also selected phenotypes with a broader spectrum of resistance, potentially due to some NTSR mechanisms. However, mixture effect was more homogeneous over time while the impact of alternation impact was more irregular. This variability in performance of alternation over the cycles may penalise alternation especially in a short time scale even if on a longer one its relevance is acted. Consequently, in presence of single mutants resistant to the AIs displayed, mixture looks more effective than alternation. Finally, when a multiple resistant genotype is present, none of these anti-resistance strategies can be considered relevant. The growth of the population was slightly lower for mixture than for alternation, but this discrepancy was insufficient to provide acceptable control of the population. This small difference might result from a fitness cost associated to a multiple mutant being expressed under alternation regimes but not comparably in mixtures (Shaw, 1993). This advantage of alternation may reverse when the frequency of the multiple resistant genotype is initially very low (Hobbelen et al., 2013). The populations exposed to these two types of selection regime showed no difference in phenotypes. However, sometimes specialist and generalist phenotypes can be differently selected by alternation and mixture: mixing two nutrients concurrently selected two specialist phenotypes, each capable of growing on only one of these nutrients, and a generalist one capable of growing on both; when those two

nutrients were alternated, specialists growing on one single nutrient were selected whereas generalists were eliminated (Kassen, 2002; Roemhild et al., 2015).

Consequently, depending on the AIs considered, on the population structure, on the time perspective and the dose of mixture (full, efficient, half), the advantage of mixture over alternation might be mitigated, which prevents the generalization of such statement and prevent the unequivocal recommendation on mixture over alternation.

## Adaptive landscapes may give insights on our evolutionary outcomes

Understanding the evolutionary trajectories toward resistance, *i.e.* the underlying dynamic mutational fitness landscapes, would improve our prediction of adaptation to pesticides and the design of sound anti-resistance strategies (Jansen et al., 2013; Steinberg and Ostermeier, 2016). More precisely, establishing fitness landscapes may potentially help us understanding the distinct evolutionary outcomes achieved under homogeneous and heterogeneous selection pressure. Fitness landscapes connect through a physical topography representation the space of different combinations of genotypes (horizontal axes) to their corresponding fitness (height) (Wright, 1932).

The fitness landscape characterizing an environment under low-dose treatment is generally described as rugged, *i.e.* with multiple peaks displaying several local optima (Raymond, 2019). Note that a low dose application is considered here as the reduction in the dose of a given treatment, and not a reduction of exposure due to a lower frequency of full-dose treatment. Resistance selected from low-dose application may generally be caused by more possible mutations than high-dose treatment, each step being associated with small fitness variation and sometimes being "off-target" (*i.e.* not affecting directly the binding site). Selection under reduced doses is also recognized for its increased mutational supply due to higher population size (Raymond, 2019). Consequently, low-dose applications may fasten the emergence of resistance, likely with low RFs at the beginning but increasing over time. Patterns of quantitative resistance dynamics may display similar fitness landscapes, *i.e.* multiple local optima peaks in a rugged fitness landscape. Under such selection pressures, the gradual accumulation of either mutations associated to the reduction of the susceptibility to fungicides or compensatory mutations, may result in an overall increase of fitness (Hawkins and Fraaije, 2021). Some of them may be "permissive" mutations, *i.e.* their acquisition allows the acquisition of others that would not have been possible in the first place (*i.e.* stepping stones to higher peaks) (Bloom et al., 2010; Szili et al., 2019). Also, immigration in a population undergoing rugged

fitness landscape would result in the accumulation of more mutations, as the high diversity allows the exploration of the best combination of mutations towards high-fitness regions (Van Cleve and Weissman, 2015). On the other hand, a smooth fitness landscape displays a single peak characterized by a fixed effect of each mutation on fitness and no fitness interaction among genes. The resulting population would then have a low phenotypic diversity as different evolutionary trajectories lead to the same peak. Finally, high-resistance risk fungicides applications may display fitness landscapes characterized either by a high fitness peak surrounded by multiple low-intermediate peaks allowing the final accession, or a smooth landscape with a unique peak easily reachable in one step. By contrast, low-resistance risk fungicides applications may display one (or a few) very high peak(s) in a less rugged (maybe even smooth) landscape, preventing or limiting the pathway to that fitness optimum, making resistance less likely to evolve. Comparable types of adaptive landscapes may be found for low-dose applications and high-dose applications of a fungicide selecting for qualitative resistance where the first, reduced dose, would be rugged and the second, higher dose, smoother.

Several mechanisms may contribute to shape fitness landscapes. For example, the accumulation of mutations can result from epistatic interactions, *i.e.* from situations when the effect of a gene mutation is dependent on the presence or absence of mutations in one or more other genes (Hawkins and Fraaije, 2021). Epistatic interactions (*i.e.* when combined fitness effects of mutations are greater or less than expected from their separate effects) modify fitness landscapes over the course of adaptation and are even more important to consider when there is a heterogeneous selection pressure (Tenailon et al., 2012). In the case of a negative epistasis between beneficial mutations, the fixation of one beneficial mutation shapes the mutational landscape and drives not only one but many other mutations, either neutral or deleterious. When sufficient, such effect might prevent or slow down the evolution of multiple resistant or generalist phenotypes (Kassen, 2002). On the contrary, a positive epistasis may lead to the selection of double mutant or generalist phenotypes. In our experiments, in order to test if the acquisition of a mutation responsible for the resistance to one MoA would favour the acquisition to another MoA, we could have compared the rate of resistance acquisition under a given straight selection regime for one isolate displaying the wild-type background and another one carrying the resistance allele to an independent MoA. The influence of epistasis is not necessarily reciprocal, *i.e.* fitness landscapes may differ according to which mutation occurred first. Such statement may be particularly significant if we consider MoAs order in alternation. Pleiotropy could also explain phenotypes resistant to multiple AIs, the acquisition of mutation responsible for the resistance to one AI conferring *de facto* the ability to resist to other stresses or a reduced sensibility to another AI (cross-resistance) (Wang et al., 2019).

Fitness landscapes are not set in stone over time and environmental fluctuations. In particular, multidirectional selection pressure may highly interfere with the selection process if the allele to-be-fixed has a high fitness in one landscape but not in others (Saakian et al., 2019). This could be particularly the case for a specialist phenotypic profile undergoing a heterogeneous selection pressure. On the other hand, a generalist phenotype would have a greater fitness, in comparison with the wild-type, in rugged as well as in smooth fitness landscapes under fungicide selection pressure. Its fixation will then result from its mean fitness in the two environments. Selecting a resistant specialist phenotype in a heterogeneous environment would require the crossing of multiple fitness valleys in order to acquire resistance to all the MoAs displayed. Such scenario may occur from recombination or stochastic unlikely events or more likely thanks to environmental changes that model fitness landscapes (Steinberg and Ostermeier, 2016). A sudden environmental change would select genotypes with a lower maximal fitness compared to when a progressive change occurs and might need more adaptive changes to reach this optimum (Collins et al., 2007). Consequently, a slow increase of fungicide dose through time, as it could be the case when selecting slowly-evolving quantitative resistance, would be a worse strategy than alternation for example. However, Steinberg and Ostermeier (2016) noticed that the allele responsible for the higher antibiotic resistant phenotype was obtained through an initially negative selection (*i.e.* it outperformed the genotype obtained after positive, neutral and oscillating selection). This evolutionary pathway was only possible through the initial step where the presence of one key initially deleterious mutation underlay a positive epistasis to neutral or slightly beneficial mutation that revealed being a high fitness allele in the following environment. Even if, as suggested by the authors, such finding should be further investigated to settle if it is a general rule or a specific case, this could have important implications for the design of anti-resistance strategies. For example, negative selection occurring during a long rhythm of alternation may later provide access to new fitness peaks when fungicides change. Alternation rhythm may also allow early mutations with lower effects to be fixed when environmental changes are less frequent. Frequent environmental shifts increase the time needed for mutations with larger effects to be fixed, depending on the adaptive paths already undergone by the population, that may have led to potential neutral- or low-effect mutation steps before (Collins et al., 2007).

In the light of these developments, the debate on anti-resistance strategies is still burning. It looks like that applying a high dose less frequently and in alternation with other MoAs is more likely to induce major and sudden changes of fitness landscapes, which could result into an efficient anti-resistance strategy in comparison to more frequent treatments at a lower dose, finally defining more

homogeneous selection pressure over time (Raymond, 2019). On the other hand, mixtures at efficient-dose (and therefore reduced pesticide load, as expected from current regulation) would result in a rugged landscape allowing earlier resistance evolution, potentially stepping stones toward high multiple or generalist resistance. Constant environments (as provided by mixtures always including the same MoAs, even if different) would not sufficiently modify fitness landscapes to possibly extend the access to new mutations (Steinberg and Ostermeier, 2016). Therefore, if no generalist phenotypes or bridge between fitness valleys are available in the environment imposed by a mixture regime, there would probably not be a rapid adaptation towards such phenotypes. To decipher these evolutionary hypotheses, it would be interesting to complete the analyses achieved in this PhD with additional sequencing of the evolved genotypes and genomes, in order to appreciate the mutational diversity of our mutants and to allow a complete description of the resistance evolution process.

## Conclusion and perspectives

In this PhD, we established that the main drivers of resistance evolution were the choice of the AIs involved in the overall selection pressure and their intrinsic resistance risk, as well as the initial status of resistance in the initial population. The high variability in population responses to different AI combinations and depending on pre-existing resistances makes it impossible to close up the debate on the best anti-resistance strategy. Ideally, before being implemented, every strategy should be tested to validate the assumptions on its durability. For example, the implementation of an alternation of mixtures with several different AIs should increase even more the degree of heterogeneity of selection and would probably be effective in controlling the disease in the first instance. However, depending on the MoAs chosen, generalism, and potentially NTSR, might be strongly selected and this strategy might prove less durable than expected. Moreover, the increase of such generalist phenotypes in the population may alter the durability of future products not even on the market. To prevent a “tragedy of the commons” (Hardin, 1968), resistance management should therefore be considered on long time scales, be tailored according to field situations, and explore the trade-off between resistance dynamics and generalism of the selected phenotypes. This goal will probably not be achievable without some national coordination of monitoring, prevention, management actions and research and without rethinking agricultural systems, as can be the case in human health for antibiotic resistance management (Haywood et al., 2021). Raising awareness of the importance and complexity of resistance management among farmers, advisers, companies and public authorities could accelerate this transition toward a new paradigm. A clear distinction between resistance and

efficacy management should be made clear in all minds, as it may sometimes lead to contradictory decisions and imply contrasted time perspectives. Scientific research has a major role to play in improving anti-resistance strategies and more generally in understanding the constant adaptation of pathogens to pesticides and cultivar resistances, as well as their potential interaction. In this work, we developed a tool, experimental evolution, adapted to a fungus model, that may need further improvement and validation with empirical data but that will be useful in the future to investigate resistance on many other aspects. A direct follow-up of this work would be to test other anti-resistance strategies and resolve some of the pending outstanding issues. For example, it would be especially interesting to test the durability of field-like mixtures (higher overall dose), or the introduction of unisite MoAs not affected by resistance yet (*e.g.* QoIs recently introduced or metyltetraprole, a new QoI not exhibiting cross-resistance with strobilurins). The impact of generalism on resistance durability is still insufficiently considered and explored, especially in agricultural mycology (Hu and Chen, 2021). Its relevance could be demonstrated by considering initial populations containing strains with NTSR mechanisms like MDR into the initial population. EE could also be used to decipher the resistance risk of new products, including biocontrol AIs that have a direct effect on the fungus.

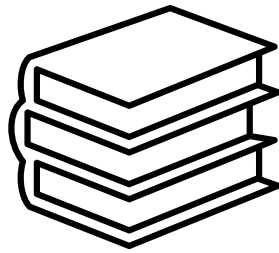
To conclude, some findings of this PhD work could be translated into practical outcomes, while tempting some answers to the following question:

*How can farmers manage resistance while complying with the recommendations to reduce and make better use of pesticides?*

- Acting as soon as possible, *i.e.* from the introduction of the AI, with sound anti-resistance strategies is essential to prevent resistance emergence then selection, and more particularly of multiple resistances which compromise the durability of MoAs, and is therefore critical.
- Increasing the heterogeneity of selection by using different MoAs either in alternation or in mixture is generally a good practice for resistance management as long as high resistance risk MoAs are avoided and even more if alternation is frequent.
- Hitting hard (full dose) but less frequently with a pluri-annual alternation (*e.g.* only one fungicide application per year) may be particularly relevant to prevent the emergence of resistance, and later limit the selection of quantitative resistance. This strategy might be more relevant for resistance management than using non adapted mixtures.

- To achieve low annual input of fungicides, it would be necessary to concomitantly use all the other methods available to control the disease (agricultural practices, varietal resistance, biocontrol) and to monitor fungal adaptations to these different selection pressures in order to quickly adapt practices to field situation.

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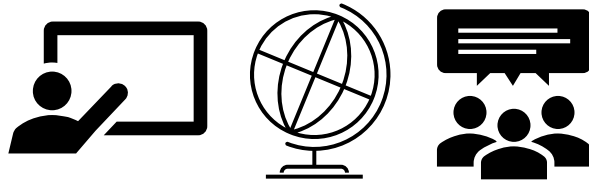
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# Appendices



# Conference presentations

- **Talk:**

**Understanding the impact of alternation on resistance evolution by the mean of experimental evolution: the case of *Zymoseptoria tritici*, the causal agent of Septoria leaf blotch**

Ballu A., Carpentier F., Torriani S., Dérédec A., Walker A-S., 2019

International Symposium on Cereal Leaf Blights, 22-24, May 2019, Dublin, Ireland

**Astract:**

Enhancing the sustainability of the fungicide active ingredients is one of the challenges that need to be faced in crop protection. Though, predicting resistance evolution and the efficacy of anti-resistance strategies for a given resistance case could help risk assessment.

*Zymoseptoria tritici* is the causal agent of Septoria leaf blotch and is the most detrimental fungal pathogen of wheat. In Europe, it has evolved fungicide resistance in the field to all unisite inhibitors, leading to variable efficacy losses depending on the modes of action and resistance frequency. This highly adaptive pathogen is then a challenging candidate for which preventive resistance management needs improvement.

This study aims to analyse the impact of modes of action alternation on resistance evolution using experimental evolution. Indeed, experimental evolution tends to accelerate in lab-standardized and miniaturized conditions the selection observed *in natura* and may help to the quick identification of operational drivers of this strategy. We expect that alternation may slow down the rate of resistance, compared to straight selection, that there is a relationship between the frequency of alternation and the rates and outcomes of resistance evolution, and that the heterogeneous selection pressure over time created by alternation may influence levels and mechanisms of resistance. Independent lines resistant to three fungicides (benzovindiflupyr, carbendazim and prothioconazole-desthio) were selected from the ancestral WT strain IPO-323, according to different alternation regimes. Resistance and fitness were measured in evolved lines. The comparison of resistance dynamics in between evolved lines was used to characterize the sustainability of the selection regimes.

**Why is fungicide mixture considered the most sustainable anti-resistance strategy ?**

**An experimental evolution approach with the case of *Zymoseptoria tritici***

Ballu A., Carpentier F., Dérédec A., Walker A-S., 2020

Réunion du groupe Réseau Écologie des Interactions Durables (REID) – Champignons, 26-27, November 2021, France

- **Poster:**

**Effect of fungicide alternation on resistance evolution: experimental evolution of *Zymoseptoria tritici*, the causal agent of Septoria leaf blotch**

Ballu A., Carpentier F., Torriani S., Dérédec A., Walker A-S., 2019

19<sup>th</sup> International Reinhardtsbrunn Symposium on Modern Fungicides and Antifungal Compounds, April07-11, 2019, Friedrichroda, Germany



# Effect of fungicide alternation on resistance evolution: experimental evolution of *Zymoseptoria tritici*, the causal agent of septoria leaf blotch

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## Introduction

In Europe, *Zymoseptoria tritici* (Fig 1.) has developed fungicide resistance in the field to all unisite inhibitors, leading to variable efficacy losses depending on the modes of action and resistance frequency. This highly adaptive pathogen is thus a challenging candidate for resistance evolution prediction and for the risk assessment of the efficacy of anti-resistance strategies.

Among the different strategies available, **alternation** is an interesting one as it allows the expression of resistance costs.



Fig 1. Septoria leaf blotch symptoms on wheat leaf, caused by *Zymoseptoria tritici*

### Alternation components :

- Fungicides involved
- Frequency (rhythm) of alternation (i.e. how often there is a fungicide change)

### Experimental evolution approach:

- Study of populations across multiple generations under defined and reproducible conditions
- "Exposing an experimental population to a stress that is lethal (or sublethal) and allowing the survivors (or the hardest) to become parents of the next generation"

(Garland and Rose, 2009)

**Advantages:** accelerate in lab-standardized and miniaturized conditions the selection by fungicides observed in the field, cheaper than field experiments, possibility of screening multiple modalities

### Research question: What is the impact of alternation of modes of action on resistance evolution?

## Experimental method

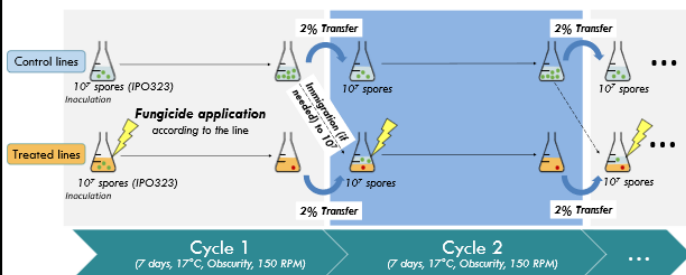
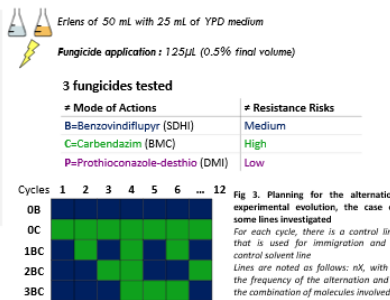


Fig 2. Protocol of the experimental evolution used to test alternation strategies



## Results

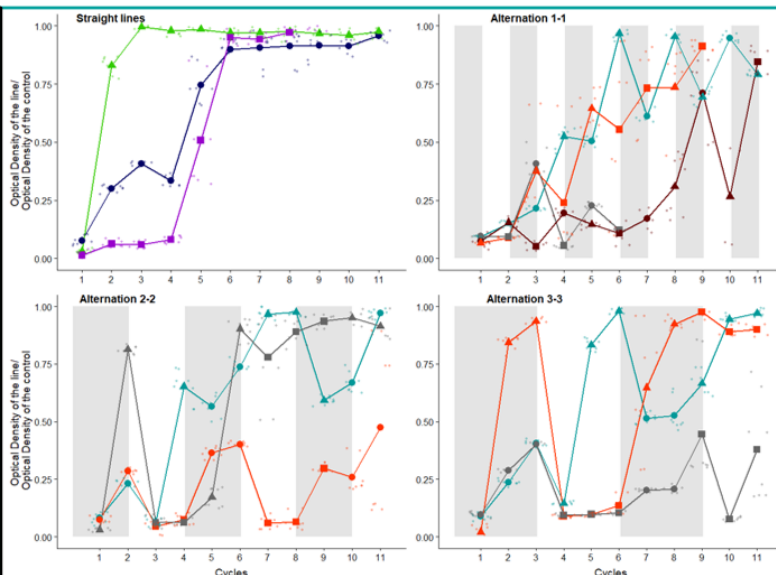


Fig 4. Evolution over time of concentrations compared to free-fungicide control of each strategy tested

Lines name	no cycle R > 10% *	no cycle R > 90% *	Relative growth mean	Total of cycles completed
OB	1	6	0.67	11
OC	2	3	0.87	11
OP	5	6	0.50	9
1BC	2	6	0.60	11
2BC	1	7	0.59	11
3BC	1	6	0.57	11
1CP	3	6	0.48	9
2CP	2	6	0.60	11
3CP	2	3	0.59	11
1BP	1	Not yet	0.17	6
2BP	2	Not yet	0.22	11
3BP	1	Not yet	0.22	11
1BCP	1	11	0.28	11

Fig 5. Summary table of number of cycles to exceed a threshold, relative growth mean (compared to control growth) and the number of cycles that have been used for calculation, given for each line

\*Number of the first cycle where relative growth exceeds the percent threshold

### Effects identified statistically:

- Strongest effect: the fungicides involved (Carb>Benz>ProDo)
- Effect of the number of ≠ fungicides involved ( $p.val < 2.2e-16$ )
- Frequency effect: negative effect ( $p.val = 0.01$ ) modulated by the fungicide used

## Conclusions

- ≠ Resistance risks / fungicide (as observed in the field)
- Alternation is efficient to delay resistance emergence (compared with no alternation), all the more so when resistance risk of fungicide is low
- Main action lever: fungicide selection, little impact of frequency in our study

### Perspectives:

- Resistance intensity, cross resistance, fitness, resistance mechanism of the obtained lines: droplet tests (Fig 6), mutant analyses (genotype, IC<sub>50</sub>)
- And what about other strategies (mixture/dose modulation)?



Fig 6. Droplet test for the effect of each fungicide on growth of Zymoseptoria tritici on different Zymoseptoria tritici strains



# French summary

## Résumé substantiel en français

### **Introduction sur la problématique de recherche (partie I) :**

La résistance aux pesticides impacte économiquement la production agricole (dégâts sur le rendement et la qualité des récoltes) et indirectement l'environnement (traitements phytosanitaires supplémentaires ou substituts plus dommageables). Sa gestion implique de maximiser l'hétérogénéité des pressions de sélection, notamment en combinant des substances actives (SA) de modes d'action différents. Malgré l'utilisation d'approches empiriques et de modélisation pour établir l'efficacité des différentes stratégies à retarder l'émergence et la sélection de la résistance dans les populations pathogènes, le débat reste ouvert. Cette thèse développe une nouvelle approche, complémentaire, pour nourrir les discussions autour des stratégies anti-résistance en abordant deux questions en particulier : (i) Comment améliorer la performance des stratégies anti-résistance et quels sont les facteurs permettant leur optimisation ? (ii) Comment le statut initial de la résistance dans les populations module-t-il la performance des stratégies ? Pour illustrer ce débat, nous avons utilisé *Zymoseptoria tritici*, agent causal de la principale maladie du blé, résistant à la plupart des fongicides en Europe.

### **Développement méthodologique sur l'évolution expérimentale mise en place dans la thèse (partie II) :**

Nous avons appliqué une approche innovante pour les champignons, l'évolution expérimentale, qui permet d'imiter et d'accélérer, en conditions contrôlées de laboratoire, la résistance aux fongicides habituellement sélectionnée au champ. En particulier, en soumettant un isolat sensible à des régimes de sélection hétérogènes par comparaison avec l'utilisation en séquence des mêmes SA, nous avons dissocié l'impact relatif de plusieurs facteurs sur la réduction de la sélection au sein de stratégies d'alternance, de mélange et de modulation de dose. Dans cette partie nous développons le protocole et les analyses utilisés pour évaluer les stratégies tant sur le plan quantitatif que qualitatif, ainsi que les mises au point qui ont été nécessaires.

### **Evaluation de la stratégie d'alternance et de ses principaux facteurs pour limiter l'évolution de la résistance (partie III) :**

Dans une première évolution expérimentation sur l'alternance, nous avons évalué cette stratégie en comparaison à un usage séquentiel d'une seule SA, et avons plus particulièrement testé l'effet de trois déterminants : le nombre de SA, le type de SA (et son risque de résistance intrinsèque) et le rythme de l'alternance. Nous avons également exploré si l'effet de l'alternance résidait dans la diminution de l'exposition de chaque fongicide ou bien dans une atténuation du taux de sélection. L'alternance s'est avérée être une stratégie bénéfique ou neutre pour diminuer le taux d'évolution de la résistance par rapport à une utilisation continue d'un seul fongicide, mais a augmenté le degré de généralisme. Nous avons démontré que ce compromis résidait probablement dans le risque de résistance intrinsèque des SA, plutôt que dans le nombre de fongicides utilisés ou le rythme de l'alternance.

### **Evaluation de la stratégie de mélange à dose efficace et de ses principaux facteurs pour limiter l'évolution de la résistance (partie IV) :**

Dans une deuxième expérimentation, nous avons exploré comment un mélange à dose efficace peut retarder l'évolution de la résistance d'une population sensible. Nous définissons un mélange à dose efficace comme un mélange avec des doses réduites de ses composants permettant, ensemble, un contrôle de la maladie similaire à celui fourni par ses composants utilisés seuls à pleine dose. Nous avons conclu qu'un tel type de mélange pouvait être, selon ses composants, préjudiciable à

bénéfique pour la durabilité. Des résistances généralistes ou multiples ont plus ou moins été favorisées en fonction des combinaisons de SA. En effet, le choix des fongicides s'est révélé comme étant le principal levier de la performance des mélanges avant le nombre de fongicides utilisés. La réduction de la dose lors d'usages séquentiels n'a pas empêché la sélection de souches spécifiques fortement résistantes, ni la résistance généraliste dans certaines lignées.

#### **Evaluation de l'effet du statut initial de la résistance d'une population sur l'efficacité des stratégies (partie V) :**

Une dernière expérimentation a été conçue pour valider les résultats précédents avec des souches portant des résistances présentes au champ. Un isolat sensible et deux populations artificielles, contenant de faibles fréquences de résistances simples ou multiples ont été soumis à des régimes de sélection contrastés par quatre sources d'hétérogénéité (type SA, nombre SA, intra *vs.* inter MoA, intra *vs.* inter cycle). Les résultats ont conclu que l'augmentation du nombre de SA et la variation des modes d'action étaient efficaces pour retarder la sélection de la résistance, que ce soit en mélange ou alternance. Toutefois cela faciliterait un certain généralisme des isolats sélectionnés, en supplément des résistances spécifiques initialement introduites. Aussi, aucune de ces sources d'hétérogénéité ne s'est avérée efficace pour atténuer l'évolution de la résistance aux fongicides utilisés lorsque la résistance multiple était présente dans les populations. Cela suggère que la recombinaison, pouvant survenir lors de la reproduction sexuée au champ, est un facteur majeur à considérer et valide expérimentalement l'affirmation que la prévention doit toujours être préférée à la gestion des résistances. Enfin, la réduction de la valeur sélective (fitness) associée à certains allèles résistants retarde légèrement leur sélection et peut moduler la structure des populations.

#### **Conclusions générales et perspectives :**

L'évolution expérimentale s'avère être un outil puissant pour enrichir le débat sur les stratégies de lutte en analysant les interactions entre ses déterminants. Les stratégies hétérogènes peuvent ralentir l'émergence et l'évolution des résistances en comparaison à un usage en séquence de fongicides, leur efficacité dépend principalement des SA choisies. Toutefois, cela sous-tend un compromis car l'hétérogénéité sélectionne couramment des résistances généralistes ou multiples. Ces résultats ouvrent de nouvelles perspectives pour optimiser la gestion des résistances et suggèrent que la prévention de l'émergence serait plus durable que de limiter la sélection.

# Abstracts



**Titre :** Évaluation des stratégies de gestion des résistances aux fongicides par une approche d'évolution expérimentale : le cas de *Zymoseptoria tritici*, agent causal de la septoriose.

**Mots clés :** adaptation fongique, déterminants de la sélection, généralisme, évolution artificielle, spécialisation écologique, variation environnementale

**Résumé :** La résistance aux pesticides impacte économiquement la production agricole et la biodiversité. Sa gestion implique de maximiser l'hétérogénéité des pressions de sélection, notamment en combinant des substances actives (SA) de modes d'action différents. Or, le débat concernant l'efficacité des différentes stratégies pour retarder l'émergence et la sélection de la résistance dans les populations pathogènes reste ouvert. Cette thèse aborde deux questions : (i) Comment améliorer la performance des stratégies anti-résistance et quels sont les facteurs permettant leur optimisation ? (ii) Comment le statut initial de la résistance dans les populations module-t-il la performance des stratégies ? Pour illustrer ce débat, nous avons utilisé *Zymoseptoria tritici*, agent causal de la principale maladie du blé, résistant à la plupart des fongicides en Europe. Nous avons appliqué une approche innovante pour les champignons, l'évolution expérimentale, qui permet d'imiter et d'accélérer, en conditions contrôlées de laboratoire, la résistance aux fongicides habituellement sélectionnée au champ. En particulier, en soumettant un isolat sensible à des régimes de sélection hétérogènes par comparaison avec l'utilisation en séquence des mêmes SA, nous avons dissocié l'impact relatif de plusieurs facteurs sur la réduction de la sélection au sein de stratégies d'alternance, de mélange et de modulation de dose.

Dans une première expérimentation, l'alternance s'est avérée être une stratégie bénéfique ou neutre pour diminuer le taux d'évolution de la résistance par rapport à une utilisation continue, mais a favorisé du généralisme. Nous avons démontré que ce compromis résidait probablement dans le risque de résistance intrinsèque des SA, plutôt que dans le nombre de fongicides utilisés ou le rythme de l'alternance. Dans une deuxième expérimentation, nous avons exploré comment un mélange à dose efficace, c'est-à-dire un mélange avec des doses réduites de ses composants permettant un contrôle

de la maladie similaire à celui fourni par ses composants utilisés seuls, peut retarder l'évolution de la résistance d'une population sensible. Nous avons conclu qu'un tel mélange pouvait être, selon ses composants, préjudiciable à bénéfique pour la durabilité. Des résistances généralistes ou multiples ont plus ou moins été favorisées en fonction des combinaisons de SA. La réduction de la dose dans des séquences n'a pas empêché la sélection de souches spécifiques fortement résistantes, ni la résistance généraliste dans certaines lignées.

Une dernière expérimentation a été conçue pour valider les résultats précédents en soumettant un isolat sensible et deux populations artificielles, contenant de faibles fréquences de résistances simples ou multiples, à des régimes de sélection contrastés par quatre sources d'hétérogénéité. Les résultats ont conclu que l'augmentation du nombre de SA et la variation des modes d'action étaient efficaces pour retarder la sélection de la résistance, que ce soit en mélange ou alternance. Toutefois cela faciliterait un certain généralisme des isolats sélectionnés, en plus des résistances spécifiques initialement introduites. Aussi, aucune de ces sources d'hétérogénéité ne s'est avérée efficace pour atténuer l'évolution de la résistance lorsque la résistance multiple était présente dans les populations. Cela suggère que la recombinaison, pouvant survenir lors de la reproduction sexuée au champ, est un facteur majeur à considérer. Enfin, la réduction de la valeur sélective associée à certains allèles résistants retarde légèrement leur sélection et peut moduler la structure des populations.

L'évolution expérimentale s'avère être un outil puissant pour enrichir le débat sur les stratégies de lutte en analysant les interactions entre ses déterminants. Ces résultats ouvrent de nouvelles perspectives pour optimiser la gestion des résistances et suggèrent que la prévention de l'émergence serait plus durable que de limiter la sélection.

**Title:** Evaluating fungicide resistance management strategies by means of experimental evolution: the case of *Zymoseptoria tritici*, the causal agent of Septoria leaf blotch

**Keywords:** fungal adaptation, selection drivers, generalism, artificial evolution, ecological specialization, environmental variation

**Abstract:** The evolution of resistance to pesticides is a major economic and environmental burden in agriculture. Resistance management involves maximizing selection pressure heterogeneity, particularly by combining active ingredients (AIs) with different modes of action. Yet, there is still no consensus on the best strategy to delay the emergence and selection of resistance in pathogen populations. Here, we aimed at contributing to this debate while addressing two questions: (i) How can we enhance the performance of anti-resistance strategies and what are the drivers of their optimization? (ii) How the initial status of resistance in populations modulates the performance of strategies? We used *Zymoseptoria tritici*, the causal agent of the major wheat disease, which is resistant in Europe to most fungicides to varying degrees, to exemplify this debate. We applied an innovative approach for fungi, experimental evolution, to mimic and accelerate, in laboratory-controlled conditions, fungicide resistance usually occurring in the field. In particular, we disentangled the relative impact in reducing resistance selection of multiple drivers of alternation, mixture and dose modulation strategies by submitting a susceptible strain to heterogeneous selection regimes as compared to the single use of the same AIs.

In a first experiment, alternation was found a beneficial or neutral strategy to decrease the rate of evolution of resistance, relative to continuous fungicide use, but resulted in higher levels of generalism. We demonstrated that the relative intrinsic resistance risk of AI probably underpinned such trade-off, more than the number of fungicides used or alternation frequency.

In a second experiment, we dissected how an efficient-dose mixture, *i.e.* a mixture with reduced doses of its components but still allowing a disease control similar to that provided by these components used alone, may delay resistance evolution of a naïve

population. We concluded that the durability of such mixtures was detrimental to beneficial, strongly depending on its components. Such mixtures possibly favoured generalist or multiple resistance patterns, depending on the combined AIs. Dose reduction in sequence strategies didn't prevent the selection of highly resistant specialist strains, nor of generalist resistance in some lines.

A final experiment was designed to validate the previous findings, while submitting a susceptible isolate and two artificial populations containing low frequencies of single *vs.* multiple field resistances to selection regimes displaying patterns differing for four possible sources of heterogeneity. Preliminary results concluded that increasing the number of AIs and varying their modes of actions were efficient to delay resistance build-up, either in mixture or alternation regimes, although it facilitated the selection of some generalism in addition to the initially introduced specialist resistances. However, none of these sources of heterogeneity were found efficient at mitigating resistance evolution when multiple resistance was introduced in populations, suggesting that recombination, occurring during sexual reproduction in field populations, should be considered for fungicide sustainability management. At last, fitness cost associated to some resistance alleles shortly delayed resistance evolution and may reshaped population structure in some situations.

Experimental evolution has proven to be a powerful tool to enrich the debate on anti-resistance strategies whilst dissecting the interplay between their drivers in realistic biological conditions. These findings open up new possibilities for tailoring resistance management and suggest that the prevention of resistance emergence would be more sustainable than the mitigation of selection.