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Multiple roads lead to multidrug resistance in phytopathogenic fungi: *Zymoseptoria tritici* as a case study

Diverses voies d'émergence menant à la résistance multidrogue chez les
champignons phytopathogènes : le cas de *Zymoseptoria tritici*

Thèse de doctorat de l'université Paris-Saclay

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Thèse préparée dans l'unité de recherche **UR BIOGER** (Université Paris-Saclay, INRAE),
sous la direction de **Sabine FILLINGER**, Directrice de recherche (INRAE, BIOGER), et le
co-encadrement d'**Anaïs LALÈVE**, ingénieure de recherche (INRAE, BIOGER)

Thèse soutenue à Paris-Saclay, le 26 janvier 2026, par

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Mots clés : Champignon phytopathogène, Résistance multidrogue, Mécanismes moléculaires, Efflux, Facteur de transcription, GWAS

Résumé : Les activités humaines favorisent l'adaptation rapide de certains organismes, notamment des ravageurs des cultures et des agents pathogènes. Puissant outil de contrôle, les substances actives (Active Ingredients, AIs), en particulier antibiotiques et pesticides, ont été largement déployées au cours des XX^e et XXI^e siècles, favorisant l'émergence de résistances chez les populations cibles. Alors que le renouvellement de l'arsenal thérapeutique ralentit et que le phénomène de résistance se généralise, il devient urgent d'adopter une approche « One Health » pour préserver l'efficacité de notre pharmacopée, pilier de la santé publique et de la sécurité alimentaire.

Dans ce contexte, nous étudions l'évolution de la résistance aux fongicides chez le champignon modèle *Zymoseptoria tritici*, agent de la septoriose du blé. En particulier, nous nous intéressons à la résistance multidrogue (MDR), un phénotype généraliste à large spectre associé à des facteurs de résistance modérés et dont l'impact sur l'efficacité des traitements en agriculture est souvent négligé. Cependant, par son caractère généraliste, la MDR est susceptible de compromettre l'efficacité des stratégies de gestion de résistance, et des AIs futurs. Ce travail de thèse présente une exploration des mécanismes et déterminants génomiques de la MDR chez *Z. tritici*, vers une meilleure compréhension de ce phénotype.

Détectée depuis 2009 au champ en Europe, la MDR chez *Z. tritici* est principalement associée à la surexpression du gène codant le transporteur Mfs1, liée à l'insertion d'éléments transposables (TE) dans son promoteur (*P_{MFS1}*), qui mène à un efflux accru non sélectif d'AIs. Toutefois, des données récentes de surveillance indiquent une augmentation quantitative des facteurs de résistance au champ, suggérant la contribution de mécanismes additionnels.

Pour identifier ces mécanismes, nous avons étudié des mutants MDR obtenus par évolution expérimentale sous différentes pressions de sélection

fongicide. Des tests d'efflux réalisés indiquent des mécanismes contrastés en fonction des mutants, impliquant ou non de l'efflux accru. Parmi les gènes candidats identifiés par reséquençage des génomes, nous avons expérimentalement validé le rôle dans la MDR de la mutation gain de fonction C740Y dans un facteur de transcription de type AtrR. Par ailleurs, des cas d'aneuploïdies associés à des profils de résistance spécifiques ont été identifiés, suggérant que la plasticité génomique participe aux dynamiques adaptatives menant à la sélection de mécanismes de MDR *in vitro*.

Nous avons également exploré les mécanismes de MDR en jeu chez les souches naturelles, en analysant 374 isolats européens (2020-2021). Le séquençage de *P_{MFS1}* a permis d'identifier un polymorphisme d'insertion de TE plus divers que décrit précédemment dans ce promoteur, partiellement associé au phénotype MDR. La quantification de l'intensité de la résistance à la terbinafine (marqueur phénotypique de la MDR) de ces isolats et de descendants issus de croisements sexués a mis en évidence la nature quantitative de ce trait. Son caractère polygénique a été confirmé à travers une étude d'association pangénomique (GWAS), révélant des gènes candidats aux fonctions putatives variées, en accord avec un déterminisme complexe observé *in vitro*.

Ce travail montre que la MDR chez *Z. tritici* peut émerger de multiples trajectoires évolutives et la sélection de mécanismes divers, incluant un efflux accru dépendant ou non de la surexpression de *MFS1*. Il met en lumière une grande plasticité génomique de ce champignon, susceptible de favoriser son adaptation rapide aux fongicides, comme documenté chez un nombre croissant d'autres champignons pathogènes. Parce que limiter la sélection de ces mécanismes représente un enjeu majeur pour un contrôle durable des maladies fongiques, cette étude souligne la nécessité de mieux intégrer la prévention de la MDR dans les stratégies de gestion de la résistance en agriculture.

Title: Multiple roads lead to multidrug resistance in phytopathogenic fungi: *Zymoseptoria tritici* as a case study

Keywords: Phytopathogenic fungus, Multidrug resistance, Molecular mechanisms, Efflux, Transcription factor, GWAS

Abstract: Human activities promote the rapid adaptation of certain organisms, notably crop pests and pathogens. Active ingredients (AIs), particularly antibiotics and pesticides, have been widely deployed over the 20th and 21st centuries as powerful control tools, favoring the emergence of resistance in target populations. As the renewal of the therapeutic arsenal slows and resistance becomes widespread, it is increasingly urgent to adopt a "One Health" approach to preserve the efficacy of our pharmacopeia, a cornerstone of public health and food security.

In this context, we studied the evolution of fungicide resistance in the model fungus *Zymoseptoria tritici*, the causal agent of septoria leaf blotch in wheat. In particular, we focused on multidrug resistance (MDR), a broad-spectrum generalist resistance phenotype. Associated with moderate resistance factors, its impact on treatment efficacy in agriculture is often underestimated. However, due to its generalist nature, MDR is likely to compromise the effectiveness of resistance management strategies and future AIs. This thesis aims to explore the mechanisms and genomic determinants of MDR in *Z. tritici*, as a basis for a better understanding of this phenotype.

Detected in the field in Europe since 2009, MDR in *Z. tritici* has been primarily associated with overexpression of the efflux transporter gene *Mfs1*, driven by transposable element (TE) insertions in its promoter (*P_{MFS1}*), leading to increased, non-specific efflux of AIs. Recent field monitoring data, however, indicate a quantitative increase in resistance factors, suggesting the contribution of additional mechanisms.

To identify these mechanisms, we investigated MDR mutants obtained through experimental evolution under different fungicide selection pressures. Efflux assays revealed contrasting mechanisms depending

on the mutant, involving increased efflux or not. Among candidate genes identified by whole-genome resequencing, we identified a gain-of-function C740Y mutation in an AtrR-like transcription factor, which we experimentally validated as contributing to MDR. Furthermore, cases of aneuploidy associated with specific resistance profiles were identified, suggesting that genomic plasticity contributes to adaptive dynamics leading to MDR selection in vitro.

We also explored MDR mechanisms in natural strains by analyzing 374 European isolates (2020–2021). Sequencing of *P_{MFS1}* revealed TE insertion polymorphism more diverse than previously described, partially associated with the MDR phenotype. Quantification of terbinafine resistance (a phenotypic marker of MDR) in these isolates and their progeny from sexual crosses highlighted the quantitative nature of this trait. Its polygenic basis was confirmed through a genome-wide association study (GWAS), revealing candidate genes with diverse putative functions, consistent with the complex determinism of MDR observed in vitro.

This work demonstrates that MDR in *Z. tritici* can emerge through multiple evolutionary trajectories, leading to the selection of diverse mechanisms, including increased efflux dependent or independent of *MFS1* overexpression. It highlights the high genomic plasticity of this fungus, which likely facilitates its rapid adaptation to fungicides, as increasingly reported in other pathogenic fungi. Because limiting the selection of these mechanisms represents a major challenge for sustainable disease control, our study underscores the need to better integrate MDR prevention into resistance management strategies in agriculture.

"Now, here, you see, it takes all the running you can do, to keep in the same place." — Lewis Carroll, Through the Looking-Glass, 1871

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ABBREVIATIONS

ABC: ATP-Binding Cassette	GluCl: glutamate-gated chloride channel
A.D: Anno Domini	GR50: Growth Reduction 50
ADP: Adenosine Di-Phosphate	GST: Glutathione S-Transferase
AI: active Ingredient	HIV: Human immunodeficiency Virus
AMR Antimicrobial resistance	HR: High resistance
AOX: alternative oxidase	IC50: Inhibitory Concentration 50
ATP: Adenosine Tri-Phosphate	IPM: integrated pest management
Ben: Benzimidazoles	LC50: Lethal concentration 50
B.C: Before Christ	LD50: Lethal Dose 50
BCA: biological control agent	LINE: long interspersed element
bp: base pairs	Log: logarithm
Car: Carboxamides	LOF: loss-of-function
CDNB: 1-chloro-2,4-dinitrobenzene	LR: Low resistance
CDR: Candida Drug Resistance	LTR: long terminal repeat
CNV: copy number variation	MAP Kinase: Mitogen-Activated Protein Kinase
COE: carboxylesterase	MAT: mating type
CYP: cytochrome protein	MATE: multidrug and toxic compound extrusion
DDT: dichloro-diphenyl-trichloroethane	Mb: mega base
DHFR: dihydrofolate reductase	MBC: methyl benzimidazole carbamates
DMI: DeMethylation Inhibitor	MDK: minimal dose to kill
DOG: DiOxyGenase	MDR: Multi-Drug Resistance
DSS: Decision Support Systems	MFS: Major Facilitator Superfamily
DTP: Drug-tolerant persister	MIC: Minimum Inhibitory Concentration
DNA: DeoxyriboNucleic Acid	MoA: Mode of Action
EC50: Effective Concentration 50	MR: Medium resistance
ECM: extracellular matrix	NBD: nucleotide-binding domains
ED50: Effective Dose 50	NTSR: Non-Target Site Resistance
EE: Experimental evolution	OECD: the Organisation for Economic Co-operation and Development
EPPO: European and Mediterranean Plant Protection Organization	PACE: proteobacterial-antimicrobial-compound-efflux
ERV: endogenous retrovirus	PCR: Polymerase chain reaction
ETC: electron transport chain	PDR: pleiotropic Drug Resistance
EU: European Union	Pi: inorganic phosphate
FPPL: first fungal priority pathogens list	Qil: Quinone inside Inhibitor
FDA: Food and Drug Administration	Qol: Quinone outside Inhibitor
FRAC: Fungicide Resistance Action Committee	QTL: Quantitative Trait Loci
fAMR: fungal antimicrobial resistance	R: resistant
GBD: Global Burden of Disease	

R&D: Research and Development
RF: Resistance Factor
RNA: RiboNucleic Acid
RND: resistance-nodulation-cell division
ROS: reactive oxygen species
R4P: the Research and Reflection Network on Pesticide Resistance
SNP: single Nucleotide Polymorphism
STB: STB Septoria Leaf Blotch
TE: Transposable Element
TIP: Transposon Insertion Polymorphism
TMD: transmembrane domain
TMS: transmembrane-spanner
Tri: Triazoles
TSR: Target Site Resistance
R6G: rhodamine 6G
S: susceptible
SDHI: Succinate DeHydrogenase Inhibitor
SDR: short-chain dehydrogenase
SGV: Standing genetic variation
SMR: small-multidrug-resistance
T1SSs: related type 1 secretion system
UGTs: UDP-Glucuronosyltransferase
USA: United States of America
USD: United States Dollar
WHO: World Health Organization
XDR: eXtensive-Drug Resistance
YPD: Yeast extract Peptone Dextrose

Introduction

Depuis le milieu du XIXe siècle, l'utilisation de substances actives (SA) s'est imposée comme un levier essentiel dans la lutte contre les agents parasites et infectieux susceptibles de compromettre la santé humaine et la sécurité alimentaire. Chez les organismes cibles, le développement de résistances comme réponses adaptatives mène à des échecs de traitement, en médecine et en agriculture, ce qui représente une menace croissante à l'échelle mondiale. La résistance aux SA se définit comme un trait stable et héréditaire conférant à un individu la capacité de survivre à un traitement qui tue ou inhibe un individu sensible et repose sur différents mécanismes moléculaires. Deux catégories de mécanismes sont distinguées, liés à la cible moléculaire de la SA et non liés à la cible. Parmi ces derniers, certains préviennent la toxicité de la SA en réduisant sa concentration intracellulaire par réduction de la perméabilité membranaire ou par intensification de l'efflux, de la détoxification métabolique et/ou de la séquestration intracellulaire de la SA. Ils confèrent ainsi un profil de résistances croisées à plusieurs SA de modes d'action différents, qualifié de résistance multidrogue (MDR). Chez les individus MDR, les mécanismes cités reposent majoritairement sur des mutations ou variations génomiques plus larges, affectant la régulation de l'expression de gènes codant des transporteurs ou des enzymes de détoxification. Chez le champignon phytopathogène *Zymoseptoria tritici*, responsable de la septoriose du blé, la fréquence de la MDR a augmenté dans les populations naturelles françaises depuis 2009 avant de se stabiliser autour de 25 % depuis 2020. De précédentes études ont attribué ce phénotype à la surexpression du gène codant le transporteur membranaire Mfs1, due à l'insertion de différents éléments transposables dans son promoteur. De récents travaux suggèrent cependant que la plasticité du promoteur de ce gène n'est pas le seul facteur impliqué dans la MDR chez ce champignon. Dans ce travail de thèse, nous cherchons à répondre à trois questions principales : (i) comment la plasticité génomique de *Zymoseptoria tritici* favorise l'émergence de la résistance multiple aux fongicides (MDR), et quels sont les déterminants génétiques sélectionnés ; (ii) quels mécanismes moléculaires et physiologiques sont concernés ; (iii) quelles leçons peuvent être tirées de ces connaissances pour alimenter les stratégies de gestion des résistances.

Chapitre I : Identification des mécanismes de MDR sélectionnés chez les souches d'évolution expérimentale

Dans ce premier chapitre, nous explorons les mécanismes et déterminants génétiques impliqués dans le phénotype MDR de 42 souches obtenues *in vitro* par différentes évolutions expérimentales antérieures à ce travail. Afin de caractériser l'implication de l'efflux dans le phénotype MDR de ces souches, nous avons effectué des tests d'efflux d'une molécule fluorescente complétés par des tests de synergie entre fongicide et inhibiteurs de transporteurs. Ces différents tests ont révélé des profils d'efflux contrastés, traduisant d'une diversité de mécanismes cellulaires, impliquant pour partie de l'efflux accru. Associées à un reséquençage du génome, ces données ont permis d'identifier des gènes candidats, notamment au travers de variants communs entre souches partageant le même phénotype. Nous avons validé fonctionnellement qu'une substitution retrouvée dans le gène *ZtAtrR* chez cinq souches évoluées est responsable de leur phénotype MDR. Ce gène code une protéine homologue du facteur de transcription AtrR, impliqué dans la résistance aux azoles et au stress hypoxique chez *Aspergillus fumigatus*. Nous avons montré que la substitution C740Y est une mutation de gain de fonction et n'est pas associée avec un coût de fitness *in vitro* indiquant une possibilité de sélection *in natura*. Nous avons également identifié, par une analyse de la profondeur de séquençage, une duplication du chromosome 7 chez certains mutants évolués, associée spécifiquement à la résistance au prothioconazole desthio, suggérant un rôle de l'aneuploidie dans la résistance aux substances actives, un résultat original dans le domaine de la phytopathologie.

Chapitre II : Identification des mécanismes de MDR chez les populations du champ

Dans ce second chapitre, nous analysons les mécanismes impliqués dans la MDR chez 374 souches naturelles collectées en Europe du Nord-Ouest entre 2020 et 2021. L'analyse de séquence du promoteur du gène *MFS1* chez ces souches a enrichi le panel d'insertions détectées dans ce promoteur de sept nouvelles insertions, soulignant son importante plasticité. Par une approche de génétique directe, nous avons validé l'implication de l'un de ces nouveaux inserts, l'indel VIII, dans la MDR. Par ailleurs, nous avons constaté une variation quantitative de la résistance à la terbinafine, indicateur de la MDR chez les champignons phytopathogènes, parmi les souches naturelles partageant le même génotype du promoteur de *MFS1*. Ces résultats suggèrent un déterminisme polygénique de la MDR chez ces populations naturelles. Afin d'investiguer ce déterminisme, nous avons conduit une analyse d'association pangénomique (GWAS) intégrant 128 souches phénotypées pour leur résistance à la terbinafine et les données de séquençage complet du génome. Celle-ci a confirmé l'association de la MDR avec le locus *MFS1* dans les populations MDR contemporaines mais a également révélé d'autres SNP candidats, confortant l'hypothèse d'un déterminisme polygénique de la MDR.

Résultats additionnels : Indices impliquant la plasticité génomique dans la MDR

Cette partie présente les résultats obtenus, suivant l'approche employée dans le chapitre I, pour 11 souches évoluées à partir d'une souche ancestrale différente. Chez celles-ci, le nombre de mutations identifiées à l'échelle des régions géniques était dix fois plus élevé comparé aux mutants présentés dans le chapitre I. Cela suggère un taux de mutation supérieur chez la souche ancestrale concernée, concordant avec de multiples mutations identifiées dans des gènes codant des protéines du système de réparation de l'ADN « mismatch repair » (MMR). Les souches évoluées présentent un phénotype MDR large, associé à des profils d'efflux contrastés et des mutations affectant de nombreux gènes, certains codant des transporteurs membranaires et enzymes de détoxification (GST, CYP450, SDR...). Par ailleurs, cinq de ces souches présentent également une duplication du chromosome 7, strictement associée avec la résistance au prothioconazole destio et confortant certaines conclusions du chapitre I. Ces résultats additionnels corroborent le rôle de la plasticité génomique du champignon dans l'adaptation aux fongicides.

Discussion générale et conclusions

La mise en perspective des résultats des trois parties et leur confrontation aux conclusions de la littérature met en évidence des facteurs clé dans les dynamiques de sélection de résistance chez *Z. tritici*. Des exemples d'implication de facteurs de plasticité génomique, tels que l'aneuploidie ou l'hypermutableté, dans l'adaptation aux fongicides ont été mis en évidence chez différentes espèces de champignon. Les résultats obtenus dans le cadre de ce travail révèlent un probable rôle de tels facteurs, identifiés à la fois chez les souches du champ et les souches d'évolution expérimentale, dans l'acquisition de la MDR en conditions naturelles et contrôlées chez *Z. tritici*. Concernant les déterminants génétiques sélectionnés, s'ils ne convergent pas vers des candidats communs entre les souches naturelles et évoluées, c'est en revanche le cas de la fonction annotée des gènes impactés. En effet, pour les couches naturelles et évoluées, les gènes candidats sont impliqués dans du transport membranaire ou de la détoxification. Ainsi les déterminants génétiques et mécanismes moléculaires associés à la MDR chez *Z. tritici* apparaissent multiples et variés, potentiellement additifs en contextes génomiques recombinés. Ces résultats questionnent l'intégration de la MDR dans l'évaluation des stratégies de gestion de la résistance. En effet, les modalités de sélection des souches évoluées, mimant des stratégies de gestion de résistance appliquées au champ, ont sélectionné ces mécanismes de résistance généralistes contrastés. Cela soulève une inquiétude quant à la pérennité de ces stratégies de gestion et des futures SA introduites, dont l'efficacité pourrait être affectée par de tels mécanismes.

INTRODUCTION

1 Escaping chemical control: a One Health concern

1.1 Historical context of pest and disease management

1.1.1 Living with pests and diseases

Since sedentarization, components of the anthropogenic biomes from human body to domesticated organisms (crops, livestock and domestic animals) represent ecological niches for pathogenic, parasitic and pest species. Highly damaging to human lives, because of their direct effect on health or famine they likely induce, their impact has been reported in the most ancient writings. Centuries of magic and religious beliefs associated with these organisms predate our scientific understanding of them. This is a testament to the concerns they have represented throughout human history. As examples, we can cite the rites dedicated to the Roman goddess Robigo, popular in the early first century A.D. and mentioned in *The Fasti* by Ovid (translation of Nasón Ovidio et al., 1850) for protecting wheat from diseases, especially rust, now known to be caused by phytopathogenic fungi (Flint and van den Bosch, 1981). Another example comes from the Book of Exodus in the Ancient Testament. The ten plagues of Egypt, a list of infestations and diseases, are presented as a divine punishment against the Egyptian people for Pharaon not letting the Hebrews go. They include notably human boils and lice infestations, livestock pestilences and locust invasions devastating the harvests (Hort, 1957). Despite lack of historical records of such events in the considered time lapse, their importance in a writing that is the cornerstone of widely practiced religions gives insight into the aureole of power and fear surrounding these organisms in the collective imagination through history.

More recent human epidemics also marked history, as the Black Death caused by the bacteria *Yersinia pestis* that ravaged Europe during the first half of XIVth century, but also viral infections as the influenza epidemic (Spanish flu) sadly known for having caused between 20 and 50 million deaths in the beginning of the XXth century (Dobson and Carper, 1996; Nelson and Williams, 2007; Sakai and Morimoto, 2022). Crop and livestock epidemics have been quite

deadly too. As an example, the potato blight outbreak caused by the oomycete *Phytophthora infestans* ("The Great famine") caused an estimated 40 % potato yield loss in Ireland between 1845 and 1846, leading to a large extent famine estimated to have caused roughly one million deaths and massive exode (Ó Gráda, 2004). These examples traduce the major driving force these agents exerted on human population dynamics and justify the will for controlling them.

Before the XIXth century, infection and infestation control mainly relied on magic and religious considerations but also empirical observations, leading to actions and recommendations with variable efficacy and outcomes.

Prophylaxis measures were reported quite early in history since the concept of contagion was integrated long before the understanding of the biological basis of infections, as evidence by systematical exclusion of lepers from society in middle age (Nelson and Williams, 2007) or recommendation of isolation of symptomatic animals in herds since Roman empire (Columella 50 A.D., Taylor et al., 2005). Crop protection was also addressed through sanitary measures. For example, the 1660 law from the legislative authorities of Rouen, France, that required the destruction of barberry bushes in wheat-growing areas to fight rust. This occurred long before the understanding of the biological cycle of *Puccinia graminis*, the wheat rust fungus, that uses barberry as an alternative host (Ebbels, 2003).

Premises of modern biological control with natural predators were also reported in ancient Egyptians papyri suggesting conscious use of cats to control rodents and serpents (Baldwin, 1975). The first mention of use of natural enemies to fight insects dates back to 300 A.D. with records of voluntary establishment of predatory ants nest in citrus orchards to control caterpillars and beetles in China (Flint and van den Bosch, 1981).

Human also didn't wait for the advent of organic chemistry to benefit from natural active substances, including to control infectious diseases and pests. Hippocrates (459–370 BC) classified 300 medicinal plants by physiological action. He reported, for example, effects of garlic against intestinal disorders that may have been caused by intestine parasites since recent studies show *in vitro* anti-parasitic activity of garlic extracts and sulfur secondary compounds they contain (Krstin et al., 2018; Petrovska, 2012). Regarding other pests, sulfur compounds were also used to control insects and mites by the Sumerian way before 2500 B.C. By 1200 B.C., Chinese farmers used seed treatment and fumigation of plant-derived insecticides and

employed mercury and arsenic to control body lice and other pests (Flint and van den Bosch, 1981).

Since Renaissance and the discovery of microorganisms, technicity of pest and disease control methods evolved along science progresses. Effective botanical insecticides such as pyrethrum, derris, quassia, and tobacco leaf infusions were introduced in Europe in the XVIIIth century. Later, in the late 1800s, the Bordeaux mixture, a combination of hydrated lime and copper sulfate, was developed as a treatment against downy mildew in vineyards and is still in use today (Flint and van den Bosch, 1981).

By the turn of the XIXth century, major conceptual approaches had been developed in pest control and are still valid today, including chemical one (Ebbels, 2003). However, despite scientific advances in agronomic and medical knowledge, only a few drugs were available to treat pests and human diseases in the early 1900s' (quinine for malaria, ipecacuanha for dysentery and mercury for syphilis treatment, Flint and van den Bosch, 1981; Ng, 2003). Major breakthroughs in pest and disease management that had a significant impact on the outcome of outbreaks across the XXth century occurred after the World War I and the advent of organic chemistry. The modern pharmaceutical industry emerged and revolutionized crop protection and modern medicine with the production and the deployment of synthetic pesticides and drugs (Flint and van den Bosch, 1981; Ng, 2003).

1.1.2 Anthropocene: green revolution and major medical breakthroughs

1.1.2.1 Discovery and use of active ingredients (AIs)

Experiments from the early 1900s provided the framework for the screening, purification, formulation, and supply of active ingredients (AIs). The cooperation between biology and chemistry in the successive discoveries of the antibacterial effects of a *Penicillium chrysogenum* strain against *Staphylococcus spp.* (Fleming, 1929) and the purification of penicillin (Chain et al., 1940) led to the commercial production of this well-known antibiotic in 1945. This set up the paradigm for drug discovery research. As a consequence, increasing numbers of antibiotics enriched the therapeutical arsenal in the following years (Fig. 1) (Aminov, 2010; Ng, 2003).

AIs can be defined as substances that have a toxic effect on target organisms. As illustrated in Fig. 1 with antibiotics, AIs discovery historically relied on two main approaches (i) focusing on screening of natural compounds from mineral and mainly biological sources,

eventually synthesized by organic chemistry later, (ii) screening a chemical library of compounds obtained through organic chemistry. Thus, molecules corresponding to this definition and used for treating infections and infestations caused by any proliferating agent, cell or organism susceptible to cause disorders in human health and/or food supply, will be referred to as AIs later on. It englobes compounds displaying toxic effect against virus (antiviral drugs), bacteria (antibiotics), fungi (antifungal or fungicide drugs), weeds (herbicides), *protozoa* (anti-*protozoal* drugs), worms (anti-helminthes drugs), insects (insecticides), mites (acaricides), mollusks (molluscicides), rodent (rodenticides) and cancer cells (anticancer drugs).

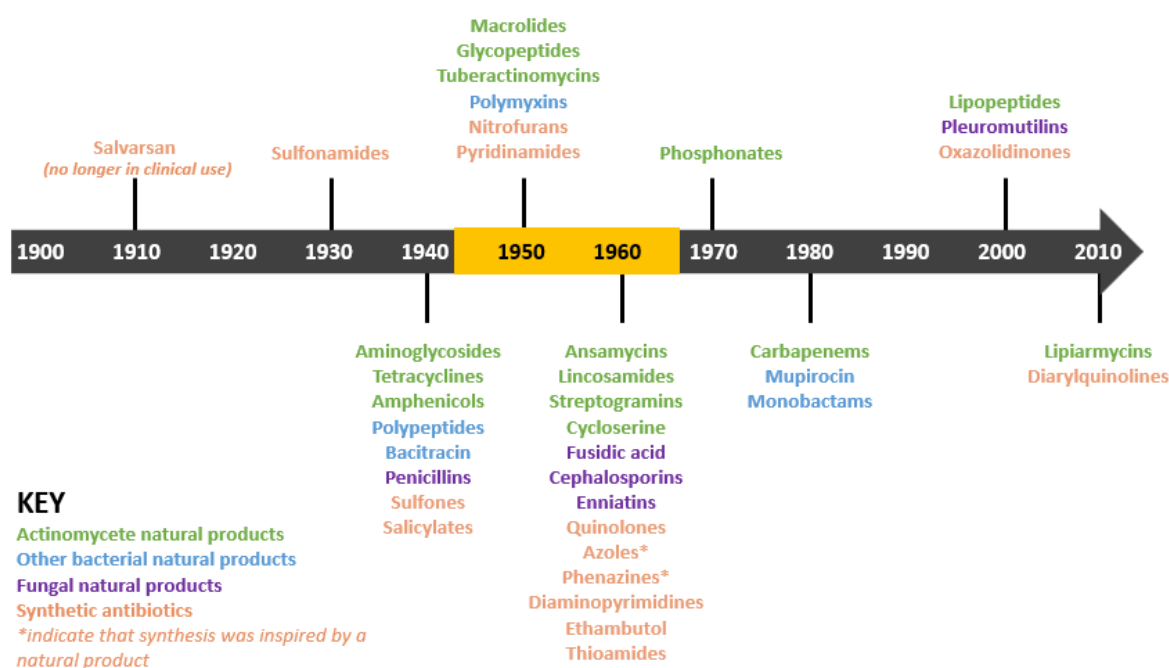


Fig. 1: Timeline of discovery of new classes of antibiotics and origin of these classes. Adapted from Hutchings et al. (2019). Period in gold is referred to as "Golden Age" of antibiotics.

Pesticide research also benefited from a similar dynamic. Major crop protection products were developed in the second mid of the XXth century (Fig. 2). After the 1940s, a large-scale screening process was initiated by the USA to test the insecticidal activity of hundreds of chemicals from international manufacturers. This led to the development of highly effective insecticidal compounds, including dichloro-diphenyl-trichloroethane (DDT), organophosphates, and carbamates. First used to control insect-borne human diseases, they were later applied in the field to control insect pests in agriculture. Similarly, 2,4-D and other selective herbicides were introduced in the 1940s (Flint and van den Bosch, 1981).

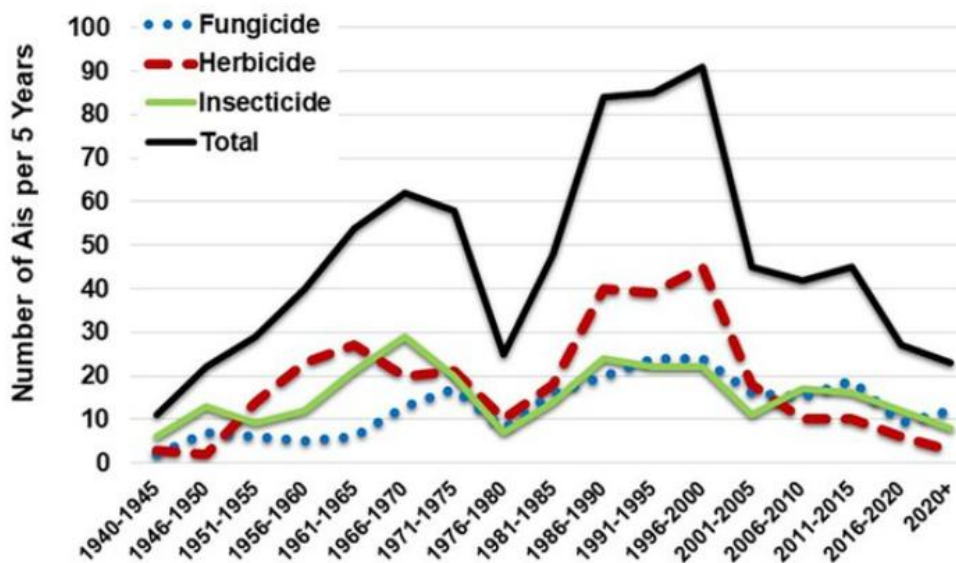


Fig. 2: Timeline of the introduction of new crop protection compounds (fungicides, herbicides, insecticides) in the 1940–2020 period. From Sparks and Bryant (2021).

The flourishing breakthrough in drug discovery was not restricted to antibiotics and pesticides. It also led to the discovery and use of AIs against various disorder agents and vectors. Some examples are listed below.

- Synthetic derivatives from quinine, isolated from the bark of the cinchona tree, chloroquine and quinacrine were synthesized in the 1930s and used for treating protozoal diseases (amoebiasis, malaria, cryptosporidiosis) (Khaw and Panosian, 1995).
- Warfarin, hemorrhagic active compound derived from dicumarol (identified in spoiled sweet clover) was registered by the early 1950s for rats and mice control. Since then, anticoagulant AIs are used for rodent control in agriculture, urban space as well as island restoration projects (Rattner et al., 2014).
- Idoxuridine, synthetic molecule originally described as a potential antitumor agent, was the first antiviral drug to be used and was approved in 1963. Since then, 90 antiviral drugs have been approved for the treatment of human viral diseases, including HIV, hepatitis B, influenza virus infection, cytomegalovirus infection, varicella-zoster and papillomavirus infections (De Clercq and Li, 2016).
- Combination of several AIs developed between the 1940s and 1960s, including nitrogen mustard, methotrexate (folic acid synthetic derivate) and vincristine (alkaloid derived from

Vinca rosea) set up the basis of cancer chemotherapy in the 1960s, with major breakthrough in both leukemia and Hodgkin's disease treatment (DeVita and Chu, 2008).

- Azole drugs identified by screening synthetic compounds (Vanreppelen et al., 2023) are used in controlling human fungal infections, like candidiasis or aspergillosis, as well as damages on crops at pre and post-harvest stages. These were introduced in the field with imazalil (imidazole) in the late 1970s (Jørgensen and Heick, 2021) and in the early 1980s for systemic clinical use of the imidazole derivative ketoconazole (Maertens, 2004).
- Ivermectin, derived from metabolites produced by the actinomycete *Streptomyces avermitilis* and launched in 1981, was initially used as an antiparasitic drug for treating nematode and arthropod ectoparasites infestations in livestock and pets. Later approved for human use, it is now used against onchocerciasis, lymphatic filariasis, scabies and, more recently, head lice (Crump, 2017).

1.1.2.2 Mode of action

Toxic effects of Als against their target rely on their disturbing effect on different cellular processes, often inhibiting or mimicking a protein involved in an essential biological pathway (Gould et al., 2018). Als can be classified in many ways, according to their chemical structure (Martin et al., 1968), their acute toxicity (WHO, 2019), their target organism or their biological target (i.e. Mode of Action, MoA). Here we chose to distinguish them based on their MoA. This introduces the concept of multi-site *versus* uni-site Als. Multi-site Als have broad cellular toxic effects, disrupting several cellular functions. Preeminent examples are fungicidal copper-based preparations or synthetic chlorothalonil, a non-systemic broad-spectrum fungicide (Tillman et al., 1973). There are also multitarget drugs for medical applications such as the antitumor drug sorafenib, a multi-protein kinase inhibitor (Wilhelm et al., 2004), the first multitarget anticancer drug to be approved by the FDA. However, in both sectors AI development has been followed the "one drug, one target" paradigm for long, stating that specific (single-target) treatments would be less toxic than multitarget treatments due to reduced off-target effects (Talevi, 2015). This led to concentrate discovery efforts on uni-site Als, specific to one target protein as it is illustrated by the number of multi-site *versus* uni-site fungicides developed since 1950 (Fig. 3).

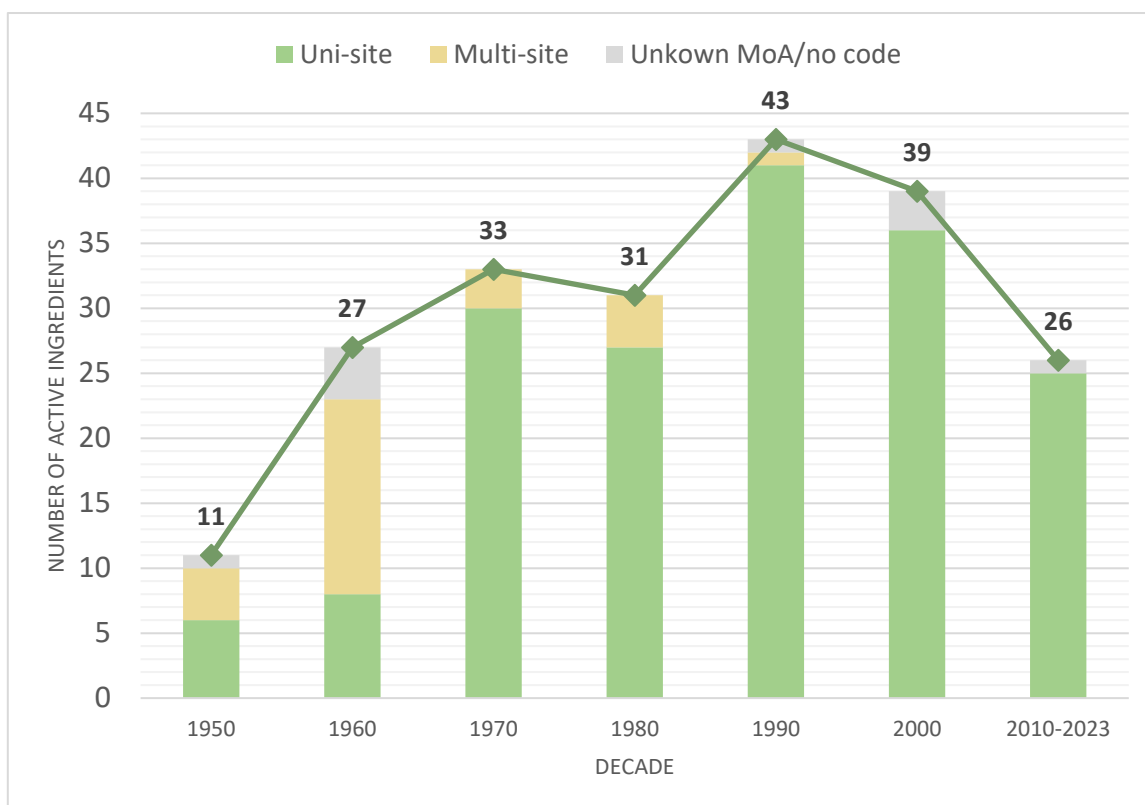
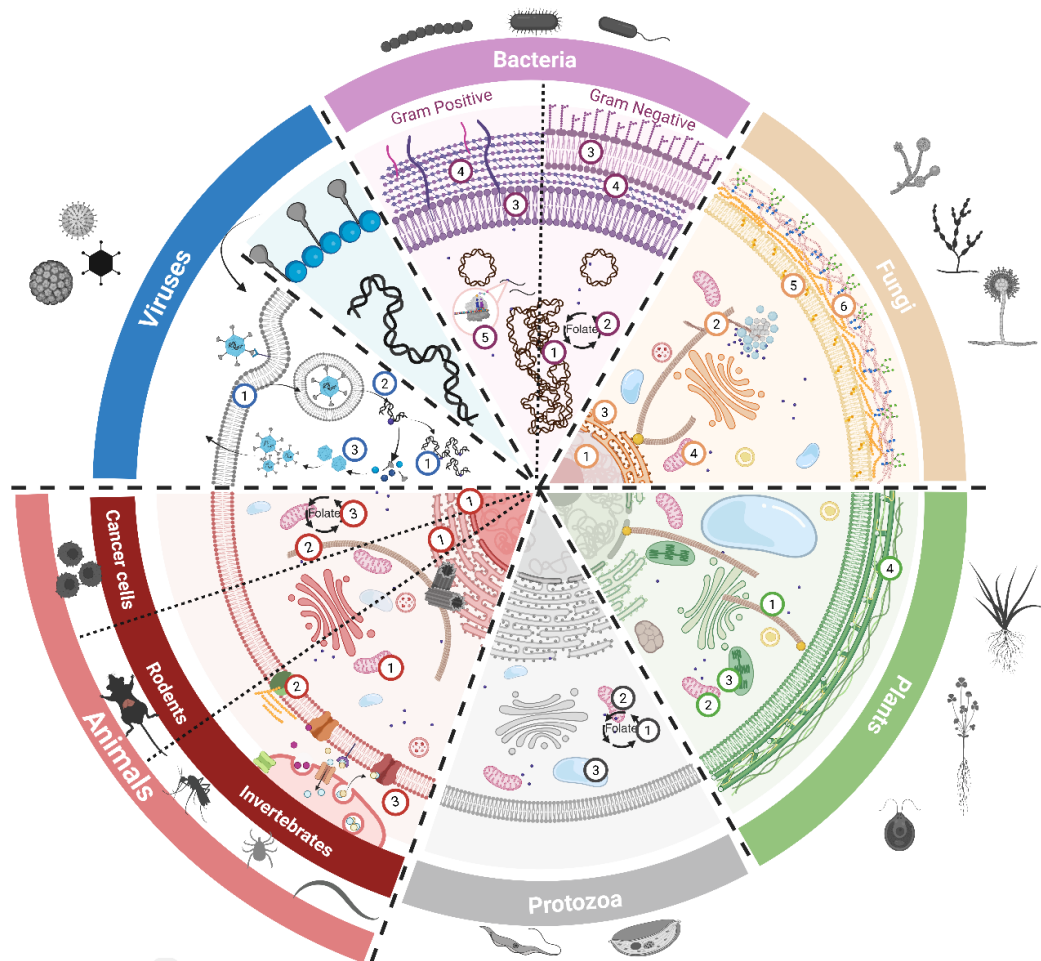


Fig. 3: Discovery and development of fungicide according to their number of cellular targets (unisite, multisite or unknown). Adapted from data of Beckerman et al. (2023).

Major cellular processes inhibited by commonly used active ingredients (AIs) against a range of infectious agents, pests and parasites are summarized in Fig. 4. This figure highlights both the specificities and shared targets among AIs developed for different pests. The number of distinct cellular targets is limited, as living organisms share fundamental pathways. Consequently, many active ingredients converge on the same essential processes, such as folate metabolism, DNA replication, or cytoskeletal dynamics, to suppress growth across diverse organisms.



	Animal			Protozoa	Plant	Fungi	Bacteria	Virus
	Cancer	Rodent	Invert.					
Nucleic acids	1 Alkylating agents cyclophosphamide Topoisomerase inhibitors irinotecan, etoposide					1 Nucleic acid metabolism inhibitors ipflufenquin furalaxyl	1 Gyrase inhibitors ciprofloxacin RNA pol inhibitors rifampicin	1 Nucleoside analogs acyclovir, ribavirin RT inhibitors tenofovir
Microtubules	2 Microtubule inhibitors paclitaxel, vinblastine				1 benzamide, dinitroaniline, benomyl, carbendazim	2		
Metabolite synthesis	3 Antifolates methotrexate			1 proguanil, sulfadoxin	2 Fatty and amino acid, carotenoid BS inhibitors sulfonylurea isoxazolidone	3 Ergosterol BS inhibitors mefenitruconazole, terbinafine	2 Antifolates trimethoprim	
Respiration		1 cyhexatin rotenone phosphine		2 ETC inhibitors atovaquone	2 dinitrophenol	4 benzovindiflupyr, azoxystrobin, methyltetraprol		
Membrane properties						5 Ergosterol binding amphotericin B	3 Phospholipid binding polymyxins	2 Attachment, penetration inhibitors maraviroc, enfuvirtide Release inhibitor peramivir
Cell wall integrity		2 CHS inhibitors lufenuron			3 CS inhibitors dichlorobenzonitrile	6 CHS and β 1,3-glucan synthases inhibitors polyoxin caspofungin	4 Cell wall synthesis inhibitors penicillin, cephalosporin	
Other	1 Anticoagulants (AVK) wafarine bromadiolone	3 Neuromuscular toxins DDT nicotin abamectin avermectin		3 Heme detoxification inhibitors chloroquine mefloquine	2 Photosynthesis inhibitors pyridinium aminotriazinone phenylpyrazole		5 Ribosome inhibitors erythromycin kanamycin	3 Uncoating inhibitors amantadine 4 Maturation inhibitors telaprevir

Fig. 4: Overview of cellular processes targeted by clinical and agriculture active ingredients in different target organisms.

In lower part the molecular targets are explicitly stated and associated with some examples of active ingredients used. BS: Biosynthesis, CHS: Chitin Synthase, CS: Cellulose Synthase, DDT: Dichlorodiphényltrichloroéthane, ETC: Electron Transport Chain, RT: Retrotranscriptase.

Data collected from <https://www.frac.info/>, <https://irac-online.org/>, <https://www.hracglobal.com/>, De Clercq and Li, 2016; De Rouck et al., 2023; DeVita and Chu, 2008; Halawa et al., 2024; Rattner et al., 2014; Sparks et al., 2020. Created with Biorender.com.

1.1.2.3 *Als-associated gains and costs*

1.1.2.3.1 *Gains on human life expectancy and food supply*

The golden age of Als' discovery participated to major breakthroughs in both medicine and agriculture. The direct gain appears quite obvious on human health. Along with hygiene conditions and vaccination campaigns, AI-based therapies contributed to dramatically decrease the human mortality rate due to infectious diseases between 1900 and 2000, as illustrated by USA data in Fig. 5. More specifically, a 23-year human lifespan gain was attributed to antibiotics (Hutchings et al., 2019).

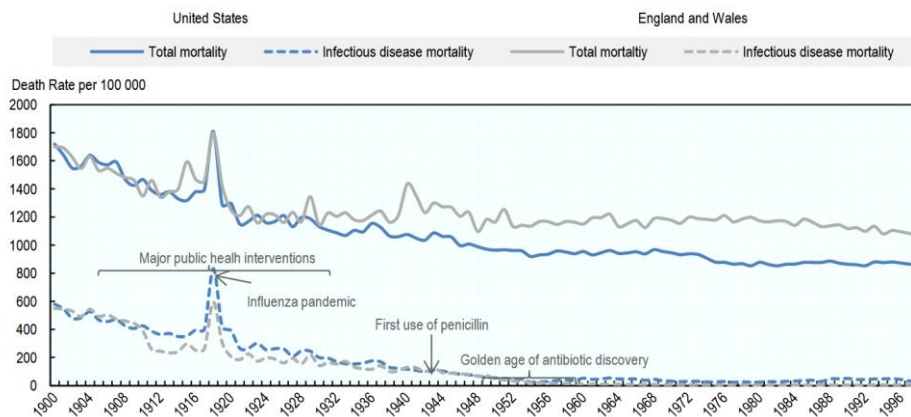


Fig. 5: Evolution of the crude mortality rates (per 100 000 people) of infectious diseases versus other death causes, in the United States of America over the XXth century. From OECD (2018).

The 1966-1985 time period referred to as the first Green Revolution also marked major advances in agricultural productivity (Pingali, 2012). Among its critical components, the development of high-yield varieties accelerated by advances in genetic knowledge was the most determinant driver, but mechanization and improved inputs including irrigation and plant protection products (pesticides and synthetic fertilizers) were also of critical importance (Pingali, 2012). Global adoption of these technologies led to important gains in major crop yields, which have roughly doubled since the 1960s (Fig. 6), notably in low-income countries (FAO, 2004), resulting in food price reductions (Hayami and Herdt, 1977; Scobie and T., 1978). In practice, the annual gain in yield rate has been accompanied by an ever-increasing use of Als until today. In 1997, Pimentel and collaborators estimated a \$40 billion USD gain for \$10 billion USD invested in pesticides annually in the USA (Pimentel, 2005). Over the last 30 years, the total pesticide use has doubled, reaching 3.7 million tons of Als in 2022: 50% herbicides, 22% insecticides and 25% fungicides (FAO, 2024).

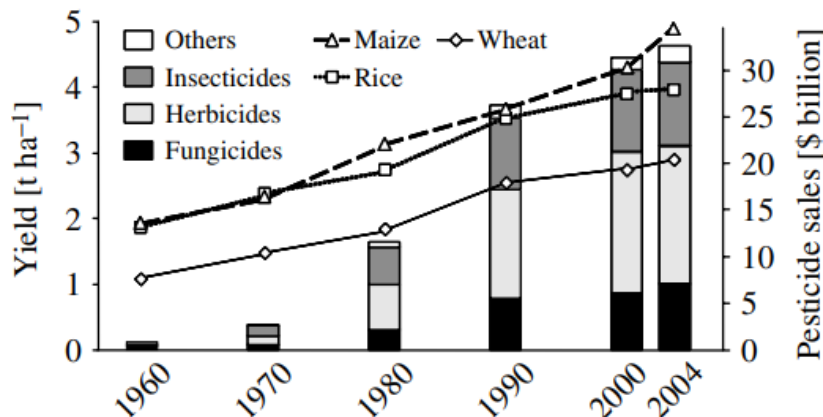


Fig. 6: Parallel evolution of yield gains and Active Ingredient (AI) use in agriculture. From Oerke, 2006.

The twentieth century witnessed a dramatic growth in the human population, rising from 1 to 7 billion individuals between 1900 and 2000 (Van Bavel, 2013). This growth was due, in part, to the effective control of two main factors of high crisis mortality: epidemics and famine, which were at least partially addressed by pharmaceuticals. However, the costs of chemicals and especially pesticides to human health and the environment offset their positive aspects and question the sustainability of their use in the future.

1.1.2.3.2 Risks and limitations of AIs

Concerns regarding consumer and environmental health arose in parallel with the Green Revolution. As a turning point, Rachel Carson's book "Silent spring", published in 1962 as reaction to the increased use of DDT in the USA, raised concerns among both the general public and the scientific community on the matter (Epstein, 2014). Indeed, it turned out that many pesticides were shown to have carcinogenic effects likely to increase cancer risk in humans. Data collected in 1987 indicated that 90% of all fungicides used at that time displayed dietary oncogenic effects on laboratory mice (National Research Council (US) Committee on Scientific and Regulatory Issues Underlying Pesticide Use Patterns and Agricultural Innovation, 1987). Their toxicity to off-target organisms was also pointed out, since their large-scale use imposes uniform exposure of macro- and microorganisms in field ecosystems and beyond (through surface runoff and soil leaching) leading to accumulation of the most persistent molecules in the environment. A recent study conducted on soils collected from European countries reported 83% of the tested soils contaminated with pesticide residues in a total of 166 different mixtures (Silva et al., 2019).

In 2005, Pimentel and collaborators intended to estimate the costs of externalities associated with pesticide use in the USA and compared them to the economic gains cited before. They identified as relevant externalities:

- Impact on human health, including acute poisoning and increased cancer risks (primarily concerning applicators) and pesticide residues in food.
- Off-target toxic effects on beneficial natural predators and parasites, wild birds and mammals, fish, microbes and invertebrates, and pollinators.
- Direct crop production losses, due to plant damage caused by inappropriate dosage of the AI or excessive accumulation of pesticide residues necessitating the destruction of harvests.
- Pollution of ground and surface water involving monitoring and clean-up and other government funds for pesticide pollution control.

They estimated a minimal cost of \$12 billion each year due to the negative effects of pesticides on these externalities, particularly offsetting the \$40 billion gain discussed before (Pimentel, 2005). Subsequent meta-analyses conducted worldwide have supported this estimate, sometimes reporting even higher values (Alliot et al., 2022; Bourguet and Guillemaud, 2016). To address this issue, public policies have been launched in several countries aiming to reduce conventional pesticide use, with different outcomes (Lee et al., 2019). However, globally, the use of pesticides is still rising over time (FAO, 2024).

Despite these costs, AIs remain essential tools for maintaining human health because of the gains described above. Therefore, one of the most critical costs associated with their use lies in their widespread application that undermines their own sustainability by promoting the rapid evolution of resistance in target organisms.

Indeed, since the beginning of the Anthropocene, the scale, intensity and connectivity of human activities have profoundly modified a substantial fraction of the world's resources (Vitousek et al., 1997). In this regard, humans constitute now the major evolutionary force on earth, influencing both the rate and direction of evolution of organisms (Latta, 2008; Palumbi, 2001). Among the outcomes of this phenomenon, the widespread use of AIs represents a stringent selective pressure imposed by humans and drives the adaptation of all target organisms, pests, pathogens and also cancer cells, in short timescales (Jørgensen et al., 2018).

In fact, in 2014, more than 11,000 cases of pesticide resistance were documented worldwide, regarding roughly 1000 species of weeds, insects and plant pathogens (Tabashnik et al., 2014). In 2020, 16,000 documented cases of insecticide resistance alone were reported (Sparks et al., 2020). Both in agriculture and clinical settings, the phenomenon is characterized by an ever-increasing number of cases over time (Fig.7).

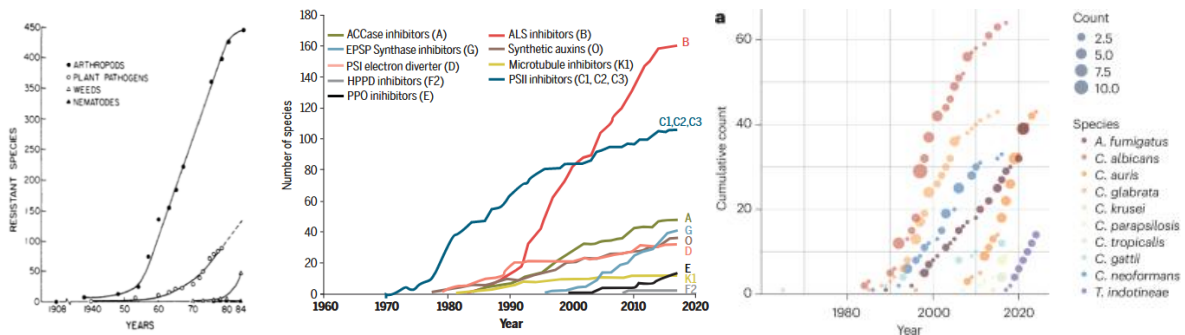


Fig. 7: Evolution of resistance against Active Ingredients (AIs). A: in arthropods, insects, weeds and plant pathogens between 1940 and 1984 (Georghiou, 1986), in weed species to the different herbicide classes between 1960 and 2020 (Gould et al., 2018) and in ten clinically relevant pathogenic fungal species between 1980-2024 (Van Rhijn and Rhodes, 2025).

In the Living with Resistance project, Jørgensen and collaborators introduced the concept of ecosystem services associated with AI susceptibility. In this framework, susceptible organisms and genotypes are viewed as a critical biological resource that contributes to the long-term sustainability of AI use. Recognizing the depletion of this resource as a major risk, the authors proposed the concept of an Anthropocene safe operating space for antibiotic and pesticide resistance (Fig. 8), which provides a framework to assess the status of susceptibility at population and ecosystem scales. Using this approach, they concluded that all six defined safe boundaries of this operating space have already been crossed (Jørgensen et al., 2018).

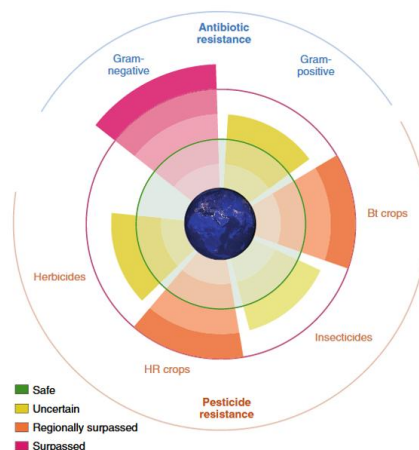


Fig. 8: State of the Anthropocene operating space of biocide susceptibility. From Jørgensen et al. (2018). For

antibiotics (blue labels), the state is assessed for Gram-negative and Gram-positive bacteria. For pesticides (brown labels), the state is assessed for resistance associated with two types of transgenic crops, as well as in general for herbicides and insecticides. Three of the six variables are surpassed regionally or globally; none are in the safe zone.

This work emphasizes the global urge to address and manage the rising burden of AI resistance.

1.2 The burden of AI resistance

1.2.1 Actual and future socio-economic costs

Today, some bacterial and fungal infections are resistant to all AIs available for their treatment. Antimicrobial resistance (AMR), primarily referring to antibiotic resistance, was estimated to be associated with 1.27 million deaths worldwide in 2019 (Murray et al., 2022). AMR in the six leading resistant pathogens (“ESKAPEE” group: *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*) was estimated to be directly responsible for 929,000 (660,000–1,270,000) deaths the same year. Overall, infections caused by resistant bacteria claim around 79,000 deaths annually across the 34 high-income countries included in the 2023 OECD analysis (OECD, 2023). The projection of the impact of AMR on human life varies considerably across studies. As one example, the Global Burden of Disease (GBD) project estimated that 1.91 million deaths worldwide could be attributed to AMR by 2050 and four to five times as many deaths potentially resulting from indirect effects (Naghavi et al., 2024).

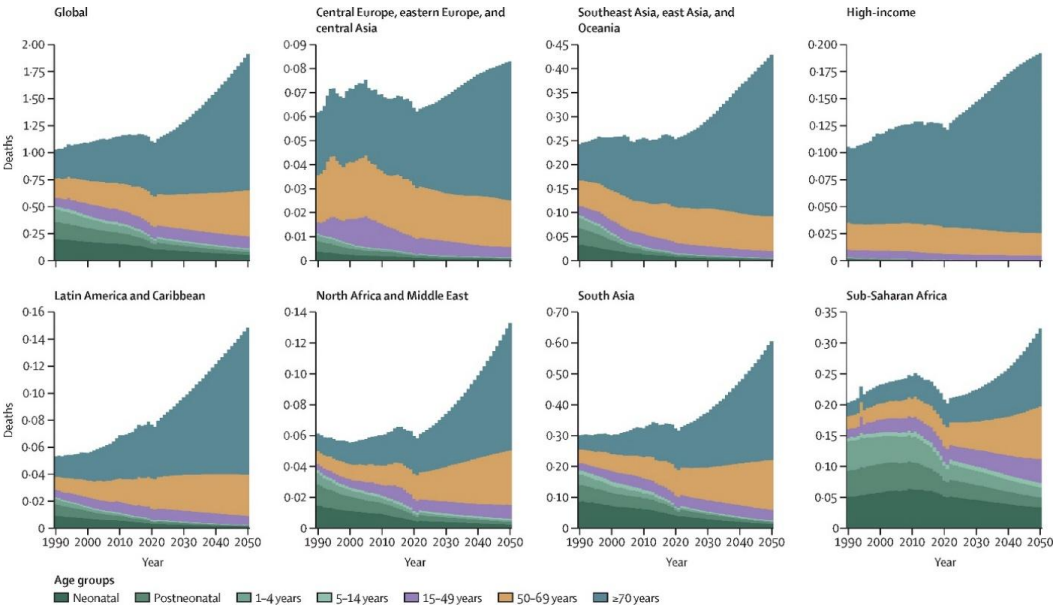


Fig. 9: Recorded and projected deaths attributable to bacterial AMR by age group and location. From Naghavi et al. (2024)

Beyond bacteria, fungi also pose a major threat to human health and fungal antimicrobial resistance (fAMR) is of increasing concern (WHO, 2022). In 2023, over 3.8 million people died from fungal infections (Mudenda, 2024). In its first fungal priority pathogens list (FPPL) published in 2022, the World Health Organization (WHO) pointed out several species as critical, namely *Cryptococcus neoformans*, *Candida auris*, *C. albicans* and *Aspergillus fumigatus*, associated with increased morbidity and mortality and known to develop fAMR (Fisher et al., 2024). For example, Lockhart and collaborators (2017) reported a 59% death rate in a cohort of 54 patients from three different continents infected by *Candida auris*, associated with 41% fungal isolates resistant to two classes of medical antifungals and 4% to three classes (Lockhart et al., 2017). Resistance to antiprotozoal drugs matters too. In Sub-Saharan Africa, malaria deaths increased threefold between 1980 and 2004 concomitantly with the spread of chloroquine resistance (Murray et al., 2012). Under the scenario of widespread artemisinin resistance, an annual excess of 116,000 malaria deaths may be reached (Lubell et al., 2014).

Several studies intended to evaluate the economic costs associated with accelerated human-driven evolution, in both medical care and agriculture. In high-income countries (USA, EU or OECD countries), AMR in healthcare is estimated to cost annually > \$20 billion (Centers for Disease Control and Prevention (U.S.) et al., 2013; OECD, 2023). Losses in agriculture were estimated at \$7-35 billion in the USA in the early 2000s (Palumbi, 2001).

Retreatment is an important component of these direct financial costs, estimated to account for 10% of costs due to resistant pests through respraying fields (Palumbi, 2001). It is also the case in response to clinical failure, notably because second-line treatments are more expensive than first-line ones (Centers for Disease Control and Prevention (U.S.) et al., 2013).

AI resistance also leads to indirect costs. These are difficult to address and predict, since AI resistance is associated with many externalities with varying degrees of implications and potential feedbacks. For example, retreatment in case of control failure increases the dose of AI delivered or even leads to choose more toxic compounds in lack of other solutions. Both may accentuate the selection for resistance and increase the exposure of humans and the environment to toxic compounds, aggravating the risks described.

AMR is also a threat to the management of chronic diseases, including cancer, and to surgical procedures in the future. Patients undergoing chemotherapy or major surgeries have

weakened immune systems, putting them at a higher risk of developing infections, including AMR ones. Medical AMR may also impact the quality of life of healthy individuals. According to the 2018 OECD report, non-life-threatening procedures may be limited in the future due to the high risk of AMR-related infection and mortality (OECD, 2018).

The large-scale and cross-sector nature of AI resistance makes it difficult to fully understand in its intrinsic dynamics, which may involve feedback effects of unintended impacts. Therefore, it makes it difficult to estimate its costs precisely. However, some contributing risk factors have been identified and discussed in the forthcoming sections.

1.2.2 Factors Accelerating the pace of AI resistance

1.2.2.1 A restraint diversity of mode of action

1.2.2.1.1 Limited by costs and withdrawal

The risk of resistance selection was identified as early as 1914 when Melander reported decreased efficacy of sulfur-lime against resistant insect pest populations (Melander, 1914). Alexander Fleming himself warned of this risk after his discovery of penicillin (Fishman, 2012). Initially, this risk was offset by the increasing rate of discovery of new classes of antibiotics, reaching a golden age in the 1950s-1960s. However the 1990s marked the beginning of the discovery void for antibiotics as the pace of discovery slowed (Fig. 10A, Silver, 2011).

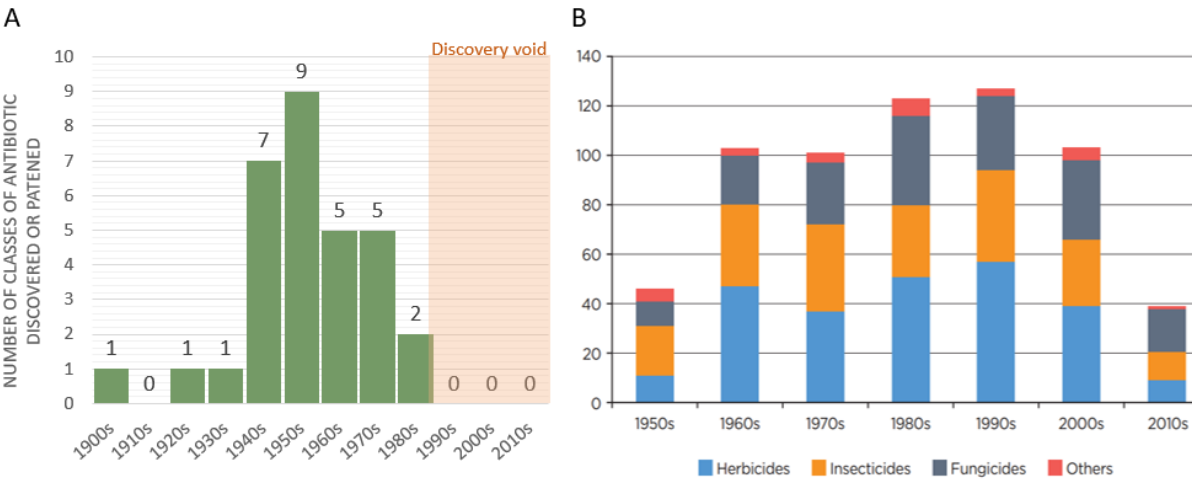


Fig. 10: Chronology of discovery or patenting of new antibiotic classes (A, adapted from Renwick and Mossialos, 2020) and of active ingredients for crop disease control (B, from McDougall, 2018).

This slowdown can be explained by a combination of financial, regulatory, and scientific barriers, and because the focus of R&D has shifted from antibiotics to other therapeutic areas (Renwick and Mossialos, 2018). Concomitantly, the global call for reducing the use of

antibiotics for preserving their efficacy also decreased the interest of pharmaceutical industry to invest in their development (OECD, 2018).

The discovery rates of pesticides showed a similar pattern (Fig. 2, McDougall, 2018; Sparks and Bryant, 2021), particularly for herbicide development, with no new modes of action commercialized in the last 30 years (Gould et al., 2018). The evolving regulatory framework is considered to play an important role in this phenomenon. With growing evidence of health and environmental impacts of pesticides, the regulatory guidelines were extended to include toxicological and ecotoxicological risk assessments during the developing pipeline (e.g. Regulation (CE) No 1107/2009 for pesticides) in order to prevent deleterious outcomes of pesticides on human health and the environment (Sparks and Bryant, 2021). Thereby, as estimated by McDougall's study (2016), the overall cost for research and development of a new AI doubled between 1995 and 2010. In the meantime, the time from discovery to launch of a new AI was extended by three years and the fraction of screened compound that eventually reach the market was reduced by a factor of three (McDougall, 2016).

Resistance coupled with increasingly stringent regulations, especially in Europe, has resulted in the withdrawal of many current AIs, justified by reduced efficacy or health and environmental concerns (Hawkins et al., 2019). By 2011, one third of the original arsenal of crop protection AIs (350 out of 900-1000) remained in the pesticide market (Leadbeater, 2011).

The unbalanced flux between AI input and withdrawal on the pesticide and drug market results in a contraction of the number of available modes of action (MoAs). This contraction is likely to intensify resistance emergence due to increased selection pressure on molecular targets of the remaining MoAs.

1.2.2.1.2 Dual use of MoA across sectors, against different targets

The restricted number of MoAs is a problem, because it leaves few alternatives for treating a particular disorder when resistance arises. It also implies that many pathogens and pests are controlled using the same MoAs.

Almost all AIs target living organisms sharing similar cellular features and processes (except viruses). They act by disrupting some of these processes such as DNA biosynthesis, cell division or mitochondrial energy production (Fig.4), interacting with proteins that often share homology across species. The effective use of an AI with the same mode of action against

different organisms is a common practice. This can be an opportunity in several cases. For example, streptomycin, an antibiotic first isolated in 1945 (Carter et al., 1945), was recently found to be toxic to tumor-initiated cells, revealing a new lever in cancer treatment (Guillorit et al., 2025).

However, it can also be responsible for resistance co-selection. Indeed, *in vitro* studies suggest that some anticancer drugs with antibacterial effects may drive AMR. For example, methotrexate, a dihydrofolate reductase inhibitor, is an anticancer agent acting by inhibiting dihydrofolic acid synthesis (Cronstein, 1997). Its structure is very similar to another antifolate AI targeting the same enzyme, the antibiotic trimethoprim which is widely used to treat different infections, including cystitis. Exposing *Escherichia coli* and *Klebsiella pneumoniae* to methotrexate showed that both can develop *de-novo* trimethoprim resistance (Guðmundsdóttir et al., 2021), indicating that chemotherapy may be a driver of the evolution, selection and spread of antibiotic resistance. Antifolate, including trimethoprim, are also used as antimalarial agents, raising questions about the potential co-selection of resistance in protozoan pathogens.

Resistance co-selection exists across clinical and medical sectors, raising ethical questions. Because of their phylogenetic proximity, humans, food-producing animals and pets share similar pathogens, targeted by same MoAs. Veterinary medicine is the leading user of antibiotics worldwide, with 63,200 tons of antibiotics used in 2010 in livestock production for therapeutic and prophylaxis purposes (Dadgostar, 2019). For instance, colistin, used in pig production, is also a last-line antibiotic to treat severe human infections resistant to multiple drugs. Colistin resistance is conferred by plasmid-borne *mcr* genes, capable of horizontal gene transfer and likely to spread rapidly at the human–animal–environment interface (Dadgostar, 2019; Rhouma et al., 2023). A Danish study identified a positive relationship between antibiotic resistance in humans and the use of different antibiotics in cattle (Emes et al., 2024). The withdrawal of colistin in livestock use as growth promoter is one example of a successful “One Health” approach in managing AMR. It has reduced the prevalence of colistin-resistant bacteria simultaneously in farm animals, humans and the environment (Rhouma et al., 2023), highlighting the extent to which these compartments are interconnected and how the use of an AI in one sector can influence resistance outcomes in others.

The question of 'dual-use' is also raised regarding AIs in clinics and crop protection. Evidence has been acquired linking the use of antifungal molecules sharing the same molecular target in agriculture and clinics (Fig.11) with fungal AMR. *Aspergillus fumigatus*, an opportunistic pathogen, is largely present in the environment and responsible for invasive aspergillosis, especially in immunocompromised people (Denning, 2024). The hypothesis of azole resistance selection in this fungus under exposure to agriculture fungicides is supported by many direct and indirect evidence (Fisher et al., 2024), notably the high genomic conservation and the identity of the azole resistance allele between resistant field and clinical isolates (Camps et al., 2012; Rhodes et al., 2022; Rocchi et al., 2021). The co-occurrence of resistance against other agricultural fungicides MoAs including methyl benzimidazole carbamate, quinone outside inhibitors and succinate dehydrogenase inhibitors in clinical isolates constitutes another piece of evidence (Fraaije et al., 2020; Kang et al., 2022). This fungus has been found to evolve cross-resistance to the medical antifungal olorofim under *in vitro* exposure to the agricultural fungicide Ipflufenquin. Both AIs share the same MoA, the inhibition of the dihydroorotate dehydrogenase (Fig.11). While olorofim approaches clinical deployment, ipflufenquin is now available for farmers since its approval by the US Environmental Protection Agency (van Rhijn et al., 2024).

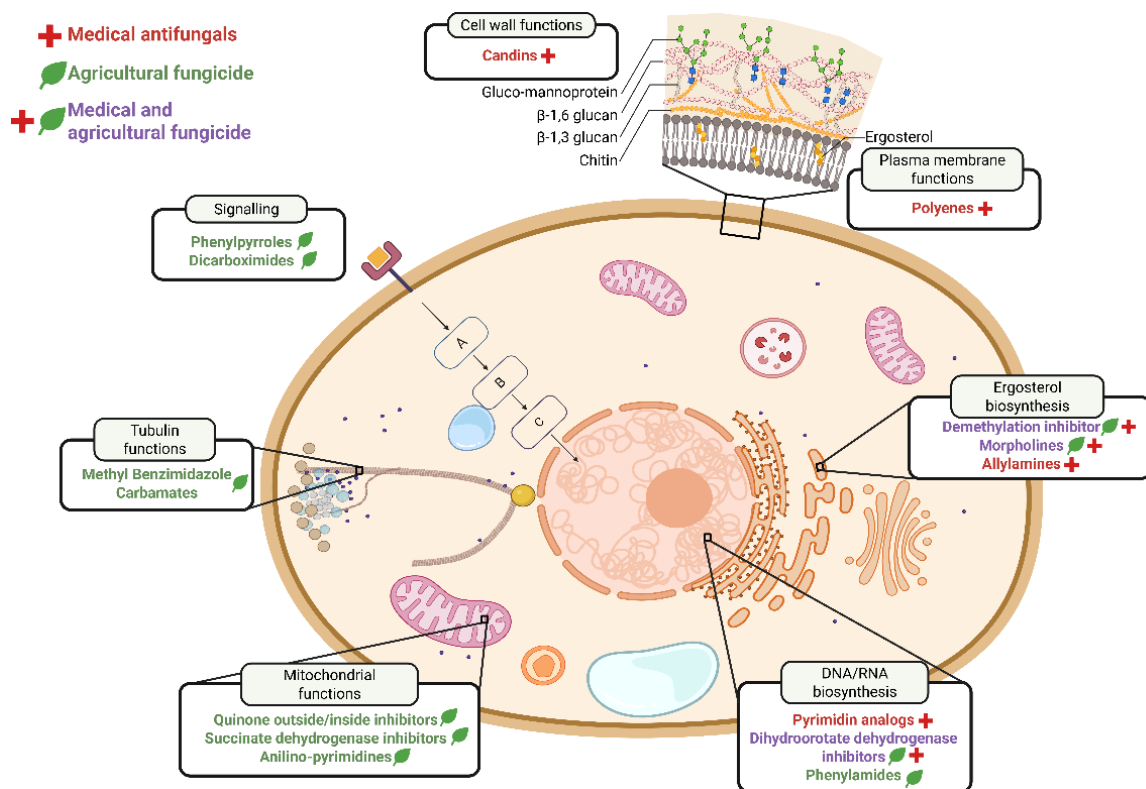


Fig. 11: Cellular targets of principal antifungals used in medicine and in agriculture. Adapted from Sanglard

(2019). *Mode of action of cited classes: polyenes bind ergosterol; phenylpyrroles and dicarboximides inhibit MAP/histidine-kinases; azoles, morpholines and allylamines are sterol-biosynthesis inhibitors; azoles target sterol 14- α -demethylase and morpholines both, sterol Δ 14-reductase and Δ 8- Δ 7-isomerase; allylamines target squalene epoxidase; pyrimidine analogues interfere with nucleic acid biosynthesis whereas phenylamides target RNA polymerase I; benzimidazoles bind to β -tubulin; strobilurins and succinate dehydrogenase inhibitors inhibit both the electron transfer chain of mitochondrial respiration by inhibiting complex III and complex II, respectively; anilinoimidazoles target mitochondrial functions and methionine biosynthesis; candins target the biosynthesis of β -1,3 glucans. Created in Biorender.com.*

In this context, dual use raises the ethical question of the balance between food supply and health security and the trade-offs that may be necessary between them. This emphasizes the need to carefully consider the use of AIs in both sectors, in order to identify overuses and misuses that accelerate the spread of resistance, while protecting health and yield as effectively as possible.

1.2.2.2 *Overuse and misuse*

The current global economic model contributes to a uniformization of the lifestyle-associated demand and the means of production to reach this demand. In particular, the use of antibiotics increased by 65 % between 2000 and 2015 worldwide to fulfill a growing demand for animal protein in fast-developing middle-income countries (Klein et al., 2018, Binsker et al., 2021).

The demand for crop production is also a source of constraints on cultivation practices, triggering pesticide use and favoring overuse. The business model of crop production adopted during the green revolution for yield purposes is associated with monoculture of economically advantageous cultivars (Gould et al., 2018). However, this model came with uniform fields of genetically homogeneous plants grown densely to maximize yield, adversely providing favorable conditions for pathogen development (McDonald and Stukenbrock, 2016). In this model, farmers are subject to market demand and their income directly depends on their production yield and quality, which in turn is threatened by plant pests. Some crop fungal pathogens produce toxins harmful for both human and cattle health (Pandey et al., 2023). Their presence in the harvest reduces its economic value for the farmer, ultimately leading to its destruction (Goda et al., 2025). Visual quality of products, likely to be altered by plant pests, is another market constraint on farmers' production.

These constraints have led to farmers' reliance on available pesticides for controlling pests. Farmers' decisions to treat were shown to be influenced by a combination of factors that

may lead to overuse and misuse of pesticides. For example, key drivers for farmers' overuse of pesticides include overconfidence in personal experience, peer behaviors and pesticide retailers' advices, giving marketing preference over technical advice (Fig. 12, Narmadha et al., 2025; Sadat and Chakraborty, 2017; Xu et al., 2008). Other contributing factors include a high perceived risk that reduced treatment would be insufficient to maintain a good yield.

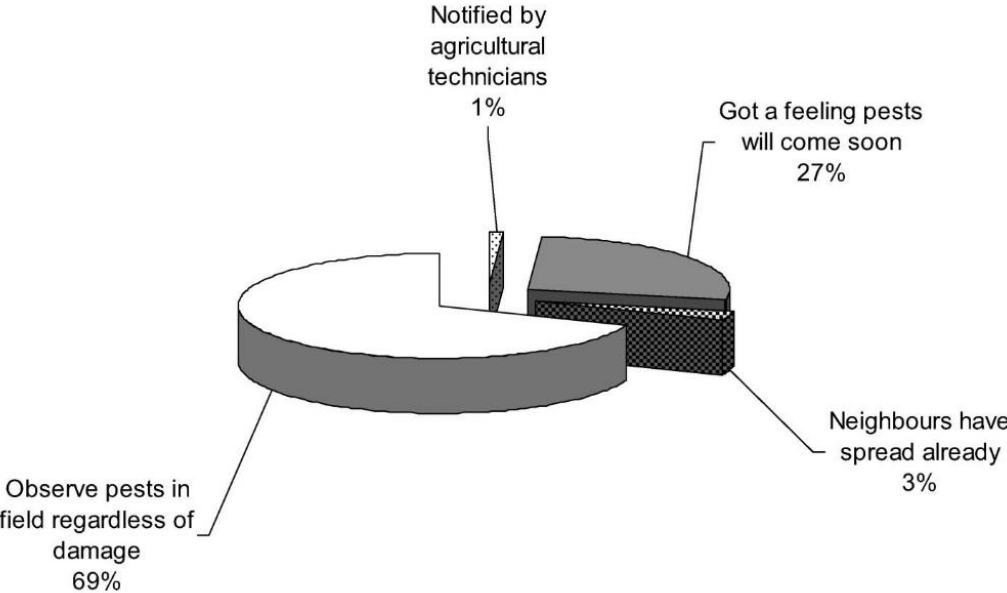


Fig. 12: Farmers' motives for applying pesticides in the provinces of Yunnan and Guizhou in China, based on a poll. Total number of respondents: representatives of 204 farmer families of different ethnical groups. From Xu et al. (2008).

Economic anxiety and vulnerability may exacerbate the perceived risks, leading farmers to prioritize short-term production over long-term sustainability (Buralli et al., 2021). In areas with limited resources, farmers frequently use illegal or unregistered pesticides due to financial constraints (Benaboud et al., 2021).

The situation in clinics is similar where overuse and misuse of antibiotics for human consumption is a trigger for AMR. Patients' poor knowledge about AMR risks and cognitive biases account for a large part of these misuses. Global surveys indicate that many patients around the globe believe that antibiotics can cure colds, coughs and fevers caused by viruses (WHO, 2015). In several countries, particularly in Asia, low regulation in drug sales leads to about 60% of total antibiotic sales not involving medical prescription (against 2 to 8% in the majority of European countries (Safrany and Monnet, 2012)). It allows patients to use antibiotics in self-medication for treating diseases that are not necessarily of bacterial origin. Also, in some

OECD countries, in 40% of the cases, antibiotic use is associated with self-medication with leftovers, drugs provided by friends or pharmacies, without a prescription (Morgan et al., 2011). Promotional efforts may also have impacted the common conception of these “miracle drugs” and promoted misuse of antibiotics (Ma et al., 2003).

Antibiotic misuse can also arise from the clinical side, such as when microbiological examinations to determine the nature of the infection are neglected (Hu et al., 2003) or when physicians perceive a social pressure to meet patients’ requests (OECD, 2018; Cole, 2014). Such prescriptions of inappropriate treatments may represent between 30% and 50% of all prescriptions (Centers for Disease Control and Prevention (U.S.) et al., 2013; Luyt et al., 2014).

Overall, conflict between individual and public goods in misuse and overuse of AIs is an aggravating factor in resistance selection because it leads to increase the exposure of pests and disease agents to the AI, facilitating resistance adaptation.

1.2.2.3 *Interconnected environment*

Through the technological revolutions and industrialization, the international movement of goods, agricultural products and people have been strongly intensified over the last decades (Fig. 13A). This global connectivity facilitates the dispersal of pests and pathogens (McDonald and Stukenbrock, 2016) and eventually the resistance genes they carry (Fig. 13B). Globalization is then an aggravating factor, increasing the scale and speed of AI resistance outcomes that threaten health and food production and erode the resilience of susceptible communities (Bokhary et al., 2021; Jørgensen et al., 2018). This international spread also concerns resistance to antimalarial drugs (Pierre-Louis et al., 2024). Regarding crop pests, there is also evidence of the spread of resistant strains or genotypes all around the world due to exchanges in agricultural products. For example, introductions of two biotypes of the polyphagous *hemiptera Bemisia tabaci* were reported in Brazil, originating from the Middle East (in the 1990s) and from the Mediterranean Basin (2010s) (de Moraes et al., 2017). The latter biotype is particularly problematic due to its resistance to neonicotinoids observed worldwide (Horowitz et al., 2005; Nauen and Denholm, 2005).

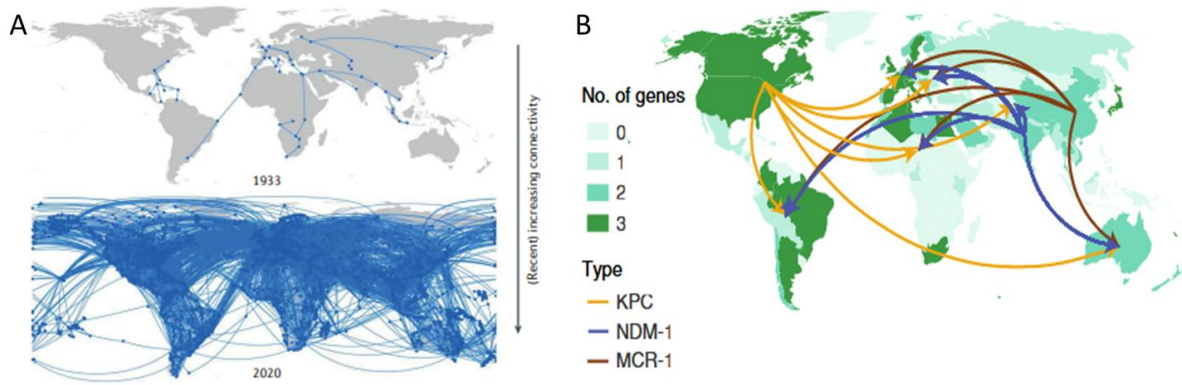


Fig. 13: The global international air travel network in 1933 and 2020 (A, from Baker et al., 2022) and intercontinental spread of antibiotic resistance genes (B, from Jørgensen et al., 2018).

1.2.2.4 Climate change

Climate change, another consequence of the anthropogenic lifestyle, is also mechanistically linked to AI resistance, mainly through its effect on pest and pathogen distribution and prevalence. Elevated temperatures, ecosystem alterations, and extreme weather events alter transmission of infectious diseases and population dynamics of disease vectors. In turn, higher disease prevalence drives greater antibiotic reliance and, thus, resistance selection in a synergistic trend between climate and the AI resistance crisis (reviewed by Aslam and Aljasir, 2025).

The global distribution of pests is also influenced by climatic factors. In their study, Bebber and collaborators showed that, since the 1960s, plant pest and pathogen geographic distribution has been shifting poleward at an annual rate of 2.7 km (Bebber et al., 2013), threatening crops in new areas. Warmer temperatures and increased humidity also provide favorable conditions for pest development; more severe fungal infections (Waheed et al., 2023) and insect infestations (Jones et al., 2017; Patterson et al., 1999) are anticipated in wetter conditions. This may also be associated with modified pesticide application protocols.

Moreover, climate change-related geochemical soil modification, rising temperatures, and altered precipitation patterns can also impact the mobility and persistence of agrochemicals, through increased runoff and leaching (Tudi et al., 2021), thereby increasing their potential to alter biodiversity and select for pesticide resistance.

AI resistance is a rising issue and by-product of the Anthropocene lifestyle that requires a holistic approach for its understanding and management. This underlines the need for comprehensive studies unraveling evolutionary drivers, molecular basis and implications in field cases of AI resistance. In the next part we propose to provide an extensive overview of resistance mechanisms, especially generalist resistance mechanisms that constitute the main focus of this thesis.

2 Drug resistance

2.1 Definitions

2.1.1 Resistance

Across sectors facing AI resistance, namely clinics and agriculture, **biological resistance** is defined at the individual level as a stable and heritable decrease in sensitivity to an active ingredient under treatment conditions (doses, frequency and duration) that would kill sensitive individuals from the same species (Fig. 14A). Given the fitness advantage it confers under drug or pesticide treatment, this trait may be selected, allowing the individual to benefit from the ecological niche emptied from susceptible individuals and increase in frequency in the population. This may eventually result in decreased effectiveness of a drug treatment in curing a disease, or of a pesticide in reducing pest impact on crop yield, when the frequency of the resistant individuals in a population overcomes a given threshold. When it occurs, it is referred to as **resistance in practice** and it ultimately results in the withdrawal of the active ingredient from treatment protocols for the pests concerned by generalized resistance (Fig. 14.B).

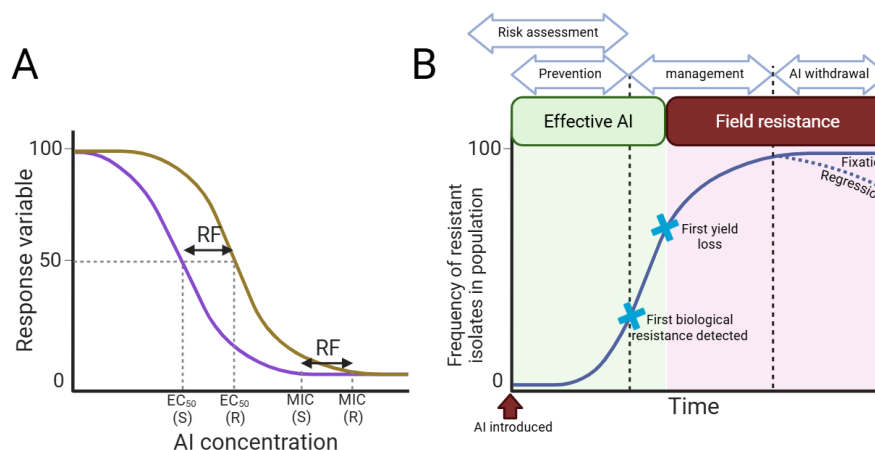


Fig. 14: Biological resistance (A) and resistance in practice (B). A: Estimated parameters for active ingredient resistance characterization form dose-response curves at individual level (clonal strain). EC₅₀: half maximal effective concentration, MIC: Minimal Inhibitory Concentration, S: susceptible, R: resistant. B: Evolution of a resistant genotype in a population and its impact on fungicide efficacy. AI: Active Ingredient. Adapted from Fouché (2021). Created in Biorender.com.

In order to identify biological resistance cases, according to the previous definition, it is necessary to define the following criteria (i) qualitative or quantitative parameters to measure the toxic effect of the AI; (ii) a comparison group to assess the decrease in sensitivity (*i.e.* susceptible wild type); (iii) how heritable the resistance trait is across clonal and/or sexual reproduction.

A typical way to quantify resistance is to first measure a response variable (*e.g.* growth, mortality) as a function of AI concentration. Several parameters can be estimated from the resulting dose-response curves. The most commonly used parameters of AI resistance across research fields are recapitulated in table I, in a non-exhaustive manner. One of the most commonly used parameter across studied organisms is the effective concentration leading to 50% of the maximal AI effect (EC_{50}) on the response variable (*e.g.*, growth compared to control). Another is the MIC, the minimum inhibitory concentration required to achieve 100% of growth inhibition. It is usually used for assessing antibiotic resistance in clinics, according to the European Committee for the Study of Antimicrobial Susceptibility guidelines (Díaz et al., 2022), but also for clinical fungi like *Candida spp.* (Clinical and Laboratory Standards Institute (CLSI), 2022). Both parameters are presented in Fig. 14A.

Table I: Common active ingredient resistance parameters estimated in different target organisms.

Parameter	Organism	Response variable	Definition
EC_{50}/ED_{50}	Fungi	Germination, germ tube elongation, mycelial growth, yeast growth or sporulation, Disease symptoms on plantlets (Siah et al., 2010)	Concentration in the media (EC) or applied orally or dermally (ED) that causes 50% of the maximum effect on the response variable.
	Virus	Viral replication or symptoms (Buss and Cammack, 2001; Kocabas and Uslu, 2021)	
	Protozoa	Parasitic load (Genetu Bayih et al., 2017) ^a	
	Weeds	Biomass (Burgos et al., 2013) ^b	
	Cancer cells	Cell death or ATP activity (Brooks et al., 2019)	
IC_{50}	Virus	PLpro deubiquitinase inhibition (Kocabas and Uslu, 2021) MEK1/2 inhibition (Hoffmann et al., 2023)	Concentration of an inhibitor at which the response (binding) achieves 50% of its activity
LC_{50}/LD_{50}	Insects	Mortality (Blouquy et al., 2021)	Concentration in air or water (LC), or applied orally or dermally (LD) that kills 50% of the exposed individuals.
	Weeds	Mortality (Burgos et al., 2013)	
GR_{50}	Cancer cells	Cell death (Brooks et al., 2019)	Concentration that reduces cell growth rate by half

MIC (EC ₁₀₀)	Bacteria Fungi	Bacterial growth (Andersson et al., 2025) Yeast or mycelial growth (Berkow et al., 2020)	Lowest concentration that prevents visible growth <i>in vitro</i> under defined experimental conditions and within a defined time period
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^a: measured from SYBR GREEN fluorescence bioassay on hemolyzed blood cells suspensions inoculated with *P. falciparum*. The fluorescence is correlated with parasitic DNA concentration, and so parasitic load. The estimated parameter is referred to as IC₅₀ in the literature of protozoal disease drug resistance, but it corresponds to EC₅₀ in given definitions, since it is not a direct measure of enzymatic activity inhibition. ^b: also referred to as GR₅₀ (Effective dose that causes 50% growth reduction assessed through biomass reduction and/or visual ratings). ED₅₀: Effective Dose 50, EC₅₀: Effective Concentration 50, IC₅₀: Inhibitory Concentration 50, LD₅₀: Lethal Dose 50, LC₅₀: Lethal Concentration 50, GR₅₀: Growth Rate Inhibition 50, MIC: Minimum Inhibitory Concentration.

The level of resistance is often quantified using the ratio of these parameters, *i.e.*, AI concentrations required to reach the same inhibitory effect in resistant (R) and susceptible (S) strains (or populations). In this thesis, we will focus on the EC₅₀ parameter and the resistance factor defined as:

$$RF = \frac{EC_{50} R}{EC_{50} S}$$

For establishing a RF, another crucial criterion for defining drug resistance is the need for a susceptible reference (population or strain) carefully selected.

Some qualitative assays are also useful for comparing AI susceptibility or resistance intensities between groups in large-scale screens, such as disk diffusion assays for bacteria (Martins et al., 2020), growth scoring at discriminatory doses (*i.e.*, the MIC of the susceptible strain) in drop dilution assays for yeast and yeast-like fungi (Ballu et al., 2021) or azole agar screening for *Aspergillus fumigatus* (Guinea et al., 2019).

As we consider that biological resistance is acquired by some individuals in an overall sensitive population, it is properly defined as **acquired biological** resistance, opposed to **natural resistance**. This latter term is used to qualify a species that is inherently resistant to an AI at treatment concentrations, due to intrinsic or induced resistance shared by all individuals, either lacking the target of the AI or harboring a resistant isoform of the target.

Finally, resistance is a stable and heritable trait, meaning that it is genetically encoded and transmitted to all individuals across clonal multiplication and into progeny mainly following Mendelian inheritance. There are some exceptions to that last point, like maternal inheritance of insecticide resistance or more generally, resistant alleles of mitochondrial genes (Van Leeuwen et al., 2008), but overall the heritability is a component of resistance definitions across the different research sectors.

2.1.2 Tolerance

The term **tolerance** is widely used but not necessarily properly defined. Resistance and tolerance are often used interchangeably in the literature, and when a distinction is made, it depends on the research field and is not always based on the same parameters, leading to inconsistencies in terminology and misleading conclusions (Leon, 2024).

In herbicide research, a tolerant species corresponds to a naturally resistant species according to our previous definitions (Leon, 2024). This definition is not satisfactory, since it defines two different concepts based on an arbitrary timepoint, the “before” and “after” treatment, while they may be of the same molecular nature. As we have discussed in the Part 1., AI resistance is likely to spread very quickly in populations of susceptible pests. As acquired resistance can become prevalent, we prefer the term natural resistance over tolerance, since acquired resistance may become natural if fixed in the population after pesticide treatment (see Part 2.4.2 and the concept of fitness costs).

The **tolerance** concept can be defined based on specific criteria mainly for organisms with clonal multiplication that can be grown *in vitro* for determination of clonal lines resistance parameters. Namely, it concerns bacteria, cancer cells and fungi. A common feature among these clonal cell populations under AI treatment is that they may include a fraction of tolerant cells. These tolerant cells are able to transiently survive or slowly grow under AI concentration higher than the MIC (for fungi and bacteria) without increasing this parameter for the strain (El Meouche et al., 2024). Despite differences in definitions between these organisms, a shared rationale is the lack of genetic differences among cells showing phenotypic differences in response to drug treatment. Response to killing (cidal) drugs *versus* drugs inhibiting growth (static) is also different. For bacteria and fungi exposed to cidal drugs, tolerance is quantified by an increase in the time required to kill 99% of the cells (MDK₉₉, Fig. 15A) in comparison to a non-tolerant isolate. For fungi, tolerance also results in a substantial growth under fungistatic concentrations higher than the MIC, revealed by slow colony growth into the inhibition halo on disk diffusion assays or e-tests (Fig. 15B). **Persistent** cells refer to a small fraction of drug-tolerant cells in the population able to grow at very high concentration of the AI. Functionally, they are often associated with persistent infections and cancer relapse (Amich et al., 2025; Berman and Krysan, 2020; El Meouche et al., 2024). The different definitions of tolerance and persistence varying according to the considered organism are available in Table II.

Table II: Resistance, tolerance and persistence definitions in cancer cells, fungi and bacteria, from El Meouche et al., 2024

	Resistance	Tolerance	Persistence
Cancer cells	Increased Minimal Inhibition Concentration (MIC) and cell growth in the presence of the anti-cancer drugs	<ul style="list-style-type: none"> - Occur when rare cells in the naïve population express a few drug-resistance genes, and thus, survive through the initial course of drug treatment - Persistent and tolerant terms are used interchangeably in the cancer literature eg. Drug-tolerant persisters (DTPs) - Able to slowly replicate - Arise due to non-genetic determinants - On prolonged drug treatment, persistent/ tolerant cells acquire resistant traits through genetic mutation/evolution 	
Fungi	Reduced susceptibility of fungal cells to a drug due to heritable genetic mutations	<ul style="list-style-type: none"> - Generally owed to a substantial fraction of the population (5–90%) - Ability to survive longer times to drug exposure and to grow slowly at inhibitory drug concentrations, without an increase in MIC - Can be due to genetic and nongenetic determinants - Reservoir for genetic changes 	<ul style="list-style-type: none"> - Involves rare cells (generally less than 1%) that are able to grow at drug concentrations that are at least 8 × the MIC - Can be due to genetic and non-genetic determinants - Reservoir for genetic changes
Bacteria	Increase of the MIC and the ability of bacteria to grow in presence of antibiotics	<ul style="list-style-type: none"> - Occurs when a population temporarily survives longer without growing in presence of lethal doses of bactericidal antibiotics - Characterized by a slower killing rate without a change in the MIC - Can be due to genetic determinants or growth-restrictive environments - Reservoir for genetic change 	<ul style="list-style-type: none"> - Occurs when a small number of bacteria acquires a drug-tolerant state - Can be due to genetic and nongenetic determinants - Reservoir for genetic changes

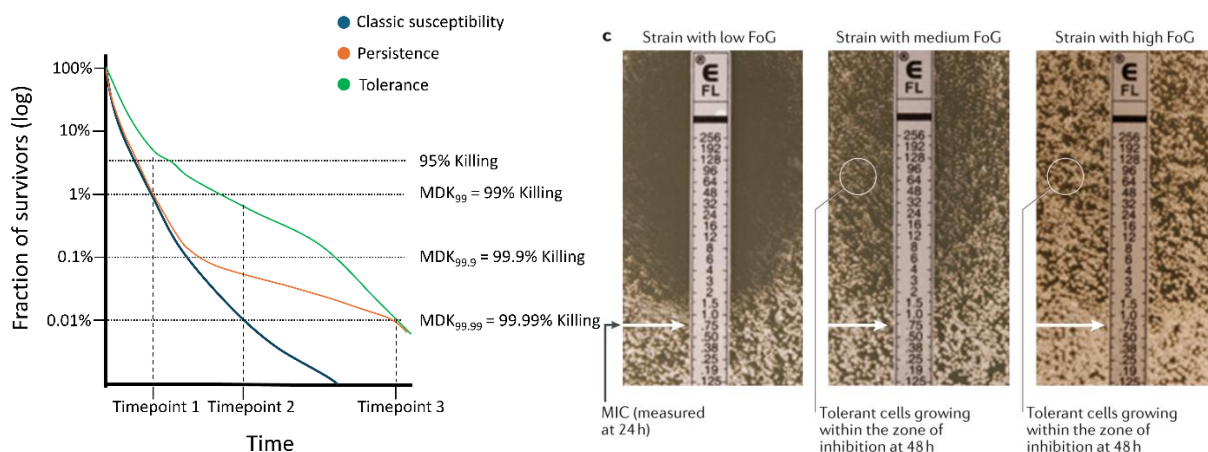


Fig. 15: Tolerance identification metrics in a fungal isolate against fungicidal (A) and fungistatic (B) drugs. Adapted from Amich et al., 2025 and Berman and Krysan, 2020 respectively.

At the end of this definition section, it appears clear that resistance and tolerance are different concepts. However, the molecular mechanisms and the genetic and non-genetic bases involved remain to be dissected. Below, we summarize current knowledge of the molecular mechanisms primarily linked to resistance.

2.2 Molecular mechanisms

For the rest of this introduction, we will only consider uni-site AIs, following the “one-drug, one-target” paradigm. Molecular mechanisms that lead to biological resistance toward these uni-site AIs can be divided in two categories: target and non-target site resistance, which are associated with distinct consequences, notably in terms of resistance intensity.

2.2.1 Target-site resistance

The first and best described resistance mechanisms are associated with the molecular target of a given AI. As we described above, the MoA of an AI principally relies on its specific inhibitory interaction with a molecular target, often an enzyme, involved in an essential cellular function of the pest or the pathogen. A modification of this target through single nucleotide polymorphism(s) in its encoding gene, modifying the AI binding site, can lead to a loss of affinity between the two molecules. It is referred to as **Target Site Resistance (TSR)**. Case examples are reported in all organisms that have been cited before, with common points between them. For instance, a single amino acid substitution in the dihydrofolate reductase (DHFR) of *Staphylococcus aureus* and *Streptococcus pneumoniae* confers resistance to

trimethoprim, while single and multiple mutations in the homologous *dhfr* gene of *P. falciparum* are linked to its resistance to antimalarial antifolates, pyrimethamine and cycloguanil (Dale et al., 1997; Sirawaraporn et al., 1997).

Among insects and mites treated with pyrethroids, many cases of target-site resistance have been reported, associated with substitutions in the voltage-gated sodium channel, resulting in resistance to the neurotoxic effect of permethrin in several parasites and vectors such as human scabies mite (*Sarcoptes scabiei*), dog tick (*Rhipicephalus linnaei*) or mosquitoes (*Aedes aegypti*) (Pasay et al., 2008; Ranathunge et al., 2021; Takhampunya et al., 2025). As an illustrative example of target-site resistance through reduced target affinity for the AI, we can cite the work of Cai and collaborators (2024). Through a genotypic screen of the crop pest fall armyworm (*Spodoptera frugiperda*) from various locations in China, they identified both known and unknown variants of common insecticide targets. Among the new ones, molecular docking revealed different binding affinities to insecticides. The binding affinity of abamectin to the glutamate-gated chloride channel (GluCl) variants G326E and G314D is decreased compared with the wild-type and associated with resistance to this insecticide (Fig. 16).

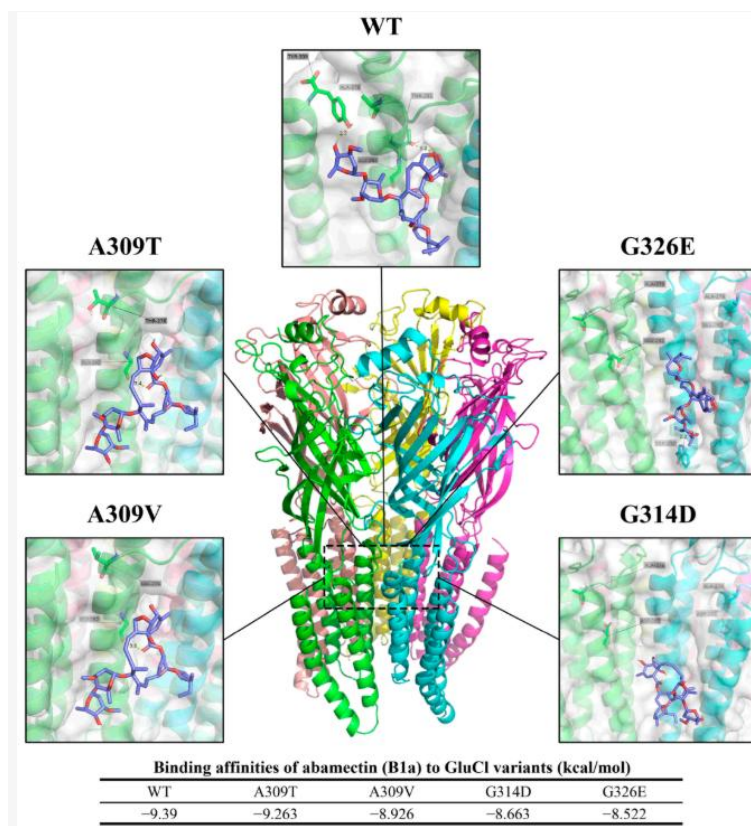


Fig. 16: Predicted binding orientations of abamectin (B1a) to wild-type and mutated GluCl channel from fall armyworm and corresponding binding affinities. From Cai et al. (2024).

Dual use of azole fungicides, also called DMIs (from DeMethylation Inhibitors), is associated with the selection of resistant isolates carrying substitutions in the target enzyme sterol 14- α demethylase (CYP51) in clinical isolates of *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* and field isolates of *Blumeria graminis* (powdery mildew in wheat and barley), *Puccinia triticina* (brown rust in wheat), *Erysiphe necator* (powdery mildew in grapes), *Mycosphaerella fijiensis* (black sigatoka in bananas) and *Zymoseptoria tritici* (septoria leaf blotch in wheat) reviewed by Parker et al., 2014. Target-site resistance is also the most widespread and only type of resistance reported in rodents against anticoagulant rodenticides, due to substitutions in the VKORC1 target protein involved in vitamin K recycling, a cofactor crucial for the activation of coagulation factors (Grandemange et al., 2009; Marquez et al., 2019). Target-site is also the only type of resistance known in viruses, intrinsically due to their mode of multiplication and extremely reduced genome (Condra et al., 1995).

TSR can also arise from overexpression of the target molecule. Increasing the titer of the protein target *versus* the AI leads to a greater amount of functional free target able to accomplish its cellular function. This overexpression can be due to (i) *cis* modifications in the promoter of the gene, (ii) overexpression or gain-of-function mutation of a transcription factor (iii) copy number variation of the target gene. Examples of each case have been reported for azole resistance associated with *cyp51* expression, sometimes in combination with coding sequence variations. Transposable element insertions in the *cyp51* gene promoter are involved in its overexpression in several fungal pathogens such as *Plenodomus biglobosus*, *Leptosphaeria maculans* or *A. fumigatus* (Albarrag et al., 2011; King et al., 2025; Yang et al., 2020). Besides, *C. glabrata* gain of function mutation in the transcription factor Upc2 in clinical isolates leads to its constitutive activation and upregulation of *cyp51* expression (Dunkel et al., 2008). Finally, *cyp51* overexpression can also arise from copy number variations (CNV) of the gene, reported as an adaptation to azole treatment in some fungi like *E. necator* with variations in CNV among isolates exposed or not to DMI fungicides (Jones et al., 2014). Outside of the fungal kingdom, other examples of these TSR mechanisms are also frequent. As an example, the amplification of *dhfr* gene in cancer cell lines is responsible for resistance against the antifolate methotrexate and was also reported in patients suffering from acute lymphoblastic leukemia relapse (Göker et al., 1995).

2.2.2 Non-target-site resistance

In opposition to target-site resistance, other heritable molecular mechanisms also lead to decreased AI sensitivity, and are referred to as Non-Target Site Resistance (NTSR). Both are recapitulated in Fig. 17. NTSR can involve a variety of mechanisms that are more or less specific to a MoA. For example, bypass of target inhibition possibly allows resistance to AIs sharing the same cellular target. Rarely observed, there are only a few reported examples in fungi. The most documented is the overexpression of an alternative oxidase (AOX) enabling some fungi to bypass inhibition of the complex III of the mitochondrial respiratory chain by QoI fungicides (of the chemical class of strobilurins). In this alternative oxidative pathway, AOX provides an alternative electron transport pathway, thereby maintaining electron flux and ATP production. Induced AOX activity under QoI treatment was notably detected in *Z. tritici* field isolates resistant to QoIs (Miguez et al., 2004). Another example is the defective sterol $\Delta 5,6$ -desaturation in clinical isolates of *Candida albicans*. Under azole treatment, the lack of $\Delta 5,6$ -desaturase activity prevents the cytotoxic accumulation of desaturated sterol 14 α -methylergosta-8,24(28)-dien-3 β ,6 α -diol while enabling accumulation of the non-toxic metabolic intermediate 14 α -methylfecosterol which support growth (Kelly et al., 1997).

Other common NTSR are associated with resistance to several AIs with unrelated MoAs (*i.e.* targeting unrelated biological pathways). We refer to these resistance phenotypes as Multi-Drug Resistance (MDR, Fig. 17). Further definitions and implications of this term will be outlined in the next paragraph.

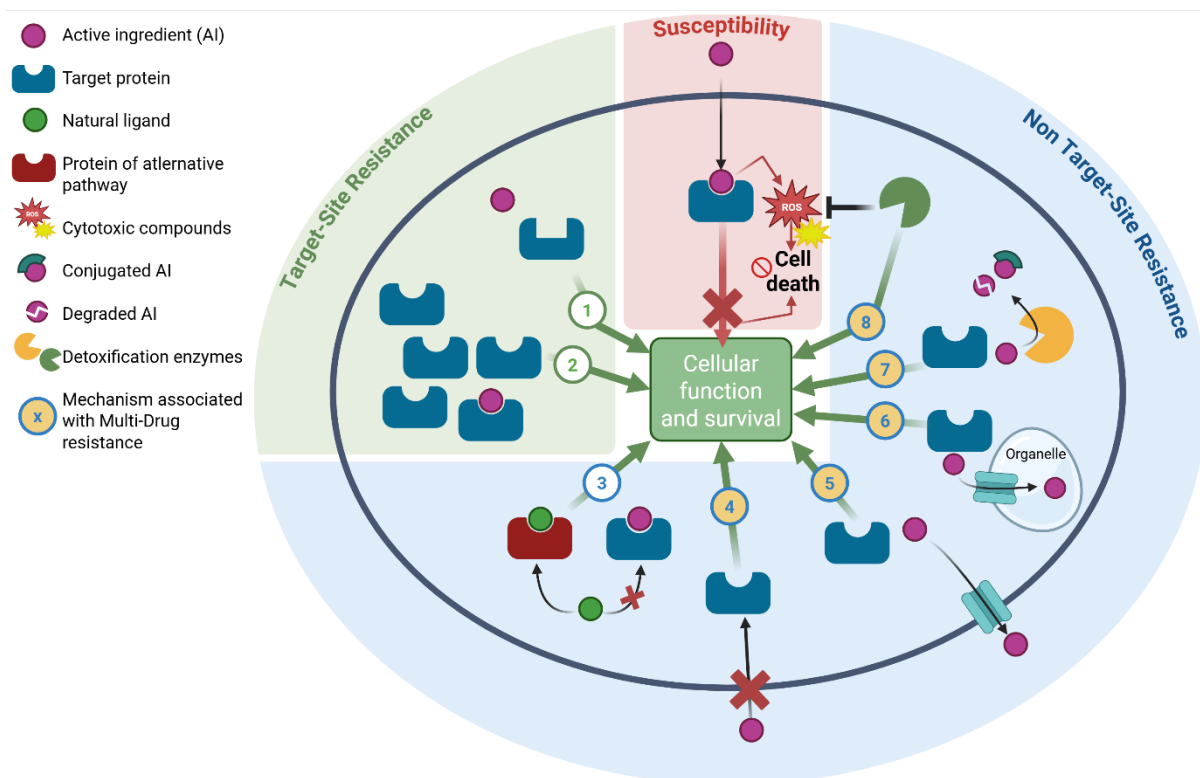


Fig. 17: Major target site (TSR) and Non-Target site (NTSR) resistance mechanisms against active ingredients (AIs) (pesticides and drugs). TSR: 1) Modification of the AI target binding site, 2) Overexpression of the AI target. NTSR: 3) Bypass of AI inhibition by an alternative pathway, 4) Reduced AI uptake, 5) Enhanced AI efflux, 6) AI sequestration, 7) Detoxification by enzymatic degradation or conjugation, 8) Detoxification of cytotoxic damages downstream of AI target inhibition. Adapted from Ballu (2021); R4P Network (2016). Created in BioRender.com.

2.2.3 Multidrug resistance

2.2.3.1 Definition

Given the semantic discrepancies between research fields of the term **MultiDrug Resistance** (MDR), we must define this concept in the context of the present work. Shared rationale between reviewed definitions is that multidrug resistance refers to a broad-spectrum resistance phenotype. In the medical community, especially considering antibiotic resistance, the terms MDR, XDR (eXtensive-Drug Resistance) and Pan-Drug Resistance are used for designing different categories of this resistance spectrum, *i.e.*, the range of AIs to which the individual is resistant. An international group of experts proposed a standardized international terminology for these different terms in the context of AMR (Magiorakos et al., 2012). They proposed arbitrary interval limits in the number of AIs and antibiotic categories concerned by the resistance (*i.e.*, the number of AIs and MoAs), varying for each medically relevant bacterial species. For instance, in *Pseudomonas aeruginosa*, (i) MDR is defined as a resistant phenotype

to ≥ 1 agent in ≥ 3 MoAs, (ii) XDR: resistant to ≥ 1 agent but ≤ 2 categories and (iii) PDR: resistant to all agents clinically available.

Similarly, Mitra and collaborators defined MDR for clinical fungi as resistance to AI from at least two classes of antifungals (Mitra et al., 2024). These definitions are useful to characterize new resistance spectra and to adapt treatment and resistance management for AMR regarding public health. However, only based on phenotypic characteristics, they do not consider the molecular and genetic basis of the phenomenon, yet important for addressing the resistance mechanisms and the long-term sustainability of chemical control. The Research and Reflection Network on Pesticide Resistance (R4P) defined different concepts associated with broad resistance spectra (R4P Network, 2016). The first one, multiple resistance, refers to a resistance phenotype due to the co-occurrence of multiple resistant alleles, each conferring resistance to one AI, stacked in the same genetic background (Fig. 18). It has to be differentiated from positive cross-resistance, which is the acquisition of resistance towards different AIs, with the same or different MoAs, due to a single mutation. MDR in this work is considered as a particular case of positive cross-resistance associated with non-target site resistance and leading to resistance of at least two AIs of different MoAs. In this way, we stick to the definition proposed by Paul and collaborators referring to a simultaneous acquisition of resistance to a range of drugs *via* single or a limited number of genetic changes (Paul and Moye-Rowley, 2014), and add that this range must include more than one MoA. This definition implies that MDR involves potential resistance to a new MoA even before the MDR organism has been exposed to it. In that sense, MDR phenotype can be theoretically distinguished from multiple resistance phenotype through resistance to MoA that has not previously been used against the organism and did not exert selection pressure. This is done for example for MDR assessment in *Zyloseptoria tritici* using the medical allylamine fungicides (Fig. 11) tolnaftate and terbinafine for the MDR screening of field isolates (Leroux and Walker, 2011; Omrane et al., 2017, 2015).

It has to be added that in many scientific publications, as well as in the R4P Network definition, MDR is exclusively defined as due to non-selective efflux of AIs and other drugs from cells; probably because it is the most widely described mechanism. However, sticking to the definition we gave before, we will consider in this work MDR mechanisms arising from NTSR that prevents the inhibitory effect of AIs of different MoAs on their targets by (i) reduced uptake, (ii) enhanced efflux, (iii) detoxification *via* enzymatic degradation, conjugation and/or

eventually sequestration, (iv) enhanced general stress response helping to cope with AI-associated cytotoxic effects. All these mechanisms are reviewed in detail in the next part and presented as a schematic overview in Fig. 17.

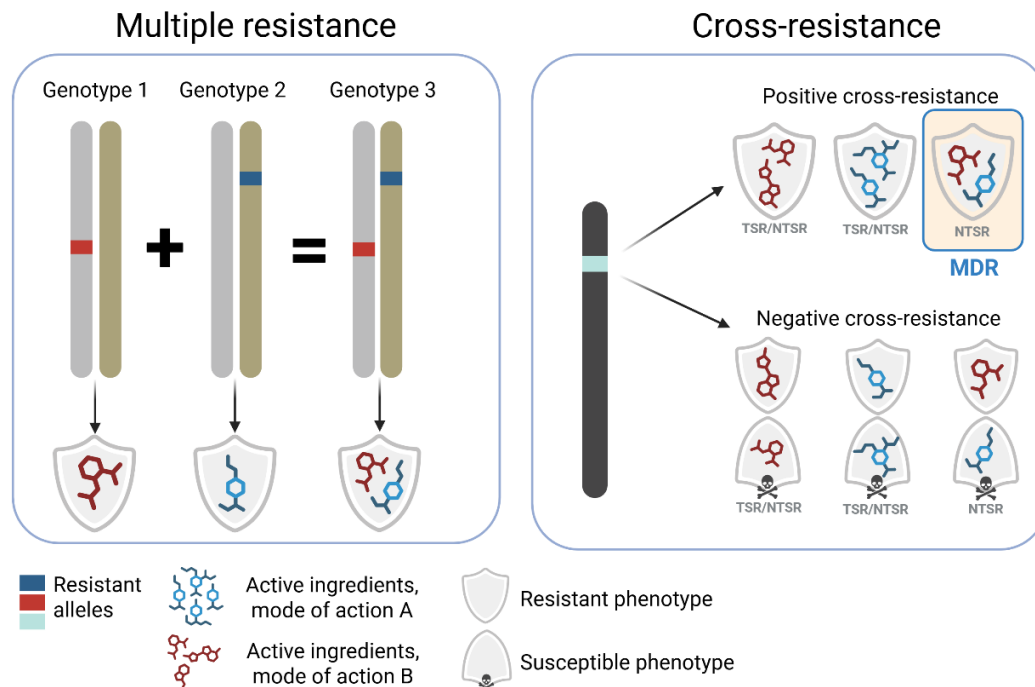


Fig. 18: Genetic basis underlying multiple, cross and multidrug resistance. TSR: Target-Site Resistance (affecting the target of the active ingredient), NTSR: Non-Target Site resistance, MDR: multidrug resistance. Created in BioRender.com.

2.2.3.2 Mechanisms

The main objective of this chapter is to give a broad overview of the different MDR mechanisms observed *in vitro* or *in natura* across organisms targeted by drugs and pesticides, in order to highlight both their universality and specificities. This will serve as a critical foundation for subsequent investigations into the characterization of novel MDR mechanisms in a phytopathogenic fungus, the focus of this thesis.

2.2.3.2.1 Introduction to detoxification pathway

In all living organisms, pathways for xenobiotic detoxification have been selected long before pesticide or drug treatments. They are essential for survival in adverse conditions, for detoxifying natural organic and inorganic toxic compounds (Després et al., 2007; Sheehan et al., 2001). Indeed, a large part of discovered antibiotics is actually derived from microbial metabolites, such as penicillin, produced by microorganisms as advantageous weapons against

other microorganisms in their conquest of ecological niches and resources (Davies and Davies, 2010). Thus, to benefit from these compounds, microorganisms first need to limit their toxic effect on their own cells. As their toxic effect exerts a selective pressure on other organisms, a functional detoxification system is then a fitness advantage. More generally, every living organism, micro- or macro-organism, is confronted with toxic compounds in its environment, explaining the ubiquity of detoxification pathways, selected in all living species. These systems include expression of enzymes and transporters. In eukaryotes, especially plants and insects, xenobiotic detoxification is often described as a structured sequence of three phases (i) "functionalization" (oxidation, reduction, or hydrolysis), usually involving cytochrome P450 enzymes (ii) conjugation (coupling with an endogenous substrate) involving, for example, Glutathione S-Transferases (GSTs) and (iii) transport (efflux or sequestration) (Amezian et al., 2021; Singh et al., 2016; Stanley, 2017). These three phases may occur independently, for example a xenobiotic can be transported without prior modification. The schematic view of these processes is displayed in Fig. 19. The detoxification enzymes also help to cope with reactive oxygen species and associated cellular damage produced by the xenobiotic or its metabolization products (Singh et al., 2016).

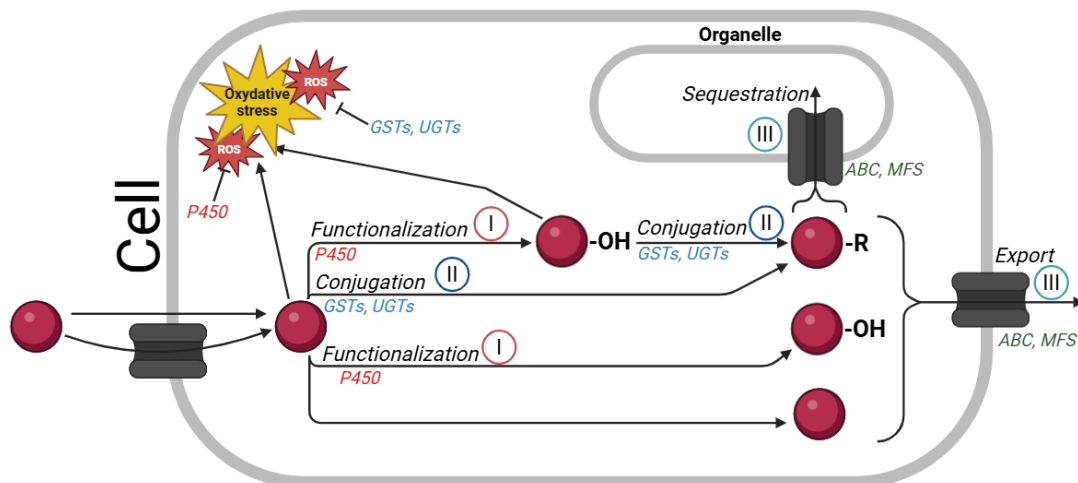


Fig. 19 : Generic model of the eukaryotic xenobiotic detoxification systems. Adapted from Amezian et al., (2021) and Singh et al. (2016). The xenobiotic (red sphere) can enter the cell by passive diffusion or through transporters. Once in the cell, it can be directly exported or translocated in organelles through phase III components (transporters ABC or MFS). Phase I (P450 for example) and Phase II enzymes (GST or UGT for example) may act sequentially or independently to modify the xenobiotic into a less toxic compound or a compound that is easily transported. The oxidative stress and associated cellular damages generated by the xenobiotic or its derivate are counteracted by detoxification enzyme from phase I and II. ROS: reactive oxygen species, P450: Cytochrome P450, GSTs: Glutathione S-Transferase, UGTs: UDP-Glucuronosyltransferase. ABC: ATP-Binding Cassette transporters, MFS: Major Facilitator Superfamily transporters. I: Phase I, II: Phase II, III: Phase III of the detoxification system. R: endogenous substrate. Created in BioRender.com.

Even if the sequence and structure of this pathway is not always the same across organisms, detoxification enzymes and transporters are key components of xenobiotic management in all living species. In other words, a system optimized for coping with toxic compounds already exists in the organisms we try to control with drugs and pesticides.

Therefore, multidrug resistance may occur through modifications in the selectivity or the intensity of this detoxification system. In the next parts, after addressing the prevention of active ingredient uptake as a MDR mechanism, we will review how different levers of the detoxification system are activated in MDR pathogens, pests and cancer cells. A synthetic overview of the examples is provided in Table III.

2.2.3.2.2 *Reduced penetration*

Also referred to as reduced uptake or influx, reduced penetration acts as a primary MDR mechanism by preventing the AI from entering the cell. It can arise through modification of the cell wall or cuticle (insects and plants) composition or permeability, alteration of the active import of the AI, and also *via* biofilm production or other phenotypic adaptations.

Reduced uptake in pluricellular organisms

According to the R4P Network definition, reduced uptake may be a prevalent MDR mechanism in weeds, but leading to low resistance factors and thus difficult to assess individually because often associated with other mechanisms ("Glossary – R4P," n.d.). Some examples of impaired translocation inside the plant have however been described. For example, in *Lolium rigidum*, resistant biotypes display modified glyphosate and paraquat translocation, preventing the systemic toxic effect of these molecules, although the mechanisms involved are likely different for both herbicides (no evidence for cross resistance) (Yu et al., 2007).

Regarding insects, epicuticular thickening is considered to virtually confer multidrug resistance because of the lipophilic nature of most insecticides (Nkya et al., 2013; Scott, 1989). In *Anopheles gambiae*, overexpression of two epicuticular hydrocarbon biosynthesis enzymes (CYP4G16 and CYP4G17) has been linked to reduced penetration rate of ¹⁴C deltamethrin and pyrethroid resistance (Balabanidou et al., 2016).

Extracellular matrix as a physical barrier (bacteria, fungi, tumor cells)

In unicellular organisms and cancer cells, other mechanisms can prevent AI from entering the cell. At the community level, biofilm formation has been involved in resistance in bacteria and pathogenic yeasts (*Candida sp.*) (Bink et al., 2011; Li et al., 2015). Biofilm establishment is accompanied by the production of a thick extracellular matrix (ECM) that forms a physical barrier drastically reducing the amount of AI entering the cells. The ability to form a biofilm varies among species, but some mutations were shown to stimulate biofilm formation. For example, mutations of the Agr system (involved in quorum sensing) in *Staphylococcus aureus* lead to increased biofilm formation and thereby resistance to antibiotics (He et al., 2022). The extracellular matrix in tumoral microenvironment plays a similar role in drug resistance, reducing the diffusion of chemotherapeutic agents and their delivery to cancer cells (Darvishi et al., 2022). Schütze and collaborators demonstrated that recombinant overexpression of lysyl oxidase LOXL2 (an enzyme responsible for crosslinking collagen molecules in the ECM) strongly reduced doxorubicin diffusion into multicellular tumor spheroids (Schütze et al., 2015).

Reduction of influx transport activity

Finally, AI can also enter cells *via* import, especially hydrophilic molecules that are imported through porins. Downregulation of these porins or mutations affecting their transport selectivity have been widely reported in many clinical and laboratory bacteria, as reviewed by Zhou et al. (2023), and are associated with resistance to β -lactam and fluoroquinolones. As an example for MDR, the deletion mutant of the major *Mycobacterium smegmatis* porin, MspA, displays resistance to β -lactam (cephalosporins), glycopeptide (vancomycin), and macrolide (erythromycin, rifampin) antibiotics (Stephan et al., 2004). Meanwhile, the main factor underlying pentamidine and melarsoprol cross-resistance in African trypanosomes is the loss of function of aquaglyceroporin AQP2, a channel responsible for drug uptake (Baker et al., 2012; Graf et al., 2013; Quintana et al., 2020).

2.2.3.2.3 Enzymatic detoxification

As we mentioned before, detoxification enzymes play a major role in xenobiotic detoxification systems. Their upregulation or acquisition *via* horizontal transfer (see 2.2.4.2) can lead to drug and multidrug resistance.

Bacterial detoxification enzymes

While bacteria do not possess a three-phase detoxification system, they still harbor detoxification enzymes that enable them to overcome the toxic effects of antimicrobial compounds. A major example of this mechanism refers to β -lactamase enzymes, which are able to cleave the β -lactam ring of the antibiotic class of the same name, including penicillin, cephalosporins, and thereby preventing their inhibitory effect on peptidoglycan biosynthesis and thus bacterial cell wall production (Bush, 2018). These enzymes confer resistance only to the β -lactam class, because of their specificity for the β -lactam ring and thus, while conferring resistance to a wide range of antibiotics, they do not confer MDR. However, a bifunctional enzyme has been described in *Enterobacteriaceae*, which occurs naturally and is encoded by the plasmid-associated *qnrA* gene. It catalyzes aminoglycoside detoxification through acetyltransferase activity and also acetylation of certain fluoroquinolones (Robicsek et al., 2006). In this case, the bacterial detoxification enzyme is likely to confer MDR.

Detoxification enzymes in eukaryotes (phase I and II enzymes)

Pesticides and drugs can be metabolized through oxidation, reduction or hydrolysis to limit their toxic effect or providing functional groups for further conjugation, eventually facilitating their sequestration or excretion (Casida, 2018). In insects and mites, overexpression of phase I and phase II enzymes is a prevalent MDR mechanism. Phase I cytochrome P450s, involved in a range of reduction-oxidation reactions, play a central role (De Rouck et al., 2023). For example, in the plant parasite spider mite *Tetranychus urticae*, acequinocyl and bifenthrin resistant strains were found to overexpress the cytochrome P450 CYP392A11, later shown to be able to metabolize different acaricides (Lu et al., 2023; Riga et al., 2015). Likewise, in *Tetranychus cinnabarinus*, another phase I enzyme has been unveiled, the esterase TCE2, for which gene expression correlates with resistance to abamectin, fenpropathrin and cyflumetofen. Its functional analysis, *via* heterologous expression in *E. coli*, showed that TCE2 is able to effectively decompose two of these acaricides (Shi et al., 2016; Sun et al., 2010). In the insect tobacco pest *B. tabaci* (whitefly), it appeared that neonicotinoid resistance correlates with the constitutive overexpression of *CYP6CM1*, able to detoxify the neonicotinoid imidacloprid by hydroxylation, but also pymetrozine, another insecticide with a distinct chemical structure and of an unrelated mode of action (Karunker et al., 2009, 2008; Nauen et

al., 2013). Cytochrome P450s also play a key role in herbicide detoxification. Transcriptomic data revealed an overexpressed gene encoding such an enzyme, CYP81A10v7, in a MDR biotype of *Lolium rigidum* compared with a sensitive one. Heterologous overexpression of this gene in rice conferred high resistance level to several herbicides with five different mode of action, *i.e.*, inhibitors targeting acetyl-coenzyme A carboxylase, acetolactate synthase, hydroxyphenylpyruvate dioxygenase, photosystem II and tubulin (Han et al., 2021).

Besides phase I enzymes, Glutathione-S-transferases (GSTs), as phase II enzymes, can act either on unmodified AI or on products of enzymatic action of phase I enzymes and are also crucial in insect and mite pesticide metabolism. Like phase I enzymes, they can display antioxidant activity and cope with pesticide-associated oxidative stress, *via* their peroxidase activity (reviewed in De Rouck et al., 2023). However, their major role in detoxification is due to their conjugation activity with glutathione that improves the water-solubility of the toxic compound, and can also neutralize its electrophilic site(s). In mites, GSTs are associated with resistance to different acaricides, such as cyflumetofen, flumethrin and phoxim (Feng et al., 2019; Pavlidi et al., 2017; Zhang et al., 2018) but the resistance spectra associated with GSTs are not wide enough qualify as MDR in mites (reviewed by De Rouck et al., 2023). Expression data also indicate that other yet underexplored enzymes may play a role in acaricide resistance, such as intradiol ring cleavage dioxygenases (DOGs), lipocalins and short-chain dehydrogenases (SDR), but their individual contributions to resistance phenotypes remain largely unknown (De Beer et al., 2022; Kurlovs et al., 2022; Xue et al., 2020). GSTs play an important role in MDR acquisition in several other organisms. In some cells of human lung adenocarcinoma, downregulation of Glutathione S-transferase P1 (GSTP1) led to increased effects of the chemotherapeutic agent camptothecin (Ishii et al., 2004), while its overexpression is associated with resistance to docetaxel and paclitaxel in breast cancer (Arai et al., 2008). They also play similar roles in MDR acquisition in weeds. The GSTP1-related *AmGSTF1* is indeed overexpressed in multi-herbicide-resistant biotypes of black-grass (*Alopecurus myosuroides*), and a transgenic *Arabidopsis thaliana* line overexpressing *AmGSTF1* shows resistance to herbicides with two different MoAs, atrazine, chlorotoluron (inhibitors of photosystem II) and alachlor (inhibitor of very-long-chain fatty acid synthesis) (Cummins et al., 2013).

To conclude with this part, detoxification enzymes are major players in MDR acquisition. Overexpression of individual enzymes can lead to a very broad MDR spectrum. However, it is

very difficult to clearly assess if their protective role relies only on AI metabolism or if their antioxidant activity is also involved by helping the cell to cope with oxidative damage associated with the toxic effects of AIs. For example, the cap 'n' collar isoform C/Kelch-like ECH-associated protein 1 (CncC/Keap1) signaling pathway has been explored in *T. cinnabarinus* (Shi et al., 2017) and appears to be a master regulator of xenobiotic and oxidative stress response in multiple insect species (Amezian et al., 2021). However, the direct contribution of the genes and enzyme families implicated in these pathways to the stable overexpression of detoxification genes in resistant strains is largely unknown (Amezian et al., 2021).

2.2.3.2.4 Enhanced transport: Increased efflux and sequestration

The most widely described mechanism leading to MDR across *phyla* is associated with active efflux of toxic compounds out of the cell through membrane transporters, reducing their intracellular concentration. It is also associated with the widest MDR spectra and constitutes phase III of the detoxification pathway described above.

This common feature is essential in any living cell for extruding toxic compounds. Among the multidrug transporters that have been reported, some are restricted to bacteria, such as resistance-nodulation-cell division (RND), small multidrug resistance (SMR) and proteobacterial antimicrobial compound efflux (PACE) transporters. On the other hand, ATP-binding cassette (ABC), major facilitator superfamily (MFS), and multidrug and toxic compound extrusion (MATE) transporters are ubiquitous in organisms from prokaryotes to eukaryotes (illustrated in Fig. 20).

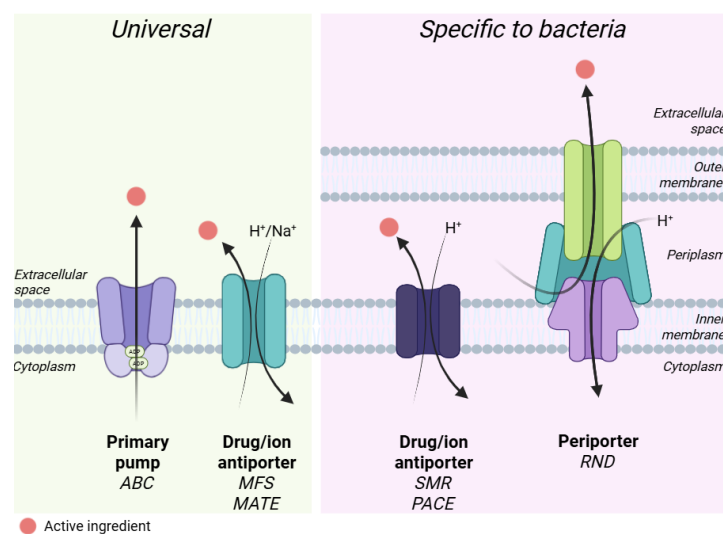


Fig. 20: Transporters involved in multidrug resistance in living organisms. Adapted from Yamaguchi et al., (2015). Created in Biorender.com

Bacterial transporters

RND transporters are specifically found in Gram-negative bacteria as tripartite efflux pumps and the related type 1 secretion systems (T1SSs). They allow transport of substances through the cell wall (Alav et al., 2021). Other tripartite efflux pumps and related (T1SSs) are found in Gram-negative bacteria, they are composed of different inner membrane transporters from ABC and MFS families. RND family comprises efflux pump that are the most clinically relevant. These transporters are fueled by proton motive force for extrusion of diverse xenobiotics including antibiotics. In *Pseudomonas aeruginosa*, the major RND-type efflux pump MexAB-OprM is of critical importance in clinical MDR. It is able to transport a wide range of antibiotics and participate to the intrinsic resistance of this pathogen (Dulanto Chiang and Dekker, 2024). Its overexpression in clinical and laboratory mutants leads to increased resistance to chloramphenicol, carbenicillin, nalidixic acid, and novobiocin (Sobel et al., 2005). In the same way, overexpression of the RND AcrAB-tolC pump is associated with multidrug resistance in clinical *E. coli* isolates (Bahaj et al., 2025)

Besides these well-known transporters, smaller efflux pumps, also using proton motive force, were described in both Gram-negative and Gram-positive bacteria. SMRs are very small proton-coupled antiporters. From this family, EmrE in *E. coli* transports a variety of antiseptic quaternary cationic compounds; its expression in a wild-type strain leads to increased resistance to ethidium bromide, methyl viologen, erythromycin, sulfadiazine, and tetraphenylphosphonium, as well as to tetracycline (Yerushalmi et al., 1995). AbeS, a SMR transporter of the nosocomial pathogen *Acinetobacter baumannii*, was studied in a MDR strain. Its deletion led to increased susceptibility to various compounds including the antibiotics erythromycin and novobiocin but also to benzalkonium chloride, deoxycholate and ethidium bromide. Expression of this transporter in a hypersensitive *E. coli* strain further confirmed its role in resistance to these compounds (Srinivasan et al., 2009).

The PACE transporter family, conserved in Gram-negative bacteria, was identified more recently with its first representant, Acel from *A. baumannii* (Hassan et al., 2015). They display a narrower substrate spectrum than other transporter families previously described. This spectrum includes primarily synthetic biocides such as chlorhexidine and acriflavine (Hassan et al., 2018). In that sense, despite their ability to transport biocides, they are not associated with MDR according to the definition we proposed earlier.

ABC transporters

ABC transporters are ubiquitous proteins that can have various functions. They have been described as transporters fueling active transport of substrates by the hydrolysis of two ATP molecules, from the outside (almost exclusively in bacteria), or inside the cells (Fig. 21). MDR naturally arises from the involvement of the second category (Wilkins, 2015).

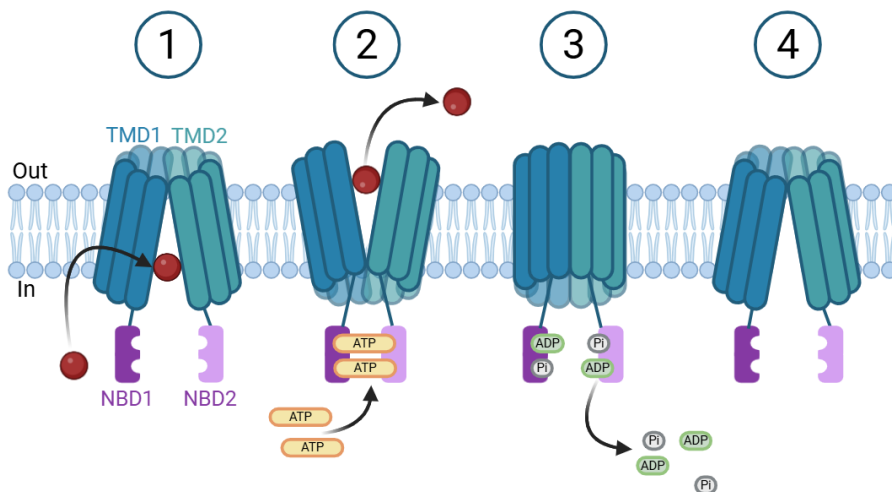


Fig. 21: ATP-switch model (Higgins and Linton, 2004) for the transport cycle of an ABC exporter with canonical structure, with two transmembrane domains (TMD) and two nucleotide-binding domains (NBD). 1: the substrate (red sphere) binds the transporter in an affinity pocket formed by the TMDs, it drives a conformational change that facilitates the fixation of two ATP molecules by the NBDs. 2: the formation of the NBD dimer due to ATP fixation drives a major conformational change of the TMDs, that turns toward the outside and liberates the substrate. 3: The hydrolysis of ATP to ADP and inorganic phosphate (Pi) releases the NBD dimer. 4: the release of ADP and Pi completely separate the NBD dimer, restoring the initial TMD conformation, open towards the inside of the cell. Adapted from (Dermauw and Van Leeuwen, 2014). Created in Biorender.com.

Their canonical structure in eukaryotes is composed of two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs). ABC transporters are mainly composed of a single polypeptide that contains these four functional units. Depending on the transporter family, each TMD has from six (the most frequent) up to ten transmembrane α -helices. The substrate specificity varies strongly between ABC transporter families and individual transporters. As an example, the vitamin B12 importer from *E. coli* does not seem to have a substrate-binding site but rather transports molecules based on their hydrophobic properties, while P-glycoproteins of the ABC-B family display overlapping drug binding sites and thus have a "polyspecificity" (reviewed by Wilkins, 2015). ABC transporters are classified according to the number of TMDs and the position of the NBDs, this organization of domains varies between organisms and ABC families of the same organism (Fig. 22). Among these families, several have

been involved in MDR. In human cancer cells, overexpression of ABC transporters of the ABC-B family, P-glycoprotein 1 (P-gp, MDR1 or ABCB1 as synonyms), ABC-C (MRP1 or ABCC1) and ABC-G (BCRP or ABCG2) play a major role in MDR acquisition against a wide range of chemotherapeutic agents, including antifolates, anthracyclines, vinca alkaloids, podophyllotoxins and taxanes (reviewed by Krishna and Mayer, 2000; Robey et al., 2018).

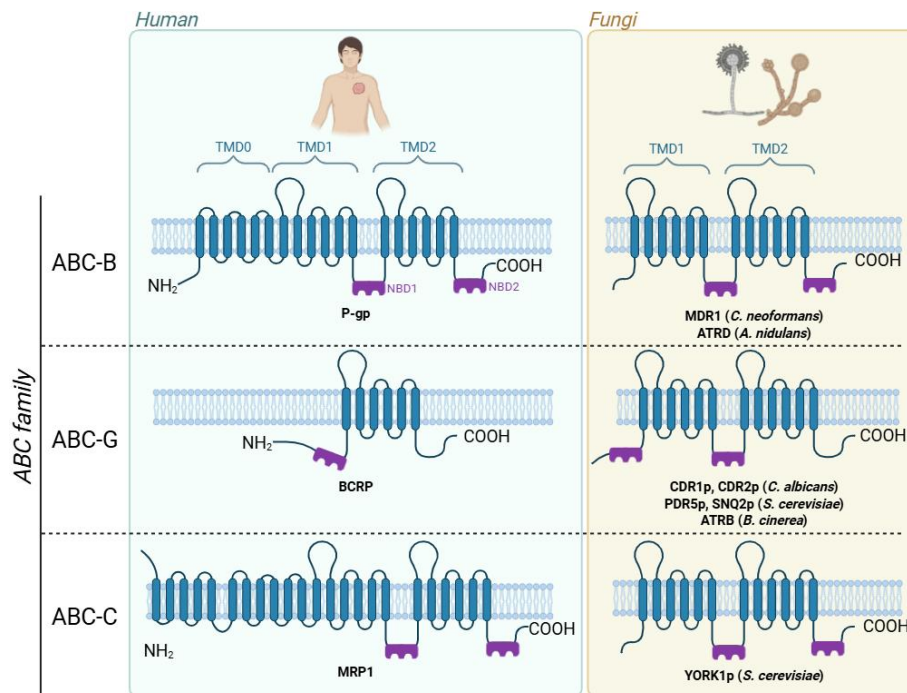


Fig. 22: Structure of major ABC transporter families, involved in multidrug resistance acquisition in human cancer cells and fungi. TMD: transmembrane domain, NBD: Nucleotide-binding domain. Adapted from Fonseca et al. (2016); Hu and Chen (2021). Created in Biorender.com.

ABC transporters play a major role in antifungal and fungicide resistance acquisition in fungi (plant and human pathogens). Examples are provided in Fig. 22 and table III. Studies of the model organism *S. cerevisiae* have been particularly useful for dissecting the role of drug transporters in fungi. YORK1p of the ABC-C family, when overexpressed, was found to confer resistance to rhodamine 6G (R6G) and to doxorubicin and weak protection against tetracycline, verapamil, eosin Y and ethidium bromide in this yeast (Grigoras et al., 2008). Besides, some fungal species also display ABC-B transporters, with homology to the human transporter P-gp, such as AtrDp of *A. nidulans*, involved in resistance to several toxic compounds (Andrade et al., 2000). Among the ABC-G family, both transporters from *C. albicans* CDR1p and CDR2p confer multidrug resistance to a large range of fungicides such as terbinafine, azole drugs and cycloheximide, but also to anticancer agents like doxorubicin or other toxic compounds such

as R6G. Both genes are overexpressed in some clinical isolates (Tanabe et al., 2011; Tsao et al., 2009). Likewise, in the plant pathogen *B. cinerea*, *BcatrB* confers resistance to pyrimethanil, fluodioxonil and interestingly, to several phytoalexins involved in plant defense (Bulasag et al., 2023; Vermeulen et al., 2001; Wang et al., 2025). This example hints to the original, physiological function of ABC-transporters and indicates that a MDR mechanism can also provide benefits for fungal fitness in nature through an increase in pathogenicity.

Beyond the fungal kingdom, ABC transporters are also involved in MDR in protozoa. Several mutations in the P-gp homolog (ABC-B) encoding gene *Pfmdr1* have been associated with resistance to major antimalarial agents like quinine, mefloquine, lumefantrine, and artemisinin in the malaria parasite *P. falciparum* (Capela et al., 2019).

While increased efflux from cells is relatively easy to assess, increased translocation into organelles has been less frequently reported. However, in some cases, overexpression of transporters located at the membrane of an organelle also leads to MDR. This is the case for two plant ABC-C transporters, *AmABCC1* and *AmABCC2* identified from MDR blackgrass *A. myosuroides* populations. Transient expression of these transporters tagged with GFP showed a tonoplast localization. Their heterologous expression in yeast led to reduced sensitivity to the sulfonyleurea herbicide mesosulfuron-methyl and the model GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) (Goldberg-Cavalleri et al., 2023).

MFS transporters

The other type of ubiquitous transporters involved in multidrug resistance refers to the Major Facilitator Superfamily (MFS) transporters, among the largest transporter families on earth. It contains antiporter and symporter of solutes, coupled or not with cations, as importers or exporters. Among the 17 families of MFS transporters, two are linked with drug transport, DHA12 (or DHA1) and DHA14 (or DHA2). They are composed of substrate/proton coupled antiporters, whose export mechanism is illustrated in Fig. 23. DHA12 and DHA14 transporters display structures of 12-transmembrane-spanner (TMS) and 14-TMS α -helices, respectively. Their structure and examples of relevant MFS in different species are displayed in Fig. 24.

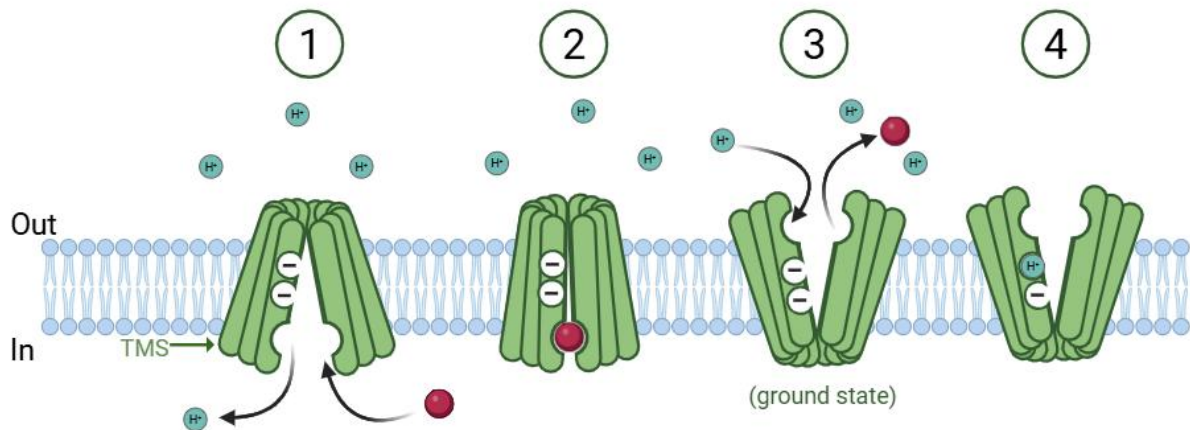


Fig. 23: Schematic representation of MFS (DHA12) driven multidrug substrate/proton (Fluman et al., 2012). The release of proton enables the substrate (red sphere) to bind in affinity pocket at the inner surface of the membrane (1). This binding is followed by conformational change (2), leading to the delivery of the substrate at the outer surface (3) before the binding of a new proton (4). TMS: TransMembrane Spanner. Adapted from Du et al., 2015. Created in Biorender.com.

MFS transporters in MDR have been identified in many species, especially in bacteria and fungi. Bacterial DHA12 and DHA14 include several pumps, such as LmrP and LmrS, respectively from *Lactococcus lactis* and *Staphylococcus aureus*. The latter is a major pathogen, responsible for important infections worldwide. Both transporters are able to export a wide range of substrates, including antibiotics (lincosamides, macrolides, streptogramins and tetracyclines for *L. lactis*; linezolid, trimethoprim and chloramphenicol for *S. aureus*) (Floyd et al., 2010; Putman et al., 2001).

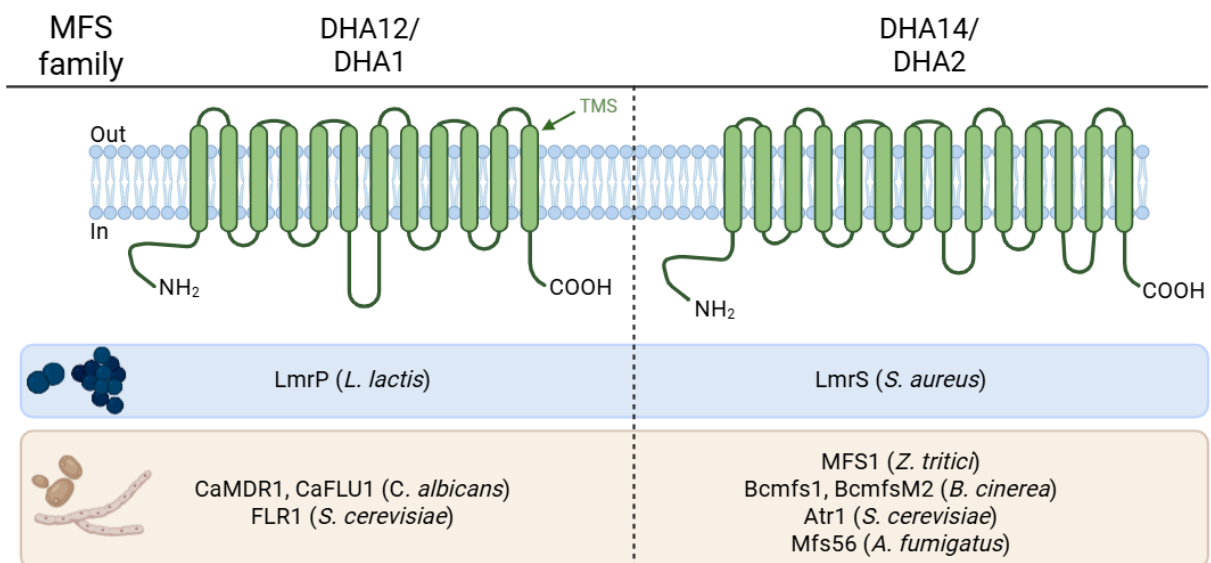


Fig. 24: Structure of major MFS transporter families, involved in multidrug resistance acquisition in bacteria and fungi. Based on references listed in the Table III. Created in Biorender.com.

Numerous MFS transporters have been associated with increased efflux and MDR in a range of fungal pathogens of plants and humans. CaMDR1 and CaFlu1 are two major transporters of *Candida albicans* belonging to the DHA12 family. The former is associated with a resistance to antifungals (e.g., fluconazole) as well as to antifolate chemotherapeutic agents (e.g., methotrexate) and other toxic compounds such as cerulenin and brefeldin A (Ben-Yaacov et al., 1994; Hiller et al., 2006; Kohli et al., 2001). Its overexpression in clinical isolates has been reported to correlate with fluconazole resistance (Lopez-Ribot et al., 1998). CaFlu1 mediates fluconazole resistance, and its expression in *S. cerevisiae* also drives resistance to unrelated antifungals and toxicants such as cycloheximide, terbinafine, sulfomethuron, cerulenin, and brefeldin A (Calabrese et al., 2000; Sanglard et al., 1997).

Two damaging plant pathogens, *Botrytis cinerea* and *Zymoseptoria tritici*, have shown increased efflux associated with MDR in the field due to overexpression of DHA14 MFS transporters. In *B. cinerea*, BcMfs1 has been shown to mainly transport natural toxic compounds (Hayashi et al., 2002). Meanwhile, the impact of BcMfsM2 on the MDR phenotype in the field was clearly demonstrated and associated with resistance to a wide range of unrelated fungicides (Kretschmer et al., 2009). Likewise, overexpression of *MFS1* in *Zymoseptoria tritici*, the causal agent of Septoria leaf blotch on wheat, has been linked to a rising MDR phenotype in the field in Northwestern European countries (Omrane et al., 2017, 2015). This will be detailed in paragraph 3 of this introduction.

MATE transporters

Among MDR transporters shared between prokaryotes and eukaryotes, MATE are important representatives, despite less studied than ABC and MFS families. The most characteristic feature of these proton or sodium ion-coupled antiporters is their 12-TM structure displaying long N- and C-terminal extensions. Across the different organisms in which they have been described, their functions vary but often include transport of xenobiotics and toxic compounds (Pastacaldi et al., 2024). In MDR *S. aureus in vitro* mutants, the overexpression of *mepA* encoding a MATE transporter is involved in the efflux of monovalent and divalent biocides and two antibiotics from the fluoroquinolone class, norfloxacin and ciprofloxacin (Kaatz et al., 2005). In clinical settings, MepA is responsible for tigecycline resistance, a widespread phenotype (Huang et al., 2023). MATE transporters are also suggested to play a

role in resistance to guazatine fungicide in the post-harvest fungal pathogen of citrus fruit, *Geotrichum citri-aurantii* (Zhao et al., 2020).

Table III: Overview of mechanisms leading to multidrug resistance in organisms from different phylogenetic origins.

Group	Species	Active Ingredient Resistance	MDR mechanism	Details	References
Mammals	<i>Homo sapiens</i> (cancer cells)	Cisplatin, doxorubicin	Reduced uptake	Altered membrane composition and fluidity	(Todor et al., 2012)
		Docetaxel, paclitaxel, camptothecin	Drug inactivation	Overexpression of GSTP1 (Glutathione S-transferase)	(Arai et al., 2008) (Ishii et al., 2004)
		Anthracyclines (e.g. dox), vinca alkaloids (e.g. vincristine), podophyllotoxins (e.g. etoposide) and taxanes (e.g. taxol).	Increased efflux	Overexpression of P-gl (ABC transporter)	(Krishna and Mayer, 2000)
		Methotrexate, mitoxantrone, topotecan, irinotecan, and flavopiridol	Increased efflux	Overexpression of BCRP (ABC transporter)	(Robey et al., 2018)
		Various neutral and anionic hydrophobic compounds and products of phase II drug metabolism	Increased efflux	Overexpression of MRP1 (ABC transporter)	(Robey et al., 2018)
Insects	<i>Drosophila melanogaster</i>	DDT, nitenpyram, dicyclanil	Drug inactivation	Overexpression of CYP6G1 (cytochrome P450)	(Daborn et al., 2002)
	<i>Bemisia tabaci</i>	Neonicotinoids, pymetrozine	Drug inactivation	Overexpression of CYP6CM1 (cytochrome P450)	(Gorman et al., 2010)
	<i>Aedes aegypti</i>	Pyrethroids	Reduced drug uptake	Epicuticular thickening via hydrocarbon remodeling due to overexpression of CYP4G16 and CYP4G17 (cytochromes P450)	(Balabanidou et al., 2016)
Mites	<i>Tetranychus urticae</i>	Cyenoxyrafen, fenpyroximate, bifentazate	Drug inactivation	Overexpression of CYP392A11 (cytochrome P450)	(Riga et al., 2015) (Lu et al., 2023)
		Fenpropathrin, cyflumetofen	Drug inactivation	Overexpression of TCE2 (esterase)	(Shi et al., 2016)
Protozoa	<i>Plasmodium falciparum</i>	Chloroquine, quinine, mefloquine, lumefantrine, and artemisinin.	Increased drug efflux	GOF mutations in Pfmdr1 (ABC transporter)	(Capela et al., 2019)
	<i>Leishmania sp.</i>	Antimonials	Increased drug efflux	Overexpression of ABCI4 and LABCG2 (ABC transporters)	(Manzano et al., 2013; Perea et al., 2016)
	<i>Trypanosoma brucei gambiense</i>	Pentamidine, melarsoprol	Reduced drug uptake	LOF mutations in TbAQP2 (Aquaglycerporin)	(Graf et al., 2013)
Weeds	<i>Alopecurus myosuroides</i>	Atrazine, chlorotoluron, alachlor	Enzymatic detoxification	Overexpression of AmGSTF1 (Glutathione S-transferase)	(Cummins et al., 2013)
		AST substrate 1-chloro-2,4-dinitrobenzene (CDBN)	Sequestration	Overexpression of AmABCC1 and AmABCC2 (ABC transporters)	(Goldberg-Cavalleri et al., 2023)
	<i>Lolium rigidum</i>	Diclofop-methyl, tralkoxydim, chlorsulfuron,	Enzymatic detoxification	Overexpression of CYP81A10v7 (cytochrome P450)	(Han et al., 2021)

		mesotrione, atrazine, chlorotoluron, trifluralin			
Fungi	<i>A. nidulans</i>	Cycloheximide, the cyclosporin derivative PSC833, nigericin, valinomycin, fenarimol	Increased drug efflux	Overexpression of ATRD (ABC transporter)	(Andrade et al., 2000)
	<i>Candida albicans</i>	Terbinafine, cycloheximide, rhodamine 6G, rhodamine 123, cerulenin, doxorubicin, cytochalasin D, latrunculine A, verapamil fluconazole, ketoconazole, itraconazole	Increased drug efflux	Overexpression of Cdr1/Cdr2 (ABC transporter)	(Tsao et al., 2009) (Tanabe et al., 2011)
		Methotrexate, fluconazole, cerulenin, brefeldin A, benomyl fluconazole, cycloheximide, terbinafine, sulfomethuron, cerulenin and brefeldin A		Overexpression of CaMDR1 /CaFLU1 (MFS transporters)	(Kohli et al., 2001) (Hiller et al., 2006) (Ben-Yaacov et al., 1994) (Calabrese et al., 2000) (Sanglard et al., 1997)
	<i>Botrytis cinerea</i>	Pyrimethanil, fludioxonil, phytoalexins	Increased drug efflux	Overexpression of BcatrB (ABC transporter)	(Wang et al., 2025) (Vermeulen et al., 2001)
		Camptothecin cercosporin azoles		Overexpression of Bcmfs1 (MFS transporter)	(Hayashi et al., 2002)
		Iprodione fludioxonil fenhexamid, cycloheximide		Overexpression of BcmfsM2 (MFS transporter)	(Kretschmer et al., 2009)
<i>Zymoseptoria tritici</i>	Azoles, SDHIs, Qols	Increased drug efflux	Overexpression of MFS1 (MFS transporter)	(Omrane et al., 2017, 2015)	
<i>Clariireedia homoeocarpa</i>	Propioconazole, iprodione, boscalid, flurprimidol	Global detoxification pathway	GOF mutation in master regulator ShXDR1 leading to overexpression of ShPDR1 and ShATRD (ABC transporters) and CYP561, CYP65, CYP68 (cytochrome P450)	(Sang et al., 2018)	
Bacteria	Gram-negative bacteria (several)	Fluoroquinolones, aminoglycosides	Drug modification (N-acetylation)	Variants of aac(6)-Ib gene (aminoglycoside acetyltransferase)	(Robicsek et al., 2006)
	<i>Mycobacterium smegmatis</i>	Ampicillin, vancomycin, erythromycin, rifampin	Reduced outer membrane permeability	LOF mutation in MspA (porin)	(Stephan et al., 2004)
	<i>Pseudomonas aeruginosa</i>	Chloramphenicol, carbenicillin, nalidixic acid, and novobiocin	Increased drug efflux	Overexpression of MexAB- OprM (RND transporters)	(Sobel et al., 2005)
	<i>Escherichia coli</i>	Ciprofloxacin, levofloxacin,	Increased drug	Overexpression of AcrAB	(Chetri et al., 2019)

		piperacillin, ertapenem, imipenem	efflux	(RND transporter)	
		Ethidium, methyl viologen, erythromycin, sulfadiazine, and tetraphenyl phosphonium, tetracycline		Overexpression of EmrE (SMR transporter)	(Yerushalmi et al., 1995)
	<i>Acinetobacter baumannii</i>	Erythromycin, novobiocin, benzalkonium chloride, deoxycholate, ethidium bromide	Increased drug efflux	Overexpression of AbeS (SMR transporter)	(Srinivasan et al., 2009)
	<i>Staphylococcus aureus</i>	Levofloxacin, vancomycin, clindamycin	Thick biofilm formation	Agr-dysfunctional quorum sensing mutants	(He et al., 2022) (He et al., 2023)
		Linezolid, tetraphenylphosphonium chloride, sodium dodecyl sulfate, trimethoprim, chloramphenicol	Increased drug efflux	Overexpression of LmrS (MFS transporter)	(Floyd et al., 2010)
		Norfloxacin, ciprofloxacin, tigecycline		Overexpression of mepA pump (MATE transporter)	(Kaatz et al., 2005)
	<i>Lactococcus lactis</i>	Lincosamides, macrolides, streptogramins, tetracyclines	Increased drug efflux	Overexpression of LmrP (MFS transporter)	(Putman et al., 2001)

GOF: Gain Of Function, LOF: Loss Of Function.

Before concluding on the individual mechanisms leading to MDR, it is relevant to end the story like we began it, addressing global detoxification pathways. Indeed, transporters and detoxification enzymes are, in many cases, under common transcriptional regulation, especially in organisms in which a clear phase I-phase II-phase III detoxification pathways have been unveiled. Thus, a mutation in a master regulator of this pathway is likely to change the expression of several components, leading to a complex multifactorial MDR phenotype. In the mosquito *Anopheles gambiae*, the transcription factor Maf-S has been identified as a key regulator of the overall detoxification pathway as knockout of the gene led to expression variation of several cytochrome P450, GST, carboxylesterase (COE), UDP-glucuronosyltransferase (UGT) and ABC transporter genes. RNAi silencing of Maf-S increased susceptibility of the resistant Tiassalé strain to DDT and the pyrethroids deltamethrin and permethrin, confirming the role of Maf-S in the regulation of the detoxification pathway and insecticide resistance (Ingham et al., 2017).

In the fungal plant pathogen *C. homoeocarpa*, Sang and collaborators identified a gain-of-function mutation in the transcription factor ShXDR1 in MDR strains, associated with constitutive overexpression of ABC transporters (*ShPDR1* and *ShATRD*) and several CYP450 genes (*CYP561*, *CYP65*, *CYP68*), leading to MDR (Sang et al., 2018). This comprehensive study unveiled that such structured detoxification system (phase I-phase II-phase III) does exist in

fungi, and that a single mutation in a master regulator is likely to lead to MDR through coordinated changes in gene expression.

Whether MDR arises through alteration of single protein's expression or activity, or of multiple proteins. The modification(s) involved are stably inherited through both mitotic and meiotic divisions. In the next part, we will further dissect the heritable basis of resistance, which is the main factor for discriminating resistance from tolerance.

2.2.4 Genetic origin of resistance

Since resistance is characterized as a heritable trait, we consider genetically encoded modifications, such as point mutations, indels, structural variants and changes in ploidy, as likely to drive resistance. While these genetic mechanisms are identical between TSR and NTSR, we will focus on the genetic drivers of multidrug resistance for illustrative purposes.

2.2.4.1 SNPs and TIPs

MDR can typically arise from mutations, *i.e.*, nucleotide substitutions, insertions or deletions. The consequences of these mutations leading to MDR may (i) modify substrate specificity or the activity of the transporter or enzyme, (ii) enhance its expression when it affects *cis* (*i.e.*, promoter) or *trans*-regulatory elements of the gene.

The first scenario can emerge from point mutations, *i.e.*, Single Nucleotide Polymorphisms (SNPs), resulting in amino acid substitutions that modulate substrate specificity and/or enhance substrate transport activity. This has been described in the BCRP (ABCG2) drug-selected variants in human tumor cells (Özvegy et al., 2002) and PfMDR1 variants of MDR *P. falciparum* (Ferreira et al., 2011).

Regarding the second scenario, expression enhancement can arise from SNPs when occurring in the promoter of the protein-encoding gene, as illustrated in other cases of cancer (Rund et al., 1999; Stein et al., 1994). Besides point mutations, insertions and deletions (indels) are also likely to affect *cis*-regulation of multidrug resistance genes. In particular, transposable element insertions are a case of large indels often reported as drivers of gene expression modification.

Transposons are repetitive and mobile genetic elements, dispersed throughout a large fraction of eukaryotic genomes (ranging from 12 % to 85 %) (Chénaïs et al., 2012). Transposon

insertion polymorphisms (TIPs) contribute greatly to the genomic plasticity and adaptation to stressful environments (Baril et al., 2025; Chénais et al., 2012; Chuong et al., 2017). The different classes of transposable elements, divided according to their mobilization mechanism and molecular intermediates, are illustrated in Fig. 25.

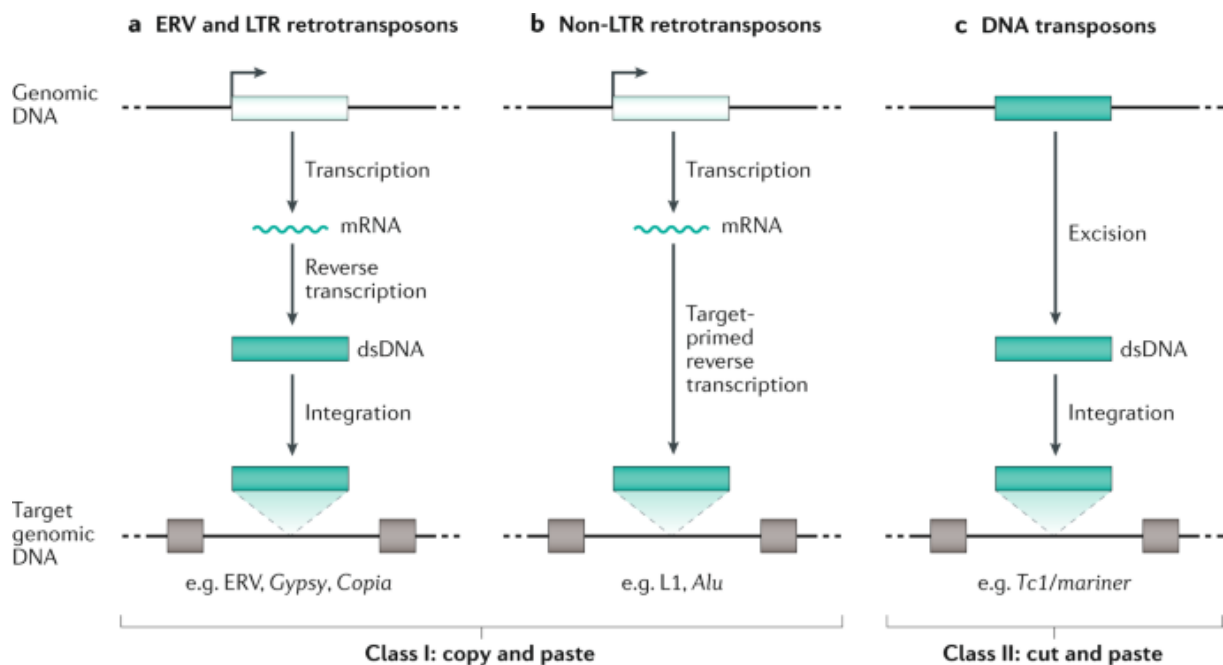


Fig. 25: Classification and transposition mechanisms of transposable elements. From Lanciano and Cristofari (2020). Class I comprises retroelements that replicate through an RNA intermediate and a reverse transcription step, the so-called copy-and-paste transposons, and comprises two main families: endogenous retroviruses (ERVs) and long terminal repeat (LTR) retrotransposons, such as Gypsy and Copia elements (a) as well as non-LTR retrotransposons, such as long interspersed elements (LINEs) and short interspersed elements, respectively (b). DNA transposons, the so-called cut-and-paste transposons, form class II (c). Their mobilization involves the excision of the transposon DNA from its original locus and its reintegration into another locus. Each class of TE comprises autonomous and non-autonomous elements. Autonomous elements encode the enzymes necessary for their own mobilization, whereas non-autonomous elements hijack the machinery encoded by autonomous elements.

Insertion of a transposable element in the promoter of a multidrug resistance gene has been described as a source of MDR through gene overexpression in many species, as in the promoter of the cytochrome P450 CYP6G1 encoding gene in *D. melanogaster* (Daborn et al., 2002), but also in the promoters of efflux transporters in numerous pathogenic and phytopathogenic fungi (Kretschmer et al., 2009; Omrane et al., 2015, 2017). In *B. cinerea*, a putative remnant of a long terminal repeat (LTR) retrotransposon was identified in the promoter of *BcMfsM2*, encoding an MFS transporter, and associated with a 600-fold increase in expression, leading to MDR (Kretschmer et al., 2009).

Meanwhile, SNPs and transposable insertion polymorphisms (TIPs) can also affect the

activity or expression of trans-regulating elements. This can arise from gain-of-function point mutations in transcriptional activators leading to constitutive activity, as described in *C. homoeocarpa* (Sang et al., 2018), *C. glabrata* (Ferrari et al., 2009) or in the transcription factor Mrr1 of *C. albicans* and *B. cinerea*, where such mutations constitutively activate transporter gene expression (Dunkel et al., 2008; Kretschmer et al., 2009). Loss-of-function mutations are also involved in MDR through repressor inactivation. As an example, loss-of-function mutations in the repressor genes *mexR*, *nalC* and *nalD* are regularly reported in clinical *P. aeruginosa* MDR isolates; these point mutations lead to overexpression of the *mexAB-opr* operon (Aguilar-Rodea et al., 2022; Daigle et al., 2007; Sobel et al., 2005; Srikumar et al., 2000).

2.2.4.2 Copy number variation

The term copy number variation (CNV) encompasses a range of genomic modifications affecting regions of different size that lead to an increase in the number of resistance genes: tandem duplication and deletion of genes to larger genomic amplifications due to whole-chromosome duplications. This eventually results in increased expression of resistance genes.

Local gene duplication is well described for TSR, such as the tandem duplication of *CYP51* involved in azole resistance in fungi (Yamada et al., 2022). It is also a mechanism in MDR acquisition, with duplications of key fluconazole resistance genes in *A. fumigatus* and *C. parapsilosis* including the multidrug resistance transporter genes (Bergin et al., 2024; Khateb et al., 2023). Conversely, gene loss or loss-of-function (LOF) events have been reported in *T. brucei* as deletion or chimeric rearrangement of the Aquaporin 2 encoding gene *TbAQP2* (Graf et al., 2013). Larger duplications, leading notably to aneuploidy, are also an important driver of resistance, especially in cancer cells (Ippolito et al., 2021) and pathogenic fungi, reviewed by Jay et al. (2025). Indeed, such genomic events lead to the duplication of multiple genes, including resistance genes, in one step at a potentially higher rate than point mutations. In *S. cerevisiae*, the mutation rate is estimated at around 10^{-10} per base pair per generation (Zhu et al., 2014) while chromosome aneuploidy rate is roughly 10^{-4} - 10^{-5} per generation (Sharp et al., 2018). Moreover, such genomic rearrangements can be driven by drug treatment itself, since some antifungal compounds were shown to alter chromosome separation, like fluconazole and methyl benzimidazole carbamates (MBC) (Altamirano et al., 2017; Bentley et al., 2000; Jeffay et al., 1996; Sarrif et al., 1994). In *C. neoformans*, disomy of chromosome 1 leads to amplification

of the *AFR1* gene, an azole transporter (Sionov et al., 2010). CNV was observed under antifungal treatment in *Candida albicans* leading to amplification of *CDR1* and *CDR2* genes as well as their transcription activator *TAC1* (Selmecki et al., 2008; Todd and Selmecki, 2020).

However, CNVs associated with aneuploidy are often not stable across cell divisions once the AI-associated selection pressure is removed. This may be explained by the high fitness cost associated with these mechanisms (Jay et al., 2025).

2.3 Tolerance and persistence: mechanisms and basis

Even if the mechanisms involved in tolerance and persistence are different between the different kingdoms, and dissected in a comprehensive review by El Meouche and collaborators (2024), some common features can be identified: While the cells are **genetically identical**, it does not imply that they are in the same metabolic state, even under constant conditions. This phenomenon is referred to as **phenotypic heterogeneity**. Tolerance or persistence can arise from cellular processes shared between bacteria, fungi and cancer cells, namely (i) asymmetric cell division, (ii) epigenetic differences among cells and (iii) stochastic biochemical reactions associated with gene expression (Fig. 26).

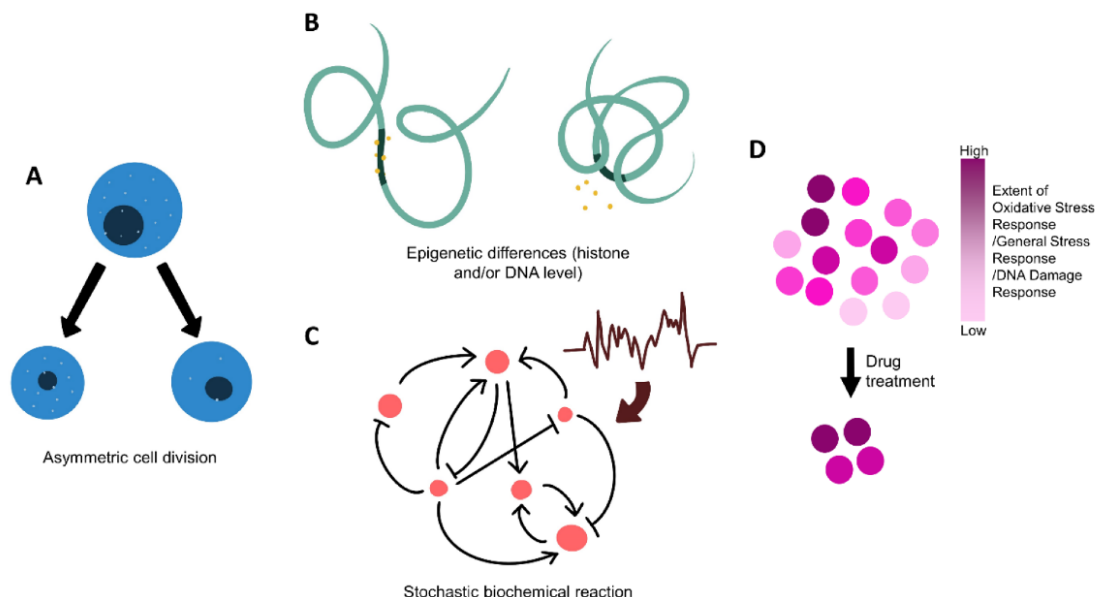


Fig. 26: Cellular processes giving rise to rare cells in the population that show persistent behavior to drugs. From El Meouche et al. (2024). A) Asymmetric cell division (asymmetric repartition of cellular content) B) Epigenetic variations between cells C) Stochastic biochemical reaction: network of biomolecular species regulating each other's level and the stochasticity involved because of small molecular counts. D) Intercellular heterogeneity in the extent of oxidative stress response, general stress response and DNA damage; and survival of cells with elevated stress response upon drug treatment.

One example is the unequal partitioning of the AcrAB-TolC efflux pump in a clonal *E. coli* population. It appears that during cell division, the mother cell inherits old cell poles enriched in AcrAB-TolC and displays increased drug efflux activity relative to daughter cells. Not only variability in gene expression but also the extent of its inheritance across successive generations play an important role for a population to survive drug treatment (Bergmiller et al., 2017). Regarding epigenetic importance, a mathematical model unveiled that the epigenetic inheritance of transcriptional rates of the AcrAB-TolC efflux pump regulatory network is necessary for bacterial adaptation to killing doses of antibiotics (Motta et al., 2015).

Beyond the cellular processes involved, several cellular pathways governing tolerance and persistence also appear to be shared (Fig. 26D), such as the involvement of oxidative stress response and activation of general stress response pathways (El Meouche et al., 2024). For instance, *C. albicans* persister cells overexpress superoxide dismutases that exert a protective effect against miconazole-induced ROS (Bink et al., 2011). Fungal persisters also produce trehalose and glycogen that help to cope with ROS-induced apoptosis by stabilizing proteins (Wuyts et al., 2018). In a review, Li and collaborators highlighted the importance of coordinated stress response in tolerance acquisition in *C. albicans* biofilm persisters, notably involving Ca²⁺-calmodulin-activated phosphatase calcineurin, the cell wall integrity pathway and the heat shock protein 90 (Hsp90) (Li et al., 2015). In cancer, persisters also respond to oxidative stress and are vulnerable to inhibitors of glutathione peroxidase (Hangauer et al., 2017).

Activated DNA damage response pathways also appear to be a shared feature. It is associated with increased mutagenesis (El Meouche et al., 2024). In yeast population, for example, slower growing persisters are more likely to accumulate DNA damage than fast-growing cells and they express a greater set of genes (Van Dijk et al., 2015). This suggests a crosstalk between chromatin state, DNA damage, stress response and growth rate in fungal tolerance and persistence. Similarly, colorectal cancer persisters selected upon chemotherapy display slow growth and an increased mutation rate under treatment (Russo et al., 2022). In bacteria, increased mutation rate in persisters was also observed and linked with efflux pump activity (Bhattacharyya et al., 2022; El Meouche et al., 2024).

Although **aneuploidy** is considered a genetic change, it has also been reported to drive tolerance in fungi. In their review addressing antifungal tolerance, Berman and Krysan conclude

that chromosome 3 segmental trisomy of *C. albicans* reported by Mount et al. (2018) as conferring fluconazole resistance, should rather be considered as tolerance because the MIC was not affected and the increased growth was detected after a 24h incubation period. Depending on the pattern of decreased sensitivity it confers, aneuploidy may act as a driver of resistance or tolerance. Another distinction should also be made. When it confers resistance, in some cases, the proportion of aneuploids is very low in the cell population, due to the high instability of aneuploidy. However, the overall MIC of the strain is affected by this small proportion of resistant individuals. This phenomenon is characterized by a biphasic dose-response curve. In that case, the term **heteroresistance** is preferred (Berman and Krysan, 2020; Yang and Berman, 2024). In the case of chromosome 1 disomy leading to *AFR1* amplification and azole resistance in *Cryptococcus neoformans* mentioned above, the term heteroresistance is therefore better suited because of this low proportion (Yang and Berman, 2024).

2.4 Resistance as an adaptative process

The generalization of AI resistance, including MDR, reported locally and globally, is the result of an underlying evolutionary process shaped by the selection pressure imposed by active ingredient use (Lucas et al., 2015). The dynamics of evolution of resistant allele frequencies under AI selection are defined by three phases, referred to as emergence, selection and stabilization phases, eventually leading to resistance in practice (Hawkins et al., 2019).

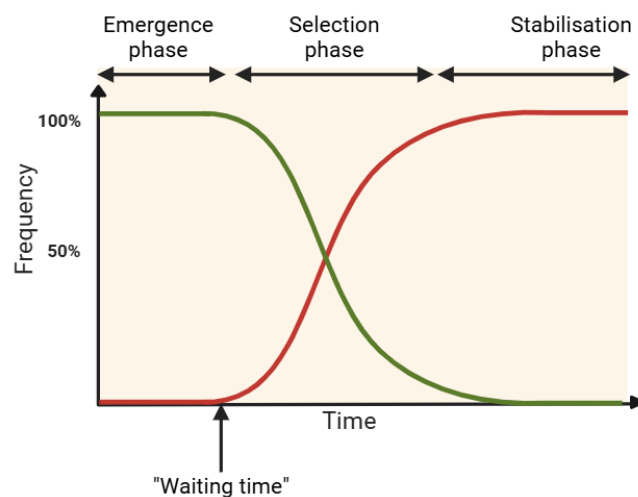


Fig. 27: Dynamics of resistance rise in population under active ingredient selection pressure. Created in Biorender.com.

2.4.1 Emergence phase

The emergence phase refers to the period for a resistant allele to arise in frequency until

a threshold upon which it is unlikely to go extinct by chance (van den Bosch et al., 2011). The time required to reach this threshold is the “waiting time”. The main driver of emergence is genetic variability, which can be divided into two categories according to timing of appearance of this diversity.

Standing genetic variations (SGV) refers to resistance mutations that pre-exist in the natural population, prior to stress exposure. Resistance mutations may have occurred randomly and have been maintained through genetic drift prior to adaptation to xenobiotics, such as host defense compounds, antimicrobial compounds produced by other organisms or toxic mineral substances. Pre-adaptation can also come from unrelated adaptations. Detoxification systems predate active ingredient applications, and are advantageous for coping with a range of natural toxic compounds. BcMfs1, which transports phytoalexins during *B. cinerea*'s interaction with host plants, can also transport fungicides. It is one example of pre-adaptation (Hayashi et al., 2002). In their review on insecticide, herbicide and fungicide resistance evolution, Hawkins and collaborators concluded that the majority of herbicide and some insecticide NTSR occur through selection of mutations from standing variation (Hawkins et al., 2019) while fungicide resistance seems more likely to emerge from *de novo* mutations (Hawkins et al., 2018).

De novo mutations (SNP, TIP or CNV) can emerge randomly after exposure to environmental stressors (*i.e.*, pesticide application) or they can be the result of the mutagenic effects of the stress in generating genetic variation. Indeed, many AI have a mutagenic effect, such as antibiotics fluoroquinolones, alkylating chemotherapeutic agents or antiviral drugs such as molnupiravir, and are likely to generate genetic diversity (Ching et al., 2020; Hunter et al., 2006; Kabinger et al., 2021). The rate of *de novo* mutation depends on the mutation rate of the organism, estimated around 2×10^{-10} per base per generation for bacteria, 1.6×10^{-10} in yeasts and 10^{-8} - 10^{-9} for multicellular organisms (Lee et al., 2012; Lynch, 2010; Zhu et al., 2014). However, due to their short generation time and large population size, microorganisms display higher mutation capacity than multicellular organisms.

Interestingly, in bacteria, tolerance and persistence have been shown to precede *de novo* mutations and the acquisition of resistance by increasing the probability that a mutation occurs

(Fig. 28). Notably, they promote longer survival, thereby extending the time window for a resistance mutation to arise. They also increase the number of surviving cells, reducing the likelihood that a resistance mutation will be lost by chance. In *Mycobacterium tuberculosis* it was shown that resistance mutations arose from antibiotic tolerant persister cells (Sebastian et al., 2017). The increased mutation rate associated with increased efflux in tolerant bacteria, as described in the previous section, may act synergistically with survival to accelerate the acquisition of resistance mutations (Bhattacharyya et al., 2022; Sulaiman and Lam, 2021; Windels et al., 2019).

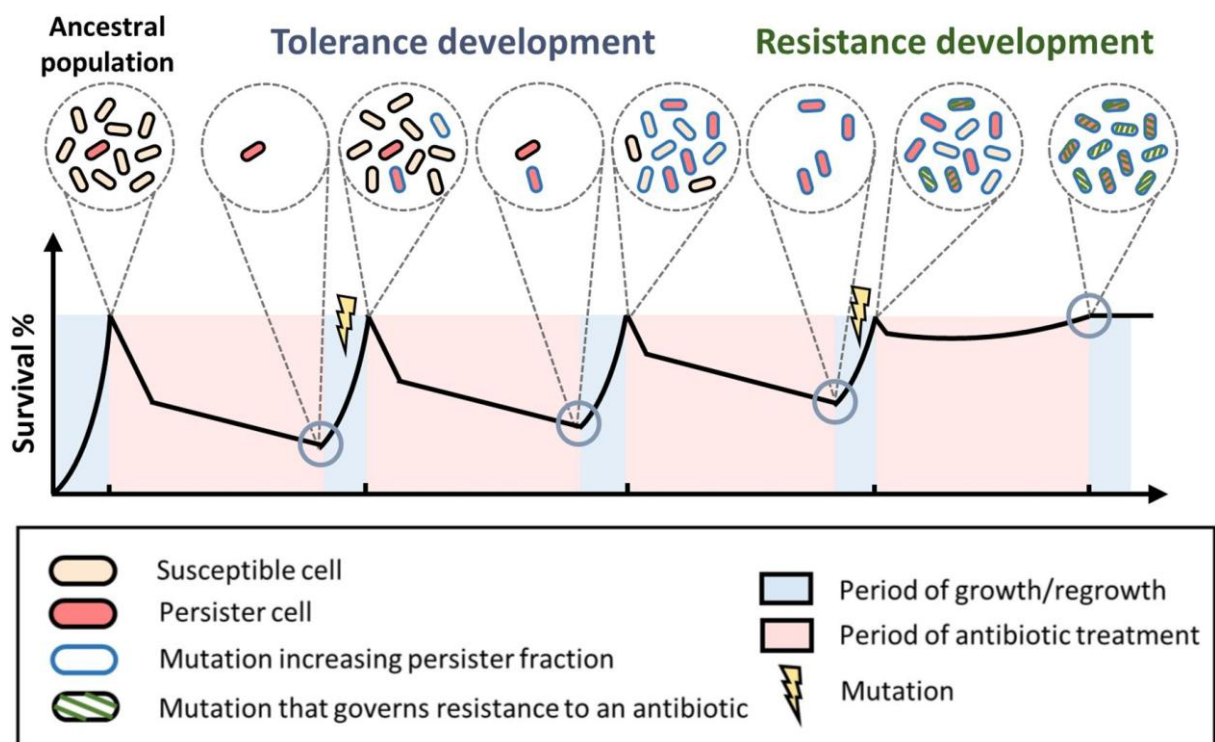


Fig. 28: Tolerance and resistance development during antibiotic treatment and regrowth. Typical evolution of bacterial populations under repetitive antibiotic treatments. From Sulaiman and Lam (2021).

Dynamics of resistance emergence are then different between *de novo* mutation and SVG, since the initial frequency is null in the former case and superior to zero in the latter one. Thus, the waiting time is longer for *de novo* variation than for SVG variation (Chevalier et al., 2019; Hawkins et al., 2019).

2.4.2 Selection and stabilization phases

While genetic variation drives the dynamics of emergence, the subsequent phases of resistance spread are mostly controlled by evolutionary forces of selection and drift, depending on the **absolute** or **relative fitness** linked to the mutation. **Absolute fitness** quantifies the

reproductive success of a genotype, depending on its survival ability and reproductive success in a given environment. **Relative fitness** refers to the comparison of different genotypes, as the ratio of the absolute fitness of a genotype to that of the wild-type (Crona and Wiesner, 2015). Both absolute and relative fitness are dependent on a given environment. Under AI selection pressure, the resistance allele confers higher relative fitness to the individual than the susceptible allele, since it enables its survival and reproduction. This is quantified by the selection coefficient (s), which is in favor of the resistance allele (Fig. 29) and supports its rise in frequency in the population under AI selection (Garnault et al., 2019).

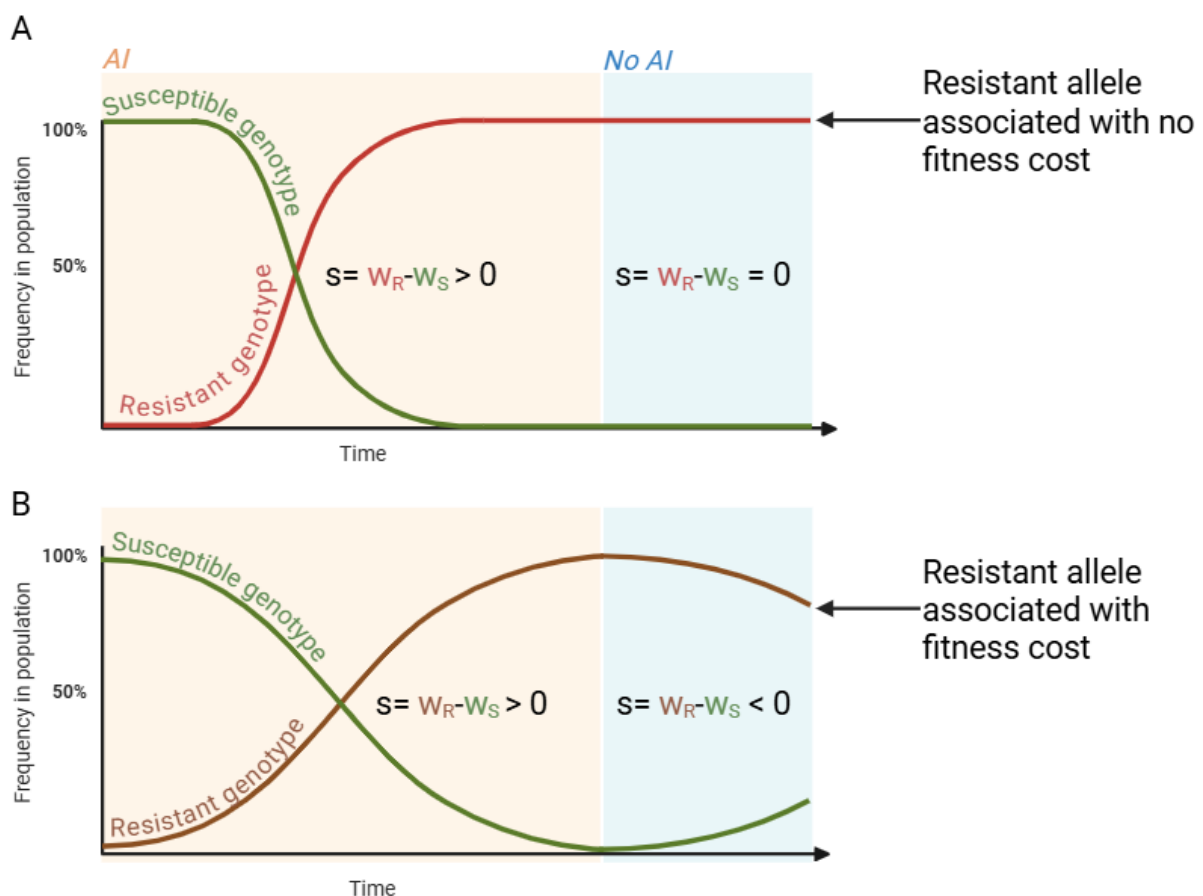


Fig. 29: Theoretical evolution of allele frequency in a population with active ingredient (AI) selection pressure (yellow area) or without (blue area). The resistant allele is not associated with fitness cost (A) or is associated with fitness cost under untreated conditions (B). w is the relative fitness of the resistant (R) and susceptible (S) alleles, s is the selection coefficient. Adapted from Ballu 2021. Created in Biorender.com

However, even if the resistant genotype is fitter than the susceptible one under AI selection pressure, this is not necessarily true when this selection pressure stops. In many cases, resistance carries a fitness cost. This is particularly true in TSR and NTSR when gene overexpression is at play, because it diverts energy toward the resistance mechanism at the expense of growth or pathogenicity (Hawkins and Fraaije, 2018). When the absolute fitness of

the resistant individual is lower than the susceptible one in an untreated environment, the “fitness cost of resistance” leads to a decrease in the frequency of the resistant allele (Fig. 29, example Fig. 30). This notion is important for management strategies, discussed in Section 2.5.

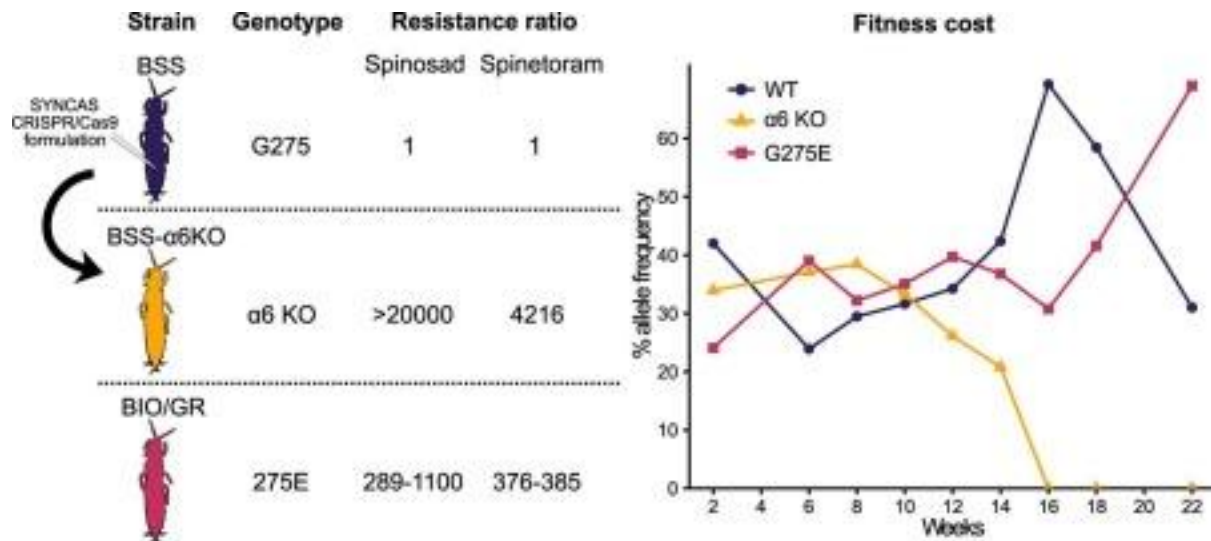


Fig. 30: Fitness cost of insecticide resistance. Resistance to spinosad conferred by two alleles of nAChR $\alpha 6$ the plant pest thrips *Frankliniella occidentalis* and frequency of the wild type and the two resistant alleles over time in segregating populations and insecticide-free media, revealing a fitness cost of $\alpha 6$ KO allele in untreated conditions. From Mocchetti et al., 2025.

It is important to note that not all resistance alleles are associated with a fitness cost, and even when it is the case, compensatory mutations may provide a rescue epistatic effect on fitness in untreated conditions (Bédard et al., 2024; Hawkins and Fraaije, 2018; Levin et al., 2000).

2.4.3 Qualitative versus quantitative resistance

The selection pressure exerted by AI exposure leads to a shift in the phenotypic distribution over time, resulting in an increase in the mean value of a resistance metric (e.g., EC_{50} or MIC) as the frequency of the resistant genotypes rises within the population. In that sense, AI exerts a directional selection pressure. However, depending on the genetic basis of resistance, the increase of resistance over time may follow different dynamics, see Fig. 31 (Lucas et al., 2015).

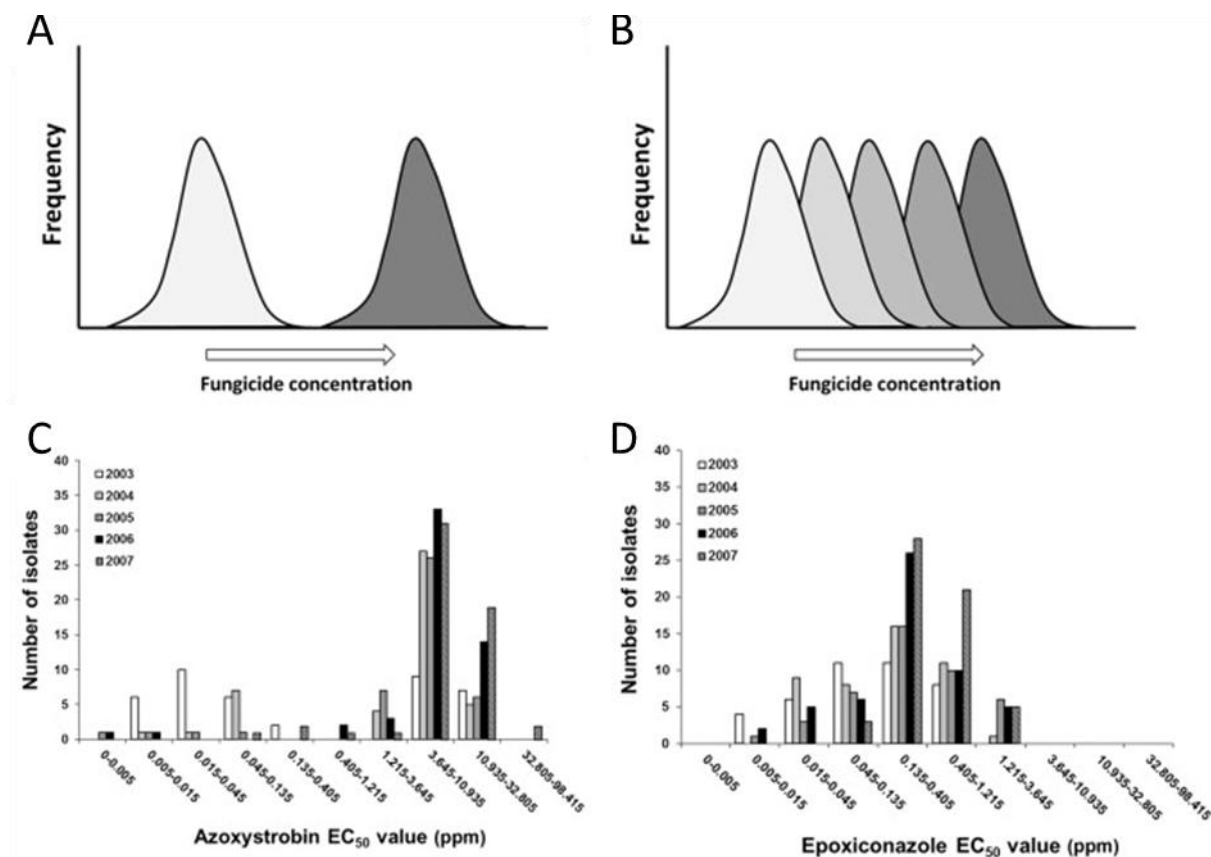


Fig. 31: Evolution of fungicide resistance in a pathogen population, shown as frequency against resistance level. From Lucas et al. (2015). (A) and (B) Hypothetical frequency distributions for discrete versus continuous sensitivity shifts. (A) Separation into sensitive and resistant subpopulations, typical of resistance due to a single genetic change of major effect. (B) Unimodal sensitivity distribution shifting toward resistance over time, due to multiple genetic changes of smaller effect. (C) and (D) Sensitivity distribution of isolates of *Zymoseptoria tritici* obtained from the field at Rothamsted in successive seasons (2003–07). (C) Response to the quinone outside inhibitor (QoI) fungicide azoxystrobin, showing increasing proportion of isolates highly resistant to the QoI. (D) Response to the azole fungicide epoxiconazole, showing a progressive shift toward resistance over the sampling period.

In that sense, the segregation of the overall population into two subpopulations of distinct resistance phenotypes is commonly referred to as **qualitative resistance**, usually controlled by a single mutation (monogenic resistance) affecting the target site and associated with a high resistance factor (discrete resistance in Fig. 31, A and C). Conversely, a unimodal resistance phenotype distribution that shifts toward a high mean over time is referred to as continuous or **quantitative resistance** (Fig. 31, B and D). In practice, it is revealed by progressive increase in resistance factor, and can be due to:

- Accumulation of **different mutations in the same resistance gene**, with different resistance levels associated with specific mutation combinations (monogenic, multi-allelic)

- Accumulation of **resistant alleles of different genes** (polygenic, mono or multiallelic) within the same genetic background.

In this last case, accumulation can arise from independent successive mutational events, recombination due to sexual reproduction or horizontal transfer (Hawkins et al., 2019; Lucas et al., 2015).

Insight into resistance dynamics and genetic and molecular mechanisms that drive them is a fundamental requirement to design robust management strategies intended to delay the onset of resistance and mitigate selective pressures.

2.5 Resistance monitoring and management strategies

Given the contraction in the number of effective AIs available, resistance risk analysis is essential for maximizing their sustainability by limiting the selection of resistance. It is defined as a two-stage process: (i) **resistance risk assessment**, which evaluates of the likelihood and impact of resistance, and (ii) **resistance risk management** which considers and implements strategies to avoid or delay practical resistance. Assessment of resistance risk and anticipation of resistance management are now prerequisites for the registration of any plant protection product (EPPO, 2015).

Specifically, in pesticide resistance analysis as defined by the European and Mediterranean Plant Protection Organization (EPPO, 2015), quantitative parameters are required for risk assessment. First, the **inherent risk** is defined as a combination of pesticide characteristics (*e.g.*, single versus multi-site mode of action, persistence, ease of metabolization), pest characteristics (*e.g.*, lifecycle, fecundity, genomic plasticity, mutation rate) and a combination of both (*e.g.*, known mechanisms of resistance identified in the species, existing cross-resistance or resistance against AIs of the same MoA without associated fitness penalty). It is defined as a score for each AI-species pair (Brent and Hollomon, 2007; Moss et al., 2019). Conversely, the **agronomic risk** is defined as the pattern of use of the AI, depending on how widely the crop is grown, routine cultural practices, number of applications needed, use of resistant or susceptible cultivars etc. This variable can be adjusted, according to the conclusions of risk assessment in evaluating the risk of **practical resistance** (combination of inherent risk and agronomic risk). If it is estimated to be at risk, possible modifiers are then

considered to determine if their implementation is likely to mitigate this risk. If such modifiers exist, the resistance risk analysis will be concluded by the designation of a resistance management strategy that can be applied when the product is marketed (EPPO, 2015). The basic components of resistance risk analysis are summarized in Fig. 32.



Fig. 32: The resistance risk analysis components. Based on EPPO (2015).

For assessment of both inherent risk and management strategy modifiers, different methodologies can be implemented. Among them, we will focus on two approaches, the generation of resistant mutants *in vitro*, via experimental evolution, and the monitoring resistance evolution *in natura*.

2.5.1 Resistance risk prediction *in vitro*: Experimental evolution

Experimental evolution (EE) is a method in which one or more ancestors of known genotype are used as the founders of a population through propagation under controlled conditions. This method is used to study underlying evolutionary processes (Cooper, 2018).

While it may virtually be implemented for every living organism, EE settings are well

suited to study microorganisms. Indeed, their large population size and short generation time make it possible to consider a large number of generations in manageable set-ups (Garland and Rose, 2009), some illustrated in Fig. 33 (McDonald, 2019).

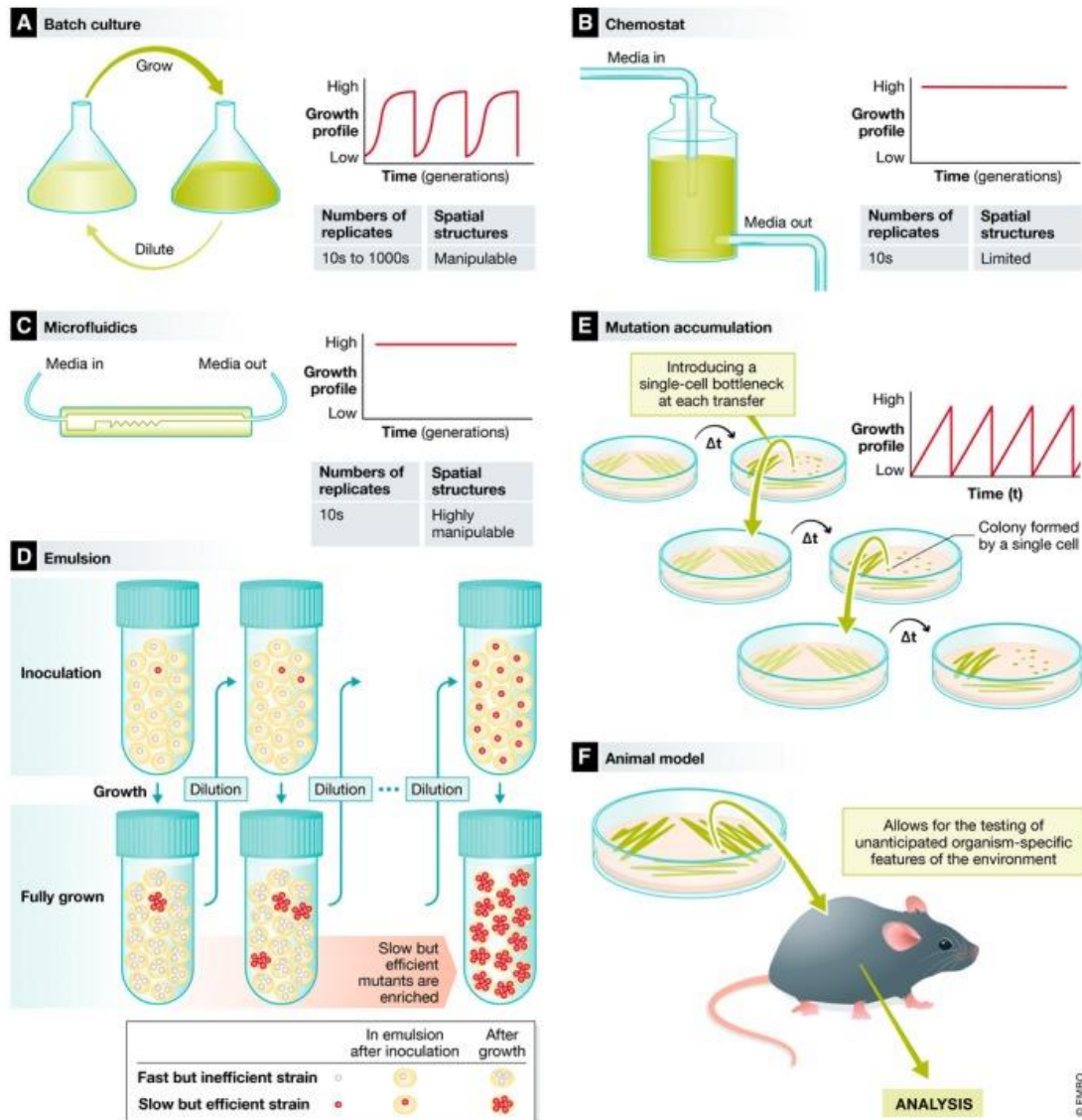


Fig. 33: Mechanisms of propagation for experimental evolution. From McDonald (2019). (A) Batch culture requires the regular dilution of culture into fresh media. (B) Chemostat culture systems include mechanisms for the constant supply of fresh medium for the continuous cultures and constant growth without 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. (D) Emulsion cultures take advantage of small cell-containing vesicles that form when mixing an oil, surfactant and cells. (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. This mode of experimental evolution allows for the testing of organism-specific features of the environment that are difficult to replicate in the laboratory.

Depending on the purpose of the study, the controlled environment can be constant, as illustrated by the long term EE carried out by Lenski and collaborators with the *E. coli* model, that has been running for more than 30 years (Lenski, 2017; Travisano and Lenski, 1996). Alteration of the environment is also achieved in other examples, for assessing adaptation to diverse stressors, such as temperature (Antunes et al., 2025; Santos et al., 2024; Tenaillon et al., 2012) or drugs and pesticides (Ballu, 2021; Lagator et al., 2013). It allows, under these selective conditions, the emergence and selection of resistant genotypes following dynamics described in section 2.4. In that sense, EE can be used for assessing intrinsic drug and pesticide resistance risks, without prior assumptions. For example, EEs under fungicide selection pressure selected resistant genotypes against Qil and SDHI fungicides in *Z. tritici* (G. Fouché et al., 2022; Gutiérrez-Alonso et al., 2017). It has also been used for assessing resistance to antibiotics (Perron et al., 2005; Trampari et al., 2020). In these experiments, pace of resistance rise is an indicator of resistance risk under unmodified use. When phenotypic characterization of selected mutants is coupled with genetic study (*i.e.* resequencing), it can also allow the identification of the resistance mechanism (TSR or NTSR), which is also important information for resistance risk assessment (G. Fouché et al., 2022; Gutiérrez-Alonso et al., 2017).

Beyond intrinsic resistance risk assessment, EE can also help to identify relevant modifiers to implement in resistance management strategies. This will be developed in 2.5.3. It has been used to evaluate the effectiveness of strategies for managing antibiotic (Kim et al., 2014; Nichol et al., 2019; Pena-Miller et al., 2013), fungicide, insecticide and herbicide (Ballu et al., 2023, 2021; Jacomb et al., 2016; Lagator et al., 2013; Sadia et al., 2024; Zoh et al., 2021) but also antimalarial resistance (Rodrigues et al., 2010).

Another positive input of EE is that it allows inter-mutant competition because of the growth in a mixed culture, resulting in the fittest mutants dominating (Gutiérrez-Alonso et al., 2017). In that sense, resistance with a high fitness cost, unlikely to occur *in natura*, is also less likely to emerge in EE due to competition. However, pathogenicity related fitness components are not often tested, since in-host EEs are of higher cost and lower speed, which may explain why some resistant genotypes identified with this method may not be viable and never detected in the field (Hawkins, 2024). Conversely, some naturally arising resistance alleles are never obtained by EE (Hawkins, 2024).

2.5.2 Resistance risk assessment *in natura*: Monitoring

While EE helps to predict resistance, **monitoring resistance *in natura*** is employed to detect resistance early in field pest or pathogen populations before control failure in practice. It can also be useful before pesticide or drug application, since it helps to define the overall susceptibility level. Monitoring resistance in other species can also be a good predictor of resistance selection dynamics and mechanisms (EPPO, 2015). For risk assessment and management, however, it is mostly an *a posteriori* approach that requires the pesticide or drug to have been introduced in clinical or field trials (EPPO, 2015; Hawkins, 2024; Hawkins and Fraaije, 2021). It can also be used to evaluate management strategies *a posteriori*, as illustrated with herbicide resistance (Comont et al., 2020). As for fungicide resistance, Garnault et al., (2021) modeled the relative efficacy of different management strategies using a dataset on *Z. tritici* fungicide resistance frequency from 13-year annual trials conducted in 70 locations.

Resistance can be monitored by phenotypic resistance testing of isolates (*i.e.*, biological assays), biochemical assays, or molecular diagnostics (example for insecticide resistance monitoring in Fig. 34).

Table IV: Advantages and disadvantages of methods for detection and monitoring of resistance in populations of insects. Adapted from Dusfour et al. (2019).

Methods	Advantages	Disadvantages
Biological assays		
<i>Diagnostic concentrations</i>	<ul style="list-style-type: none"> Standardized Simple and rapid to perform Detect resistance phenotype 	<ul style="list-style-type: none"> Lack of sensitivity No information on level or type of resistance Few diagnostic doses available for <i>Aedes</i> spp. Require live mosquitoes Require universal quality insecticides
<i>Dose-response assays</i>	<ul style="list-style-type: none"> Measure resistance levels 	<ul style="list-style-type: none"> Require large number of live mosquitoes Require a susceptible reference colony
<i>Assays using synergists</i>	<ul style="list-style-type: none"> Information on the potential mechanisms responsible for resistance 	<ul style="list-style-type: none"> Lack of sensitivity and specificity Require large number of live mosquitoes
Biochemical assays measuring enzyme activities	<ul style="list-style-type: none"> Information on mechanisms responsible for resistance Several mechanisms tested on a single individual 	<ul style="list-style-type: none"> Require a cold chain Not available for all resistance mechanisms Lack of sensitivity/specificity
Molecular assays to detect resistant alleles	<ul style="list-style-type: none"> Very sensitive Several mechanisms tested on single individuals Detect recessive alleles and provide an "early warning" of future resistance 	<ul style="list-style-type: none"> Require specialized and costly equipment Only available for a limited number of resistance mechanisms Are not always easily linked to resistance levels

For pesticide resistance management, as reviewed and detailed in the R4P Network (2016), the purpose of phenotypic testing is to identify shifts in population susceptibility to AI, regardless of the mechanisms involved. It can be achieved through measurement of

appropriate descriptors, such as mortality, survival, or growth, and determination of the resistance level on the basis of dose-response indicators such as EC₅₀ or MIC values described in Section 2.1, or by a qualitative screen using AI diagnostic doses. Some bioassays may provide additional information about resistance mechanisms such as those using synergists or efflux modulators, potentially revealing NTSR (R4P Network, 2016).

Biochemical assays can be implemented to identify metabolic enzyme activity and reveal resistance mechanisms. However, biochemical assays are often expensive and may require high-tech equipment (R4P Network, 2016).

Finally, molecular diagnostic tools are designed to detect resistant genes or mutations. It involves techniques based on nucleic acid amplification and genotyping (with or without sequencing), including isothermal amplification, quantitative PCR, PCR followed by Sanger, Nanopore or Illumina sequencing. They are fast, accurate, independent from living material and very sensitive for detecting allele frequencies in populations with high throughput. However, the main limitation of these assays is that they only reveal already characterized resistance mutations (R4P Network, 2016).

2.5.3 Modifiers in resistance management strategies

The above-described risk assessment methodologies help to identify relevant levers of pesticide (or drug) use to be implemented in resistance management strategies. These can then modulate the agronomical risk by delaying resistance emergence and/or generalization. They can involve non-chemical practices, such as quarantine measures for human pathogens, disinfection or vaccination (OECD, 2018). Against plant pathogens or pests, they include biological control, resistant varieties or other agricultural practices (*e.g.*, crop rotation, alternative host removal...) in an integrated pest management (IPM) approach (Beckie et al., 2021). Along with IPM, resistance management strategies mainly rely on maximizing heterogeneity in selection pressure in order to mitigate the directional selection exerted by an AI toward a resistance shift in the population. Heterogeneity of chemical selection pressure can be implemented over space and time. Four principal strategies combining at least two molecules over time and/or space are considered, to delay the evolution of resistance: 'Responsive alternation', 'Periodic application', 'Mosaic', and 'Combination' (table V) (Rex Consortium, 2013).

Table V: Names used to define resistance management strategies across research fields. From Rex Consortium (2013).

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

Responsive alternation refers to the use of the AI until the emergence of resistance, followed by the use of another drug molecule, used alone again, until resistance emergence, and so on. **Periodic application** refers to the temporal alternation between different AIs in different cycles, the rhythm of which can be modulated. When heterogeneity of selection pressure is achieved over space, the term **mosaic** is employed. Finally, **combination** refers to the concomitant use of at least two AIs (*i.e.*, mixture) that can be combined in different ratios and doses (Rex Consortium, 2013). The consequences of these different approaches on generational and intergenerational killing are illustrated in Fig. 34.

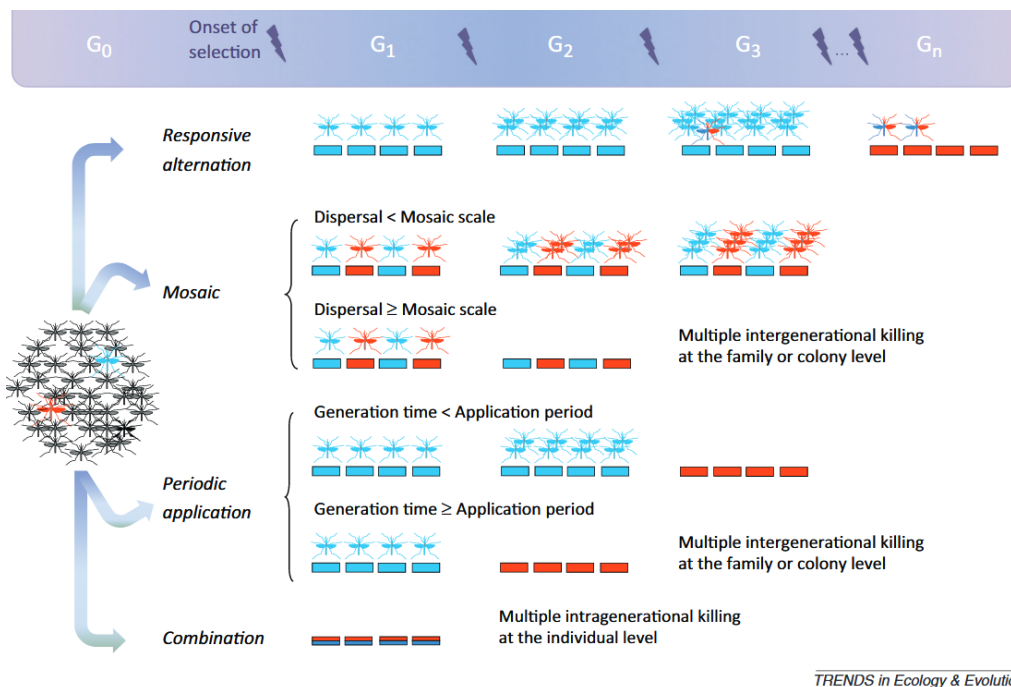


Fig. 34: Schematic representation of the effect of the different resistance management strategies (Responsive alternation, Mosaic, Periodic application, and Combination) on the targeted pests or pathogens, here, a mosquito. From Rex Consortium, 2013.

Combination is often referred to as the best management practice because it allows multiple intragenerational killing (Fig. 34) (Rex Consortium, 2013). But it depends on the set-up of the combination, notably the choice of AIs to combine, considering pre-existing resistance in the population, cross-resistance or antagonism between both molecules (Baym et al., 2016; Rex Consortium, 2013). The same applies to periodic application.

In practice, the best strategy to adapt depends on the target organism. Combinations are well established in the management of several infectious diseases, including HIV, tuberculosis and malaria (WHO, 2010a, 2010b, 2010c). In crop protection, the deployment of pesticide mixtures has likewise become increasingly common with commercial formulations in which the different AIs are pre-mixed. A survey of 590 grain farmers in Norway revealed that 89% reported using mixtures of compounds with different modes of action specifically to mitigate the risk of resistance evolution (Steiro et al., 2020). In contrast, combination approaches are not routinely adopted in antibiotic therapy, and not recommended for certain vector-control strategies, such as those targeting *Anopheles* mosquitoes, the vectors of malaria (Enayati and Hemingway, 2010).

The debate of the best strategy has led to evaluating the efficacy of these resistance management strategies through different approaches, both through EE and *in natura* monitoring as described in the previous sections. Interestingly, heterogeneous selection pressure due to combination and periodic application was shown to lead to selection of generalist resistance profiles in different organisms, e.g. MDR (Ballu et al., 2024, 2023, 2021; Comont et al., 2020; Lagator et al., 2013; Rodrigues et al., 2010; Zhou et al., 2022).

Supposed to carry a high fitness cost and to confer “only” low to medium resistance factors, MDR is often neglected in the agricultural sector (Hawkins and Fraaije, 2018). However, in some cases, MDR can be responsible for high resistance factors (above clinical breakpoints) and for increase in resistance factor against a given AI when stacked with TSR (Hu and Chen, 2021; Mitchell et al., 2014; Piddock, 2006; Vázquez-García et al., 2020). It is also not always associated with fitness cost, since some efflux pumps contribute both to resistance and virulence, probably due to efflux of both AIs and plant defense compounds (de Ramón-Carbonell et al., 2019; Lin et al., 2018; Schoonbeek et al., 2001). Even more alarming, MDR is also likely to compromise the efficacy of new unrelated MoAs. This is particularly concerning

given the declining rate at which new AIs are discovered and reach the market.

Deciphering the molecular basis of MDR is therefore critical to both understanding its evolutionary drivers and for adapting resistance management strategies. This is what we propose to address in this thesis, in the phytopathogen fungal model, *Zymoseptoria tritici*.

3 *Zymoseptoria tritici* as model species for studying multidrug resistance mechanisms in phytopathogenic fungi

3.1 Socio-economic impact of Septoria Leaf Blotch

Wheat, including mainly *Triticum aestivum* (common or bread wheat) but also *Triticum durum* (durum wheat), is a cornerstone of human food supply. It provides approximately 20% of calories to human nutrition worldwide and essential protein intake for more than 2.5 billion people from 89 countries (Gupta et al., 2024). Globally, wheat is the most widely cultivated cereal in terms of harvested area, covering around 220 million hectares in 2023, with over 798 million tons produced that same year (FAOSTAT, 2023). Recent assessments indicate that global wheat production will need to increase by approximately 35 to 56 % by 2050 in order to meet projected food demand (Kettlewell et al., 2023).

By prioritizing yield, domestication and modern breeding practices have contributed to a reduction in the genetic diversity of wheat, resulting in increased vulnerability of cultivated varieties to both biotic and abiotic stresses (Fu and Somers, 2009; Tanksley and McCouch, 1997). The threat represented by fungal diseases, estimated to cause global yield losses of about 15 to 20 % yearly is a global concern, particularly given the worldwide demographic evolution (Figueroa et al., 2018).

Among wheat foliar diseases, Septoria Leaf Blotch (STB), caused by the filamentous fungus *Zymoseptoria tritici*, is considered as the most damaging in Europe (Fones and Gurr, 2015; Torriani et al., 2015). Its symptoms are illustrated in Fig.35 A & B. By limiting photosynthetic activity of leaves, the disease impairs grain filling. It may result in yield losses of around 20% in susceptible cultivars, up to 50% under high epidemic pressure, with estimated annual yield losses in France and Germany of 350–700 and 250–500 million Euros, respectively

(Fones and Gurr, 2015). These losses can be reduced to 10% or even 5% through the choice of resistant cultivars and the application of fungicides (Fones and Gurr, 2015). But control has also a cost. In 2015, the European market for fungicides for STB control was estimated at 1.2 billion Euros annually (Torriani et al., 2015).

3.2 Biology of *Zymoseptoria tritici*

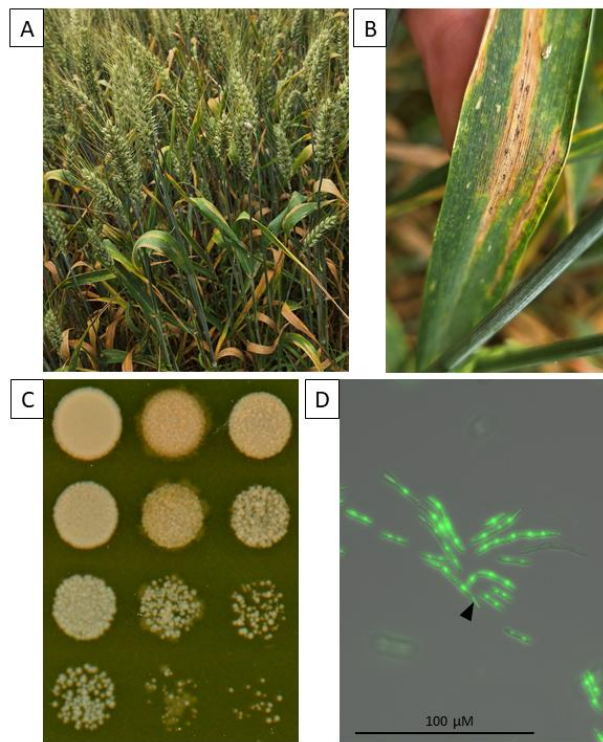


Fig. 35: Observations of *Zymoseptoria tritici* at different scales. A/ Symptoms of *Septoria tritici* blotch (STB) on wheat observed in early June 2025 in France (Poitou-Charentes, Deux-Sèvres, 79). B/ Wheat leaf infected by *Z. tritici* from the same field, showing characteristic rectangular lesions with chlorotic and necrotic areas bearing pycnidia. C/ In vitro growth of different field strains of *Z. tritici* on YPD medium. D/ Blastospores of *Z. tritici* field strain 09ASA-3apz after in vitro multiplication and DNA nuclear staining with SYBR Safe, observed under a fluorescence microscope ($\times 400$). The black arrow indicates a budding blastospore. Photo credit: Elza Neau.

3.2.1 Life cycle

Zymoseptoria tritici (anamorphe; previously teleomorph: *Mycosphaerella graminicola*) is an ascomycete fungus, belonging to the class *Dothideomycetes* and the family *Mycosphaerellaceae*, which includes numerous phytopathogenic species. Species of the genus *Zymoseptoria* are morphologically distinguishable in particular by their 'yeast-like' growth in culture (Quaedvlieg et al., 2011).

The life cycle of *Zymoseptoria tritici* (Fig. 36) is polycyclic and polyetic. It encompasses

both sexual and asexual reproduction. Primary *inoculum* is formed in late autumn and consists of different forms of fungal material (ascospores, pycnidiospores, mycelium and, possibly, chlamydospores) that overwinter on wheat residues or alternative host grasses that constitute a reservoir of the disease, such as *Anisantha sterilis*, *Festuca arundinacea*, or *Poa annua* (Suffert et al., 2011).

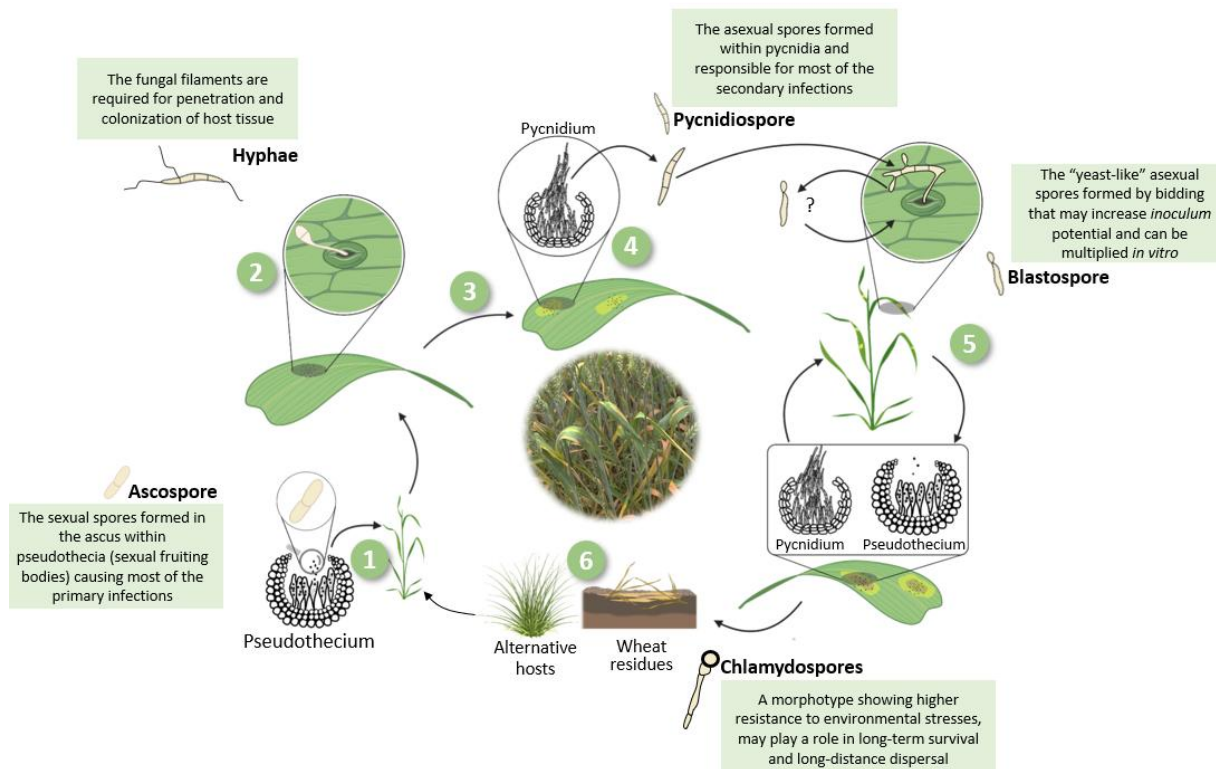


Fig. 36: Life cycle of *Zymoseptoria tritici*, adapted from Francisco et al. (2019).

1: Production and wind-driven dispersal of ascospores during the interseason, originating from sexual reproduction. 2: Ascospore germination and penetration of the germ tube into leaf tissues through the stomata. 3: Asymptomatic latent phase lasting 14 to 28 days. 4: Invasion of leaf tissues by the mycelium, leading to symptom development and the formation of asexual reproductive structures (pycnidia), which produce pycnidiospores that can in turn generate blastospores by budding. 5: Infection of surrounding plants and epidemic amplification driven mainly by pycnidiospores (dispersed by rain splash), and to a lesser extent by blastospores and ascospores. 6: Survival of the fungus during the interseason as mycelium, pycnidia, pseudothecia, and potentially chlamydospores. Created in Biorender.com, picture credit Elza Neau.

On wheat residues, the sexual reproduction arises from the interaction between individuals of two opposite mating types (MAT1-1 and MAT1-2) and leads to the production of ascospores in the sexual fruiting body, *pseudothecia* (Kema et al., 1996). These ascospores constitute the major players of primary infection. Under favorable conditions, with an optimum range of 15–25°C with low daily thermal amplitude, as well as high humidity, these spores germinate and produce germ tubes that penetrate leaf tissue through stomata (Bernard et al.,

2022; Boixel et al., 2022; Shaw, 1990). This constitutes the asymptomatic latent phase lasting 8 to 11 days (Sánchez-Vallet et al., 2015) and is accompanied by the secretion of effectors (Mirzadi Gohari et al., 2015; Thynne et al., 2024). Then, the fungal development accelerates, marking the onset of the necrotrophic phase of the life cycle and eventually results in the appearance of symptoms (necrotic lesions). On these lesions, asexual reproductive structures known as *pycnidia* are produced, harbouring pycnidiospores that generate blastospores by budding (Francisco et al., 2019). Pycnidiospores and potentially blastospores constitute the secondary *inoculum* and are dispersed principally by rain splash (Holmes and Colhoun, 1975; Shaw, 1987). Pycnidiospores dispersed from proximal wheat debris and from senescent basal leaves mark the cropping season, the fungus achieving about 5 to 6 asexual cycles a year as long as the climatic conditions are favorable (Suffert et al., 2018).

At later stages, sexual reproductive structures called *pseudothecia* form within necrotic tissues (around 25-30 days post inoculation), and produce ascospores, that participate in the global epidemic cycle and are dispersed by wind, over distances of several hundred kilometers. Recently, Suffert and collaborators demonstrated that even avirulent strains of *Z. tritici* can reproduce sexually on resistant wheat cultivars, without development of visible symptoms. Also, they showed that late sexual reproduction in the season is possible, on dead wheat tissues, but it does not contribute significantly to the *inoculum* size of the next season (Suffert et al., 2025).

Considered for long as a hemibiotroph, a study tends to review *Z. tritici* trophic classification as a 'latent necrotroph'. Indeed, during the latent phase, metabolic data indicates that fungal growth relies on killing the host cells for nutrient release, questioning the existence of a real biotrophic phase (Sánchez-Vallet et al., 2015).

The blastospores, emerging from budding pycnidiospores are particularly convenient for *in vitro* multiplication of the fungus, since they constitute a "yeast-like" form that can be induced by high nutrient availability and temperatures between 15°C and 18°C. This characteristic enables the cultivation of the fungus either in common liquid or solid growth medium, including in microassays and the use of optical density as a proxy of spore concentration of suspensions (Ballu, 2021). "Controlled" sexual reproduction, however, requires an outdoor incubation of infected wheat residues between 4 and 6 months (Orellana-Torrejón

et al., 2022; Suffert et al., 2013).

3.2.2 Adaptation capacities explained by genetic and genomic plasticity

Z. tritici exhibits high genetic variability, promoted by its ability to reproduce sexually multiple times during its developmental cycle leading to high recombination rates (Kema et al., 1996). It is also supported by a high gene flow, at regional and wider scales, due to the long distances the ascospores can be transported over (Mekonnen et al., 2020; Tucho et al., 2025).

Its fully sequenced 40 Mb genome contains thirteen core chromosomes and eight highly variable accessory chromosomes (Goodwin et al., 2011; Habig et al., 2017). This represents the highest reported number of accessory chromosomes in a filamentous fungus, comprising 12% of the genome of the reference strain IPO323. This dichotomy in the genome, between core and accessory sets of chromosomes is referred to as a “two speed” genome (Goodwin et al., 2011; Stukenbrock et al., 2010). Indeed, accessory chromosomes show higher presence/absence polymorphism, since they are easily lost during *in vitro* propagation (Möller et al., 2018), but they also undergo a higher mutation rate (Habig et al., 2021). The overall genome is particularly rich in repeated elements, including transposable elements (TE) (between 14 % and 21.5 %), that are enriched in accessory chromosomes. TEs are associated with chromosomal rearrangements, gene presence/absence variation, and effectors in several *Zygomycota* species (Lorrain et al., 2021; Oggenfuss et al., 2021). TE insertion polymorphisms are known to contribute to genome expansion and to drive adaptation, through insertions near genes. An example of adaptation in this species driven by TE insertion is host adaptation (S. Fouché et al., 2022), but also fungicide resistance, as developed in section 3.3.

Moreover, the DIM2 enzyme involved in defense mechanism against TE proliferation exhibits functional polymorphism among isolates of different geographic origin (Möller et al., 2021, Feurtey et al., 2023). Its inactivation appeared as a recent phenomenon that has spread from the center of origin of the species, *i.e.*, Middle East, and promoted hypermutator phenotype emergence due to increased TE activity (Baril and Croll, 2025). Thus, alongside with this DIM2 activity polymorphism, several recent TE bursts have been identified in the species; the most recent burst was dated about 25 years ago (Baril et al., 2025; Baril and Croll, 2025; Lorrain et al., 2021; Oggenfuss and Croll, 2023).

In that sense, *Z. tritici* displays a high genome plasticity which is currently on the rise. This plasticity favors its adaptation in selective environments and stress exposure, as it is prone to the emergence of new alleles. Large population size and important gene flow further promote maintenance and dispersion of adapted alleles (Zhan and McDonald, 2004).

3.3 *Zymoseptoria tritici* as a model to study adaptation

3.3.1 Disease control

Control of *Zymoseptoria tritici* blotch relies on different approaches for limiting yield losses. These are developed in an Integrated Pest Management (IPM) framework with the different components (Fig. 37).

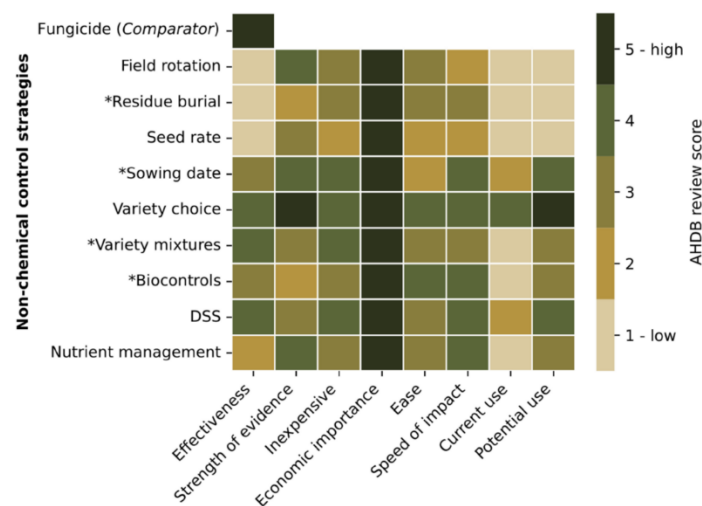


Fig. 37: Integrated Pest Management (IPM) means for *Septoria tritici* blotch control. Data are from the 2021 AHDB Research Review, and the figure from Vincent et al. (2025). Nine IPM measures applicable for the prevention of STB in wheat are identified and scored, by expert opinion, on eight qualities (including effectiveness of the control, economic factors, and ease/speed of implementation). Fungicide effectiveness is displayed in comparison. DSS: Decision Support Systems.

Agronomical practices

Among available practices, agronomical prophylactic measures can be implemented for limiting disease development, such as adjustments in seed rate and nitrogen fertilization, or practices helping to escape the disease and reducing the primary *inoculum* size, including delayed sowing and crop rotation (Almogdad et al., 2024; Ansar et al., 2010; Suffert et al., 2011). Crop rotation is recommended to disrupt the pathogen life cycle, as continuous wheat cultivation promotes the persistence of *inoculum* on crop residues and volunteer plants. According to Pederson and Hughes (1992), a 2-year rotation between wheat crops provides

adequate control of STB (Pedersen and Hughes, 1992). However, the high dispersal capacity of this fungus limits the implementation of agricultural practices aimed at reducing the *inoculum* size, since at the plot scale exogenous *inoculum* becomes higher during the cropping season than endogenous *inoculum*. For achieving an efficient limitation of primary inoculum, region-wide coordinated actions are necessary (Suffert et al., 2011). Overall, agronomical practices are minor management levers for STB control in wheat cropping systems.

Choice of resistant cultivars

Varietal resistance is an important lever for STB control. To date, 24 major resistance genes have been identified in wheat against *Z. tritici*, including 12 *Z. tritici* isolate-specific and 12 non-isolate specific genes (N. Yang et al., 2022). They confer qualitative resistance against the fungus. A gene-for-gene relationship, according to the Flore model, was unveiled only for *Stb6* and *Stb9* (Amezrou et al., 2023; Brading et al., 2002). *Stb6* and the more recently described *Stb16q*, are the only STB resistance genes cloned to date, both encoding receptor-like kinase proteins (Saintenac et al., 2021, 2018). Due to the qualitative resistance conferred by the resistant allele of these genes, their deployment is associated with strong directional selection pressure of virulent strains, overcoming the resistance. For instance, the major resistance genes *Stb4*, *Stb6*, *Stb2/11/WW*, and *Stb18* have been overcome in Australia (N. Yang et al., 2022). Despite efforts in identification of mechanisms enabling *Z. tritici* to escape varietal resistance, only three *Avr* genes have been cloned so far: *AvrStb6*, *Avr3D1* and *AvrStb9*, conferring resistance respectively to *Stb6*, *Stb7* and *Stb9* (Amezrou et al., 2023; Meile et al., 2018; Zhong et al., 2017), *AvrStb6* encoding a small secreted protein and *AvrStb9* a secreted protease-like protein.

One strategy to delay virulence emergence against resistant cultivars and preserve resistance gene durability is to maximize the heterogeneity of the selection pressure. This can be achieved by combining resistance genes in the same cultivar, through pyramiding of resistance genes (Chartrain et al., 2005; Tidd et al., 2023) or through mixture of cultivars with different resistance genes. A 2022 study reported 14% reduction in disease severity and 2% increase in yield in wheat cultivar mixture assays compared to cultivars grown in monoculture (Kristoffersen et al., 2022).

Finally, implementation of quantitative resistance is another interesting choice. Often polygenic and conferring partial resistance, this type of resistance is associated with lower virulence emergence. More than 90 Quantitative Trait Loci (QTL) involved in quantitative resistance have been reported improving protection against *Z. tritici* by up to 40% (N. Yang et al., 2022) (Brown et al., 2015).

Use of plant protection compounds

Finally, use of plant protection products represents the third main lever of STB control. Among them, biocontrol products are defined in France as “[...] macro-organisms or phytopharmaceutical products including micro-organisms, chemical mediators [...] or natural substances of plant, animal or mineral origin” (*Article L253-6 - Code rural et de la pêche maritime - Légifrance*, 2021). In France, available biocontrol solutions against STB are limited. Among them, natural algal laminarin extract (Vacciplant®, Arysta LifeScience) and chitosan hydrochloride (CHARGE®, ADAMA) are two plant defense elicitors that can be used against a variety of diseases including STB. Mineral compounds, such as sulphur and potassium phosphonates, sometimes in duo formulation (AQUICINE® DUO, Syngenta) also display efficacy against this disease (ARVALIS, 2024, 2023, 2020). However, while they have been implemented for other plant diseases, no biological control agents or organism-derived biomolecules have been registered or are commercially used against STB yet, even if studies have identified interesting BCA candidates (*Pseudomonas spp* & *Bacillus spp* strains; *Trichoderma harzianum*, *Penicillium olsonii* & *Acremonium alternatum* strains) (Suarez-Fernandez and De Francesco, 2024).

Despite all before mentioned control means, STB control heavily relies on the use of chemical control, with fungicides targeting *Zymoseptoria spp.* accounting for over 50% of the cereal fungicide market within the European Union (Torriani *et al.*, 2015). All fungicides used against *Z. tritici* are listed in the Table VI.

For a brief overview, the earliest commercialized fungicides were multisite AIs, acting on multiple cellular targets, including the fungal electron transport chain (ETC), lipid and carbohydrate pathways (Leroux, 2003). They include structurally diverse families such as dithiocarbamates (e.g., mancozeb), chloronitriles (e.g., chlorothalonil), and halogenated alkylthio compounds (e.g., folpet). Multisite fungicides are also referred to as contact fungicides

by opposition with systemic fungicides, and are often unspecific with low persistence and requiring high doses for disease control (Baibakova et al., 2019). This contributes to their ecotoxicity, a reason for their withdrawal, such as for chlorothalonil and mancozeb withdrawn in France respectively in 2020 and 2021.

Later on, unisite fungicides were developed, classified according to their cellular targets (Fig. 38). The first unisite fungicide class, Methyl Benzimidazole Carbamates (MBCs), inhibiting microtubule polymerization, was introduced in the early 1970s. Benzimidazoles bind β -tubulin, resulting in impaired cell division and growth (Zhou et al., 2016). Due to development of fungicide resistance, MBCs are not used anymore against STB (Garnault et al., 2019).

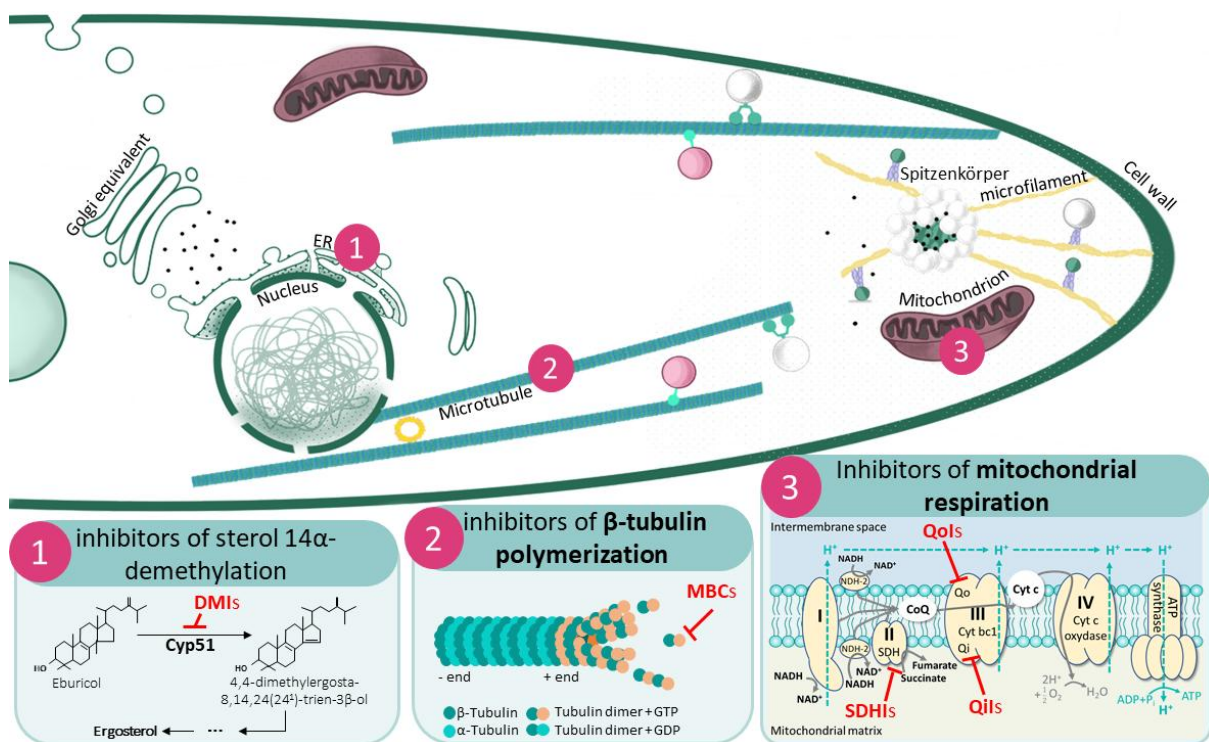


Fig. 38: Molecular targets and modes of action of the principal unisite fungicides historically used against *Zymoseptoria tritici*.

Introduced gradually between 1980 and 2006, DeMethylase Inhibitors (DMI), also commonly known as azoles, were incorporated into treatment routines. This group, comprising imidazoles, triazoles, and triazolinethiones, inhibits the sterol 14- α -demethylase, a cytochrome P450 encoded by the *CYP51* gene. They are non-competitive inhibitors of the Cyp51 protein and their effect results in accumulation of 14- α -methylated sterols and concomitant depletion in ergosterol content (Fig. 39), the principal sterol maintaining cell membrane integrity in Eumycetes (Ermakova and Zuev, 2017). Among DMIs, not all display the same affinity and

binding dynamics. For example, prothioconazole shows a competitive inhibition behavior with the natural substrate of Cyp51, which is not the case for voriconazole and prothioconazole desthio (Parker et al., 2014).

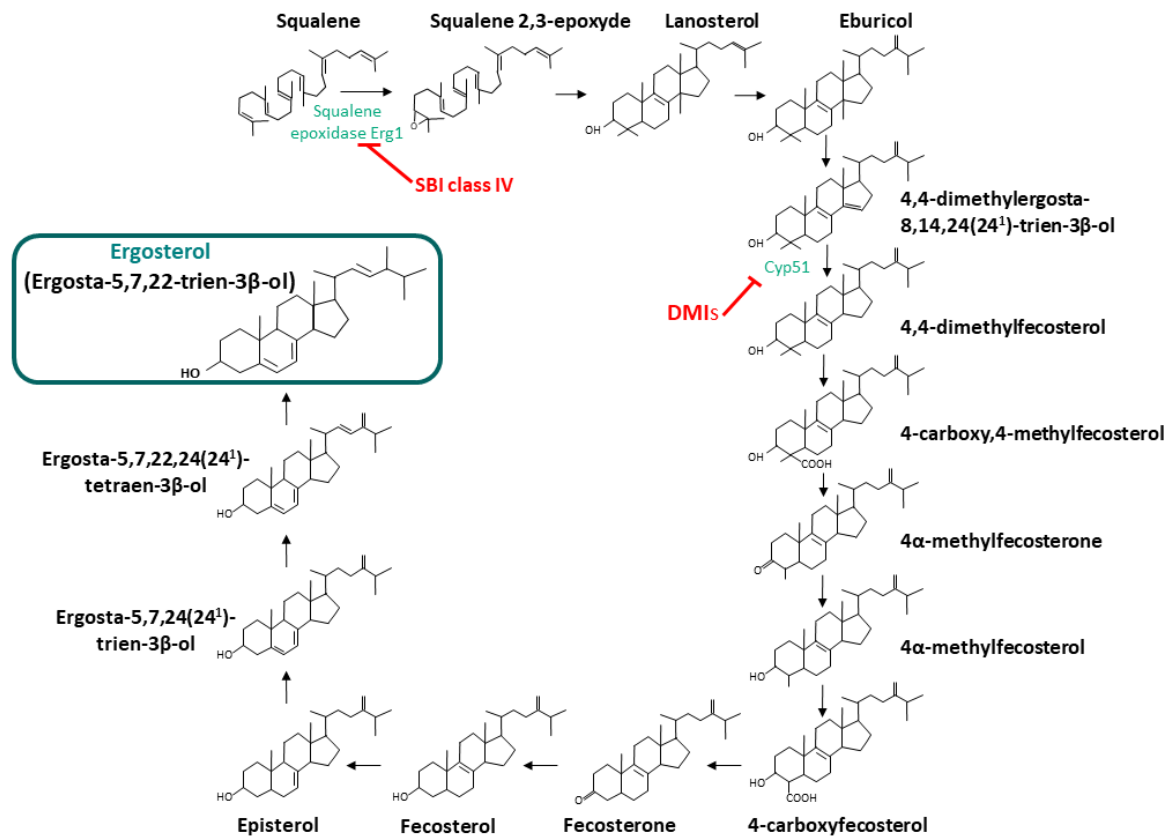


Fig. 39: Mode of action of DMI fungicide in inhibition of the ergosterol biosynthesis pathway of filamentous fungi. Adapted from Fouché (2021).

Fungicides targeting mitochondrial function were the most recently introduced: Quinone Outside Inhibitors (Qols, also referred to as strobilurins) were launched in the early 2000s, as derivatives of natural fungal toxins (Balba, 2007; Nofiani et al., 2018) followed by Quinone Inside Inhibitors (Qils) in 2021 with the release of fenpicoxamid (Owen et al., 2017). They both target the cytochrome *bc*₁ of the respiratory chain complex III, respectively in the quinone outside and inside binding sites. Succinate DeHydrogenase Inhibitors (SDHIs), authorized since the 2010s, inhibit the complex II, the succinate dehydrogenase composed of four subunits, encoded by *sdhA*, *sdhB*, *sdhC* and *sdhD* genes. SDHIs bind to the ubiquinone binding-site constituted by residues from the subunits SDHB, SDHC and SDHD (Avenot and Michailides, 2010).

Table VI: Principal fungicides and mode of action used against *Zyloseptoria tritici* and resistance. Adapted from Ballu 2021 and Garnault et al., 2019

Biochemical mode of action ¹			Chemical structures ¹		Active ingredient ²	Authorization in France ³			Target-site resistance ⁴	
Main processes	Target site and mode of action	Abbreviation	Main class	Secondary class		Registration	First year of use	Removal	Target alteration	Target overexpression
Mitochondrial respiration	Complex II or succinate dehydrogenase Binding site of ubiquinone involving the subunits SdhB, SdhC and SdhD.	SDHI	Carboxamides	Benzamides	Fluopyram	2016	2017	-		
				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	Synthetic strobilurins and analogs	Methoxyacrylates	Azoxystrobin	1996	1997	-		
					Picoxystrobin	1999	2000	2018		
				Methoxycarbamates	Pyraclostrobin	2000	2000	-		
				Oximinoacetates	Kresoxim-methyl	1996	1997	2013		
					Trifloxystrobin	2001	2002	-		
				Oximinoacetamides	Dimoxystrobin	2009	2010	-		
Fluoxastrobin	2006	2006	-							
Tetrazolinone	Metyltetraprole	?	-	-						
Mitochondrial complex III or cytochrome bc1 - fixing area for the ubiquinone on the inner face of the cytochrome b	Qil	Carboxamides	Picolinamides	Fenpicoxamid	2021	2021	-			
				Florylpicoxamid	-	-	-			
Sterol biosynthesis	Binding to sterol 14 α -demethylase (syn. CYP51)	DMI or SBI-DM	Heterocyclic compounds	Imidazoles	Prochloraz	1980	1980?	-		
				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	Binding to β -tubulin	MBC	Benzimidazole and precursors	Benzimidazoles	Carbendazim	1972	1972?	2009		
				Thiophanates	Thiophanate-methyl	1973	1980?	2021		
Multi-site activity	Fungicide acting non-specifically on multiple targets, especially respiratory enzymes	MSI	Dithio-carbamates	Alkene-bis-dithiocarbamates	Mancozeb	1999	1999?	2021		
					Maneb	1988	1988?	2017		
			Chloronitriles	Phthalonitriles	Chlorothalonil	1980	1980?	2020		
			Halogenated alky-thio compounds	Phthalamides	Folpet	2003	2003	2009 (reintroduced in 2020)		
			Sulfur-based mineral substance	-	Elemental sulphur	1942	1942?	-		

? Approximate date

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

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

3: Legal authorization 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>).

Black highlighting: TSR exists in the field. White highlighting: TSR has not been detected in the field.

Protocol for chemical control

A standard strategy for the control of septoria tritici blotch (STB) typically involves two to four fungicide applications, starting at early stem extension and extending to a final spray at full flowering (Creissen et al., 2018; Fones and Gurr, 2015). The main objective of these treatments is to protect the uppermost flag-leaves that are the main sugar source for grain filling, the main contributor to yield (Parry et al., 2011).

As extensively discussed in Sections 1 and 2, exposing populations to active ingredients, such as fungicides, typically leads to evolution of resistance, which was the case for *Z. tritici* against fungicides within a few years of deployment (Estep et al., 2015; McDonald et al., 2019).

3.3.2 Fungicide resistance

3.3.2.1 Target-site resistance

Z. tritici has evolved (*in vitro* and/or in the field) TSR against all unisite fungicide classes deployed in the field for chemical control (Table VI). The only group for which no resistance was observed is the class of multisite fungicides. The evolution of resistance frequency to the different fungicide classes in *Z. tritici* populations, in parallel with fungicide use has been studied by Garnault and collaborators (2019) (Fig. 40). Resistance appeared as generalized against MBC at the end of the 1980s and in the early 2000s for QoI. Notably, QoI resistance was detected even before market launch of strobilurins in the field, during field trials (Lucas et al., 2015). Resistance to SDHI, fungicides introduced later, was hardly detected in Garnault analysis, but has been on the rise since 2019 with global shift in overall susceptibility to both DMIs and SDHIs (Hellin et al., 2021). Resistance to MBC, QoI and SDHI are associated with TSR due to SNP mutations in the target encoding genes (*i.e.*, *tub2* E198A; *cytb* G143A; *SdhC*-H152R), leading to bimodal phenotype distribution (susceptible vs. resistant), the resistant phenotype rapidly invading the population. The fixation of MBC and QoI resistance over time also indicates that no fitness cost is associated with these resistances (Garnault et al., 2019; Hawkins and Fraaije, 2018).

Conversely, DMI resistance has evolved in a multistep way (as opposed to single-step evolution described for other fungicides). The field efficacy gradually declined with DMI use concomitantly with the rise of genotypes with increasing resistance factor as the result of accumulation of different combinations of TSR mutation in *CYP51* including SNPs in the CDS and a transposon insertion in the promoter leading to *CYP51* overexpression (Cools et al., 2013, 2012; Cools and Fraaije, 2013; Leroux and Walker, 2011). Interestingly, in triazole highly resistant genetic background (TriHR), the MDR phenotype also rose in frequency (Fig. 40).

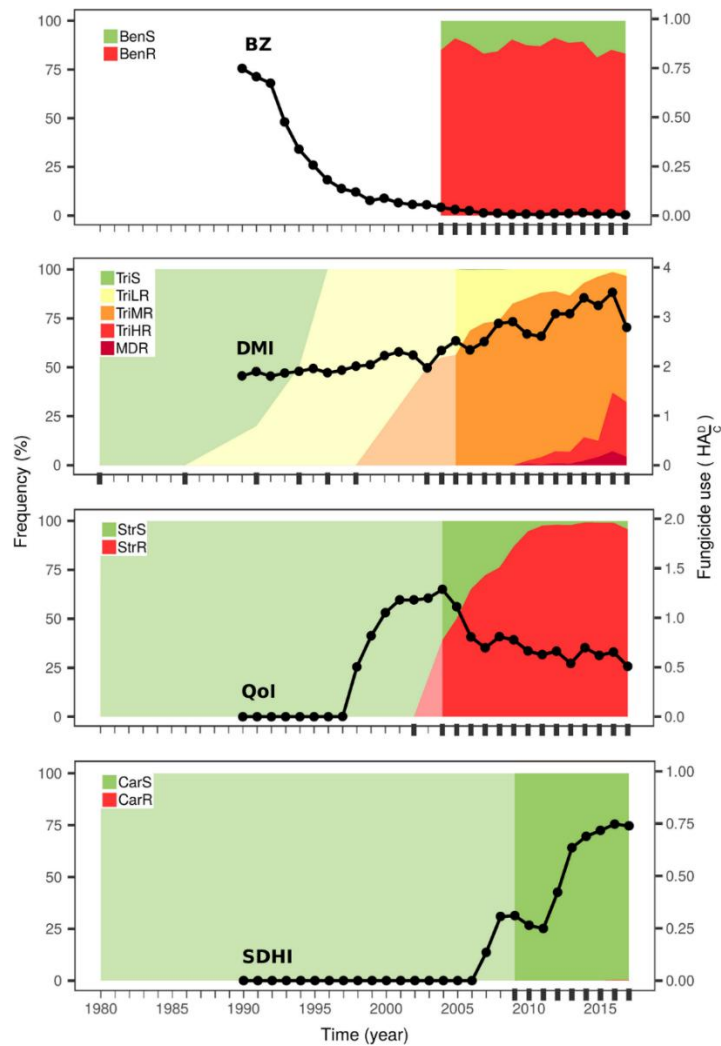


Fig. 40: Changes in fungicide use and resistance frequency in *Zymoseptoria tritici* populations in France according to Performance database. From Garnault et al. (2019). Thick ticks along the x-axis indicate years for which resistance frequency was assessed by monitoring. Lighter ticks indicate that information was obtained or extrapolated from sources other than the Performance database. BZ, benzimidazoles (antimicrotubule agents); DMI, sterol demethylation inhibitors; Qol, inhibitors of respiration complex III; SDHI, inhibitors of respiration complex II. Car: Carboxamides, Ben: Benzimidazoles, Tri: Triazoles, R: resistant, S: susceptible, LR/MR/HR: Low/Medium/High resistance.

3.3.2.2 Multidrug resistance in *Zymoseptoria tritici*

MDR in this fungus was originally studied *in vitro* only, principally through the study of ABC and MFS transporter genes (*MgAtr1*, *MgAtr2*, *MgAtr3*, *MgAtr4*, *MgAtr5* & *MgMFS1*). Expression of some of them is inducible by fungitoxic compounds such as imazalil and cyproconazole but also by natural plant defense compounds. *MgATR1* was found to reduce azole susceptibility when expressed in *S. cerevisiae* (Stergiopoulos et al., 2003, 2002; Zwiers et al., 2002). Heterologous expression of *MgMFS1* in *S. cerevisiae* resulted in a strong decrease in sensitivity to a broad range of unrelated toxic compounds while *MgMFS1* deletion in *Z. tritici*

resulted in increased sensitivity to strobilurin fungicides (Roohparvar et al., 2007a, 2008).

Walker and Leroux identified MDR isolates from the field, displaying resistance to DMI, slight positive cross-resistance with SDHIs and Qols, but also to a mode of action not used in the field, SBIs of class IV, inhibiting the squalene epoxidase in the ergosterol biosynthetic pathway (Fig. 39), namely terbinafine and tolnaftate (Leroux and Walker, 2011). The postulate was made that, since class IV SBIs (Sterol Biosynthesis Inhibitors) are not used in agriculture, resistance observed is unlikely to be due to TSR, since no directional selection against squalene epoxidase mutations has been at play, and thus NTSR is far more likely. This postulate is still a cornerstone of MDR assessment in this fungus, particularly in the present thesis work.

A few years later, Omrane and collaborators shed light on the mechanisms at play in *Z. tritici* MDR field strains and identified *MFS1* (previously named *MgMFS1*), as constitutively overexpressed in MDR isolates. A prochloraz accumulation assay with different transport modulators confirmed the increased efflux ability of these MDR strains. Applying a bulk segregant analysis (BSA) approach, they identified the responsible mutation leading to MDR, an insertion of 519 base pairs (bp), a remnant of a retrotransposon, in the promoter of *MFS1* (Omrane et al., 2017, 2015). Other insertions in the promoter of this gene (P_{MFS1}), of 369/338 (type II A/B) and 150 (type III) bp were identified in several field strains. Functional analysis through gene replacement in the sensitive reference strain led to conclude that type I, IIA and III inserts (represented in Fig. 41A) are responsible for the constitutive overexpression of *MFS1* (Fig. 41B). Different potential transcription factor binding sites were identified in type I and II inserts, leaving room for hypotheses on transcription regulation control driven by the insertions of these elements (Omrane et al., 2017). From then, MDR has been integrated in field resistance monitoring, notably through PCR genotyping of P_{MFS1} (Lavrukaitė et al., 2023; Mäe et al., 2020).

Recent surveys in European countries reported new insertions in P_{MFS1} , suggesting that the polymorphism of this promoter may be associated with diverse MDR phenotypes (Lavrukaitė et al., 2023; Mäe et al., 2020). But more interestingly, resistance monitoring programme in French populations of *Z. tritici* carried out between 2020 and 2021 unveiled a shift in MDR intensity among strains with a common P_{MFS1} genotype, suggesting additional mechanisms at play *in natura* (Patry-Leclaire, 2024) and currently emerging under fungicide selection pressure.

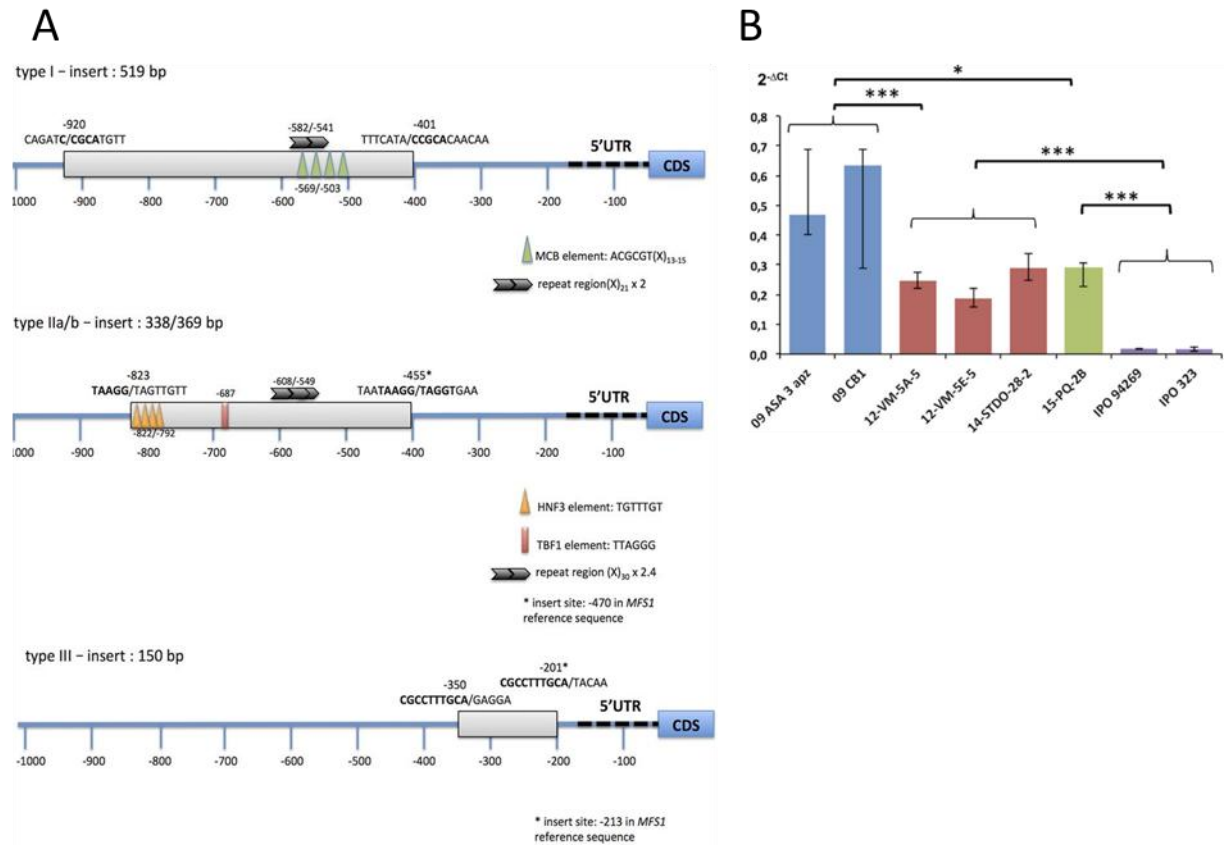


Fig. 41: *MFS1* promoter structure and *MFS1* expression in *Zymoseptoria tritici* MDR strains. A) *MFS1* promoter structure and natural insertion polymorphism B) *MFS1* expression levels associated with the different insertions (blue= type I, red = type IIa, green = type III). From Omrane et al. (2017).

Overall, *Zymoseptoria tritici* is a phytopathogenic fungus that:

- represents a global threat due to its high pathogenic potential on a major staple crop;
- has evolved resistance to nearly all single-site modes of action currently deployed in the field;
- is capable of developing multidrug resistance under diverse environmental conditions and selective pressures;
- exhibits a high adaptive potential driven by its genomic plasticity and life-cycle characteristics;
- possesses traits that facilitate cultivation under laboratory conditions, enabling high-throughput screening approaches.

Taken together, these features establish *Z. tritici* as a highly relevant model for investigating multidrug resistance, a phenomenon that represents a major global challenge in the context of One Health.

4 PhD challenges, questions, and objectives

Resistance survey of *Z. tritici* field populations indicates a recent shift in MDR levels, suggesting that Mfs1 is not the sole determinant of this resistance phenotype. Given the threat posed by MDR to both treatment efficacy and the sustainability of new modes of action, this PhD project aimed to identify the underlying molecular and genetic determinants of MDR, as a prerequisite for assessing resistance risk and designing appropriate management strategies.

As starting material, we used recent European field populations displaying heterogeneous resistance levels to terbinafine (Patry-Leclaire, 2024). Since analyzing a quantitative trait like MDR in natural populations of a highly polymorphic fungus is challenging, we complemented our approach with laboratory-evolved MDR mutants, obtained through experimental evolution (EE) under various fungicide selection pressure in our lab (Ballu, 2021; Ballu et al., 2023, 2024). EE allows following adaptation over a few generations, providing insights into short- and long-term adaptive mechanisms relevant to field conditions. Indeed, the fungicide selection conditions used in the EE experiment were designed to mimic treatment strategies in practice and is expected to shape adaptive landscapes in *Z. tritici* in ways comparable to natural populations.

Based on this dual approach, we considered experimentally evolved strains as a promising starting point for building a mechanistic framework of MDR, which can then be confronted with the dynamics observed in nature. This sequential strategy guided the transversal questions addressed in this manuscript:

- How does the genome plasticity of *Z. tritici* pave the way for MDR emergence and which genetic determinants underlie MDR?
- Which molecular and physiological mechanisms are selected under fungicide pressure and contribute to MDR in *Z. tritici*?
- What does this new knowledge teach us about the risk associated with fungicide selection pressure and about resistance management strategies?

CHAPTER I: IDENTIFICATION OF MDR MECHANISMS SELECTED THROUGH EXPERIMENTAL EVOLUTION

Existing material, objectives and approaches

Through three independent studies, Ballu and collaborators investigated the effectiveness of recommended resistance management strategies based on chemically heterogeneous selection pressures, namely alternation, mixture, and dose modulation, to delay resistance development in *Zymoseptoria tritici* (Ballu et al., 2024, 2023, 2021). These studies, conducted *via* experimental evolution, eventually led to the selection of multidrug-resistant (MDR) phenotypes under different selection regimes, particularly under alternation and mixture involving DMI, SDHI, and MBC fungicides. MDR phenotypes were observed at the population level, with global resistance levels assessed across evolved populations. In addition, the authors isolated individual strains throughout the cycles and at the end of the final cycle. These isolates were tested for fungicide resistance phenotypes (Fig. 42), revealing that those selected under heterogeneous selection pressures, vs. those exposed to a single mode of action, displayed resistance across a wide range of fungicidal MoAs, including tolnaftate resistance, a known marker of MDR. These phenotypes were not associated with length polymorphisms in P_{MFS1} , indicating the involvement of novel mechanisms distinct from those typically observed in field populations.

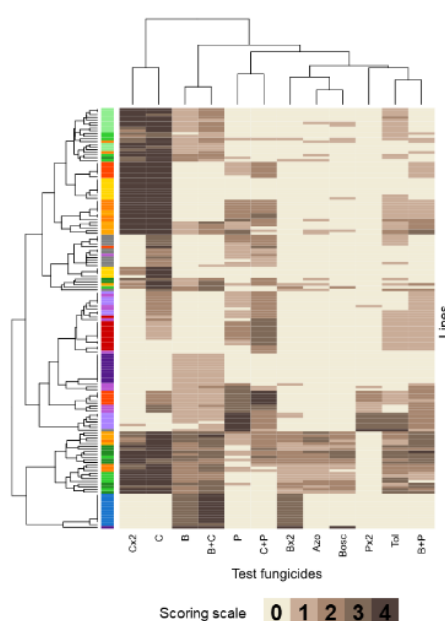


Fig. 42: Phenotypic resistance profiles selected at the end of experimental evolution. From Ballu et al.,

(2023). 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. Tol: tolnaftate.

Building on this material and data, we aimed to further characterize the underlying mechanisms and their genetic basis. In the first article of this thesis, we applied a sequential approach to these strains, including:

- Characterizing their transport activity to assess its involvement in the MDR phenotype through fluorescence accumulation and double disk synergy assays we developed for this purpose.
- Sequencing their genomes to identify candidate mutations and larger structural variations.
- Functionally validating one candidate gene using a reverse genetics approach (gene knockout and allele replacement).

In this first chapter, we focus on isolates evolved from the reference strain IPO323, while additional results obtained from isolates evolved from another ancestral strain are presented in a dedicated section later on.

Article 1: Experimental evolution unravels new genetic drivers of multi-drug resistance in *Zymoseptoria tritici* including Copy Number Variations (CNVs) and a gain of function mutation in an AtrR-like Transcription Factor

Authors: Elza Neau, Nicolas Lapalu, Alicia Noly, Sabine Fillinger, Anaïs Lalève

Abstract

The present study demonstrates that *Zymoseptoria tritici* can evolve a substantial diversity of MDR mechanisms under experimental evolution with different fungicide combinations, whether applied in mixture or alternation, and independently of the intrinsic resistance risk of individual compounds. Selected MDR phenotypes involve both increased efflux and efflux-independent mechanisms, associated with single-nucleotide polymorphisms, indels as well as copy number variations (CNVs, i.e., aneuploidy). Importantly, we provided experimental evidence that a gain-of-function mutation in the novel transcription factor ZtAtrR is responsible for the MDR phenotype of the strains harboring such mutation. Considering that this specific ZtAtrR mutation shows no fitness costs it could emerge in the field in the future. Our work demonstrates that MDR in *Zymoseptoria tritici* can be achieved through multiple evolutionary routes, consistently with the quantitative nature of this phenotype observed *in natura*.

Introduction

Over the last century, modern medicine and agriculture have heavily relied on the use of synthetic bioactive chemicals, hereafter called Active Ingredients (AIs), for controlling human, livestock and crop diseases and infestations (Aminov, 2017; Tudi et al., 2021). Efforts have been made for developing AIs with highly specific mode of action (MoA) in order to reduce deleterious off-target effects on the environment (Directive 2009/128/CE du Parlement européen, 2009; Sparks and Lorsbach, 2017) and secondary effects in human (Huggins et al., 2012; Kluwe, 2016). However, this specificity comes along with another major threat: resistance to these AIs. Indeed, the strong selective pressure exerted by these widely and extensively used compounds on target populations has promoted the emergence and spread of resistance, ultimately resulting in treatment failure. This is a universal phenomenon and concerns drugs targeting all kind of infectious diseases, harmful organisms and cells that are susceptible to evolve under selective pressure. They include, in a non-exhaustive manner, viruses (Aw et al., 2025), bacteria (Levy and Marshall, 2004), fungi (Fisher et al., 2018; Lucas et al., 2015), protozoa (Capela et al., 2019), helminths (Fissiha and Kinde, 2021), insects (Bass et al., 2015; Durand et al., 2012), mites (De Rouck et al., 2023), weeds (Powles and Yu, 2010), rodents (Krijger et al., 2023), and cancer cells (Merlo et al., 2006).

Resistance at the individual level is defined as the stable and inheritable ability of an organism

to live and reproduce under an AI dose that prevents the growth of susceptible organisms from the same species. The drivers of resistance shared between organisms include (i) mutations (SNP and indels) (ii) structural genome variations (chromosome aneuploidy, loss of heterozygosity) and (iii) epigenetic modifications (Lee et al., 2023; Sen et al., 2022; Tsai and Nelliati, 2019). The heritability and stability of these drivers differentiate resistance from tolerance and heteroresistance which refer to the ability of some cells from an isogenic population to survive for extended time or slowly grow under respectively lethal or static doses of an AI (Amich et al., 2025; Berman and Krysan, 2020; El Meouche et al., 2024; Yang and Berman, 2024).

The resistance drivers that have been most extensively described are mutations affecting genes. To describe their effects on AI protein targets and resistance spectra, and given the inconsistencies in vocabulary across research fields, we chose to adopt the terminology defined by the Research and Reflection Network on Pesticide Resistance (R4P) ("Glossary – R4P," n.d.). It defines target-site resistance (TSR) as the result of the selection of mutations affecting the gene encoding the target protein of the AI, reducing their interaction by increasing its expression or modifying the affinity of binding through sequence modification. When target-site mutations against various AIs accumulate in the same individual they lead to multiple resistance. TSR, often associated with high resistance factors (Lucas et al., 2015; Yu and Powles, 2014) and sometimes low fitness costs (Bédard et al., 2024), are highly damaging for treatment efficacy. Consequently, most resistance management strategies focus on delaying the emergence and spread of TSR in pest and pathogen populations.

Among these treatment strategies, exposing populations to heterogeneous selection pressure is a common recommendation across both medical and agriculture sectors. The most frequently advocated approach is the combination of MoAs in a concomitant treatment, as it is managed in pesticide mixture and antibiotic combination strategies. Heterogeneous selection pressure can also be achieved over time (through alternation of MoAs) or space (mosaic deployment, mixing) (Raymond, 2019; Rex Consortium, 2013; Tamma et al., 2024). However, even if diversification of selection pressure successfully limits or delays the emergence and selection of TSR, it is sometimes at the cost of selecting multidrug resistance (Ballu et al., 2024, 2023, 2021; Barbosa et al., 2021; Comont et al., 2020; Lagator et al., 2013; Zhou et al., 2022).

Multidrug resistance (MDR) refers to a broad-spectrum resistance phenotype against AI of unrelated MoA, that typically arises from non-target-site mutations (mutations that does not affect the AI's target). It can result from different mechanisms; the most widely described and conserved between organisms being drug enhanced efflux (Cannon et al., 2009; Furnival-Adams et al., 2024; Hu and Chen, 2021; Morschhäuser, 2010; Poole, 2004; Simon et al., 2022; Tian et al., 2023). Additional mechanisms include limited AI uptake (Feng et al., 2004; Sarathy et al., 2013) intracellular sequestration and/or further

metabolic detoxification through four classically described phases (i) functionalization, (ii) conjugation, (iii) specific transport of conjugates, (IV) degradation (Palli, 2020; Sang et al., 2018; Yuan et al., 2007) .

MDR should not be underestimated as it is responsible for increased resistance factor against a given AI when stacked with TSR (Hu and Chen, 2021; Mitchell et al., 2014; Vázquez-García et al., 2020) and is also likely to compromise the efficacy of new unrelated MoAs. This is particularly concerning given the declining rate at which new AIs are discovered and reach the market (Renwick and Mossialos, 2020; Sparks and Bryant, 2021). Deciphering the molecular basis of MDR is therefore critical to both understanding its evolutionary drivers and for improving practical monitoring tools, which are essential for adapting resistance management strategies.

This need is particularly acute for fungal antimicrobial resistance (AMR), an emerging global threat in both clinical and agricultural sectors that has long remained in the shadow of bacterial AMR. Since the 1980s, increasing numbers of pathogenic fungal populations have been reported to acquire resistance to commonly used antifungals (Fisher et al., 2020, 2018), a trend expected to intensify under the influence of global warming (George et al., 2025; Lurwanu et al., 2021). Notably, numerous cases of MDR have already been documented in major disease-causing fungi across both sectors (Costa et al., 2014; Kretschmer et al., 2009; Omrane et al., 2015; Sang et al., 2018).

The wheat fungal pathogen *Zymoseptoria tritici* provides an excellent model for studying fungicide resistance evolution in agriculture, including MDR. This ascomycete fungus can cause wheat yield losses of up to 50% in the absence of disease management and is mainly controlled by fungicide applications (Fones and Gurr, 2015; Torriani et al., 2015). Decades of intensive fungicide use have led to the adaptation of West-European populations through TSR against nearly all single-site MoAs, including β -tubulin polymerization inhibitors (benzimidazoles), sterol demethylation inhibitors (DMIs), and mitochondrial respiration inhibitors targeting complexes II and III (SDHs and QoIs) (Garnault et al., 2019; Hellin et al., 2021; Kildea et al., 2025). Since 2010, annual monitoring of French field populations has shown a rising frequency of MDR phenotypes, stabilizing at roughly 25 % since 2019, and detected *via* resistance to the squalene epoxidase inhibitor tolnaftate (SBI class IV), a mode of action not used in agriculture (Leroux and Walker, 2011). This phenotype is linked to enhanced efflux *via* MFS1 overexpression, conferring moderate resistance to at least three MoAs (Omrane et al., 2017, 2015).

Resistance evolution and selection in this fungus have also been extensively studied through experimental evolution, generally defined as the study of experimental populations for several generations under chosen controlled conditions, commonly used for dissecting evolutionary processes in response to these specific conditions (Kawecki et al., 2012). Using this approach, Ballu and colleagues (2021, 2023, 2024) investigated adaptation to heterogeneous fungicide selection pressures, including alternation and mixtures, and observed the independent emergence of MDR phenotypes across multiple

lines. Building on this framework, our study aimed to dissect the mechanisms underlying these MDR phenotypes selected under treatment regimens likely to be applied in the field. Through efflux phenotyping and whole-genome resequencing of individual MDR isolates, we identified distinct efflux profiles associated with mutations in several genes, suggesting that different mechanisms and genetic drivers contribute to MDR across isolates. Notably, we discovered a novel C740Y gain-of-function mutation in an AtrR-like transcription factor, which confers MDR in *Z. tritici* without detectable fitness costs under *in vitro* growth conditions, indicating a high potential for field emergence under alternating fungicide treatments. Resequencing data also revealed important chromosomal rearrangements in evolved strains. In particular, aneuploidy of chromosome 7 in some isolates selected under carbendazim treatment correlates with DMI resistance. Our work provides new insights into previously unexplored aspects of MDR phenotype in *Z. tritici* and paves the way for future fundamental studies on multidrug resistance regulatory pathways in this fungus.

Results

Experimentally evolved isolates display contrasted MDR profiles

In this study we focused on *Zymoseptoria tritici* isolates experimentally evolved from the reference strain IPO323 under different fungicide selection pressure, published in the work of Ballu *et al.*, in 2023 and 2024. The informative nomenclature of isolates' names was conserved in the present study: individual lines are characterized by unique prefix indicating the rhythm of fungicide alternation (first number), the fungicides used (letters), and the identifier of the individual line (Roman numeral). For more information about selection regimes see experimental procedures, supplementary Fig.S1, Ballu *et al.*, 2023 and 2024.

In order to select the strongest MDR phenotype candidates for identification of associated molecular mechanisms, 128 of these isolates were screened for their resistance to different MoAs. Resistance scoring was established from qualitative droplet growth tests and compared to the profiles of the corresponding ancestral strains (Fig.1). We distinguished the two ancestral strains from Ballu *et al.*, studies as they did not display the same sensitivity profile to the tested fungicides in our conditions.

Among the isolates displaying the highest resistance scores against MDR indicators terbinafine and tolnaftate (SBIs class IV, FRAC), but also to other MoAs, 29 isolates evolved from IPO323_Ancessor_2023 and 13 from IPO323_Ancessor_2024 (as verified by sequencing) were considered for this analysis and are presented in Fig.1C. Apart from the selection agents indicated in the strains' name, these isolates also showed resistance (scores between 0.5 and 1) to MoAs absent from their selection regimes. Indeed, they all displayed resistance to the complex III respiration chain inhibitors

Azoxystrobin (Qol) and/or Metyltetraprol and Fenpicoxamid (Qil). BC isolates were also resistant to a DMI, showing scores ranging from 0.75 to 1 against mefentrifluconazole 0.004 $\mu\text{g}\cdot\text{mL}^{-1}$, while having been selected under completely unrelated MoAs alternation (SDHI/MBC) selection regime. These results confirmed the multidrug resistance (MDR) phenotype of these individual isolates, confirming selection of non-target site mutations through the experimental evolution processes.

Evolved MDR isolates show contrasted efflux phenotypes

We evaluated the involvement of increased efflux in the MDR phenotype of the set of the 42 selected MDR isolates described in Fig.1. The quantitative efflux assay consisted in measuring the fluorescence of the membrane associated Nile red dye in spore suspension, inversely proportional to the efflux capacity of the cells. It was inspired from a test used for *Candida* efflux monitoring through Cdr1p and Cdr2p ABC transporters as well as the MFS Mdr1p transporter (Ivnitski-Steele et al., 2009) and set up using isogenic strains displaying different *mfs1* expression (supplementary Fig.S2).

The qualitative assay evaluated the interaction type between a fungicide (terbinafine) and transport modulators (amitriptyline, verapamil and chlorpromazine) on fungal growth in solid YPD medium. It was inspired from double-disk synergy tests (DDST), commonly used to screen synergisms between different AIs in combating bacterial AMR (Hałasa et al., 2024; Laishram et al., 2017). Modulators were chosen for their capacity to differentially antagonize transporters of *Zymoseptoria tritici* (Leroux and Walker, 2013; Omrane et al., 2015; Roohparvar et al., 2007). The synergy revealed by enhancement of the growth inhibition halo between disks containing respectively terbinafine and modulator(s) was considered as a proof that increased efflux of terbinafine is involved in the resistance phenotype. Among the three modulators tested, interaction with verapamil was consistently neutral, while synergy with amitriptyline was observed but not conclusive from this qualitative test, as it was also slightly detected in IPO323_Ancestors_2024. Only interaction between terbinafine and chlorpromazine, well contrasted between ancestral strains and evolved isolates, was further investigated.

This interaction correlated relatively well with the outcomes of the Nile red efflux assay, building robust foundations to conclude on the impact of efflux in MDR phenotypes. Results of these phenotyping assays presented in Fig.2A and 2B (and Table S1) show contrasted efflux profiles between the isolates. The first phenotype to highlight concerns the majority of BC isolates (2BC_III_12_B_a, 2BC_II_12_A_a, 2BC_IV_12_A_a, 1BC_III_12_C_b, 1BC_IV_12_A_a, 2BC_III_12_C_b, 1BC_I_12_C_a, 1BC_I_12_B_b, 1BC_III_12_A_b), two CP isolates (2CP_I_12_B_a, 3CP_III_12_A_a), the alternation evolved isolates Alt_CP_B_III_A, as well as four mixture evolved isolates (Mixt_MF_II_B, Mixt_MF_II_A, Mixt_CP_B_I_A and Mixt_CP_B_I_C). All these isolates displayed a significant 2-to-3-fold increase in Nile red efflux in comparison to their respective IPO323 ancestor (Fig.2A) associated with a clear synergy between

terbinafine and chlorpromazine. For these isolates increased drug efflux takes part in the observed MDR phenotype.

Moreover, for most isolates displaying slight but not significantly increased Nile red efflux compared to the IPO323 ancestral strain, DDST also revealed synergy between terbinafine and chlorpromazine (Fig.S2, TableS1) suggesting that increased efflux may also be at play. But in this case, the Nile Red assay did not allow us to confirm this phenomenon due to either sensitivity or to substrate specificity issues.

The monitoring of *MFS1* expression in a subset of isolates of contrasted efflux profiles showed that overexpression of this transporter contributed to increased efflux - and MDR - of some, but not all isolates (Fig.S2). These results also demonstrated the capacity of the two efflux assays to capture both Mfs1-dependent and -independent drug efflux.

Unexpectedly, DDST also revealed a decreased inhibition halo representative of antagonistic interactions between terbinafine and chlorpromazine (Fig.2B, Table S1) associated with stable Nile red efflux in six isolates from three independent lines (all 3BP isolates and 1BCP_I_12_C_b).

Taken together, these contrasted results and discrepancies between Nile red efflux, *MFS1* expression, and DDST including the unexpected antagonism detected between terbinafine and chlorpromazine, indicate that the panel of MDR phenotypes selected through experimental evolution encompasses diverse mechanisms. They include decreased xenobiotic intracellular accumulation *via* increased efflux (involving Mfs1 or not) confirmed by both efflux assays in nearly half of the isolates, as well as potential efflux-independent mechanism(s).

Evolved MDR phenotypes are associated with mutations in wide range of gene functions and larger structural variations

Resequencing data were expected to provide clues in understanding the nature of these molecular mechanisms. Given the number of cell divisions (between 49 and 84 generations (Ballu, 2021)) and the mutation rate of IPO323 of 3.10^{-10} as established by (Habig et al., 2021), the isolates were expected to carry only few mutations (between 1 to 10 per genome), restricting the number of molecular candidates and functions to mutated genes. Firstly, genetic variations to IPO323 reference genome were detected in IPO323_Ancessor_2023 (90 SNPs) and IPO323_Ancessor_2024 (152 SNPs) while both are IPO323 strains maintained in the lab. These polymorphisms, that may explain variations in sensitivity of ancestral strains towards the different fungicides, were filtered out for focusing on variations acquired through experimental evolution. SNPs and short indels detected in each evolved isolate and absent from the respective ancestor's genome (Fig.2C) ranged from 1 to 74 in the whole genome and from 0 to 9 restricted to gene containing regions (*i.e.*, gene plus 5 kb up- and downstream, respectively). All variants

called in both intergenic and non-intergenic regions are listed in Table S2.

No variant was detected for two isolates, namely Mixt_PB_II_C and Mixt_MF_III_B, in gene containing regions, indicating that the MDR phenotype may not only be explained by mutation events (SNPs, short indels) but also by other drivers such as larger indels and chromosome rearrangements not detected by variant calling. This was supported by sequencing depth analysis (Fig.3) revealing ploidy variations between isolates. The median normalized read depth across chromosomes shows that both ancestral strains and evolved isolates lack the accessory chromosomes 18 and the terminal 33 kb of core chromosome 5, while the entire accessory chromosome 15 and 100 kb of the extremity of core chromosome 7 were absent respectively in IPO323_Ancestral_2024 and IPO323_Ancestral_2023, as well as in their derivatives. More interestingly, evolved isolates from both experimental evolutions displayed aneuploidy and chromosome structural variations compared to their ancestor, regarding both core and accessory chromosomes. As an example, Mixt_CPB isolates lacked chromosome 19 and a 256 kb fraction of chromosome 10. Some isolates from 1BC, 2BC, 3CP and 1BCP treatments also displayed higher normalized sequencing depth values (of approximately 2) for chromosome 7 indicating chromosome duplication. Additionally, intermediate values suggest heterogeneity among cells of sequenced isolates in chromosome copy number, including loss (0.5) and duplication (1.5), as observed for chromosome 10 in Mixt_MF_III_C. The strong association between chromosome 7 duplication and prothioconazole-resistance (Fig.1B) indicates that aneuploidy may also play a role in DMI resistance of these isolates (all 1BC and 3CP isolates, 2BC_II_12_A_a, 2BC_IV_12_C_a, 2CP_I_12_B_a, 2CP_II_12_C_b, BCP_I_12_A_b and BCP_I_12_C_b).

We focused all further analyses on variants affecting genes potentially involved in MDR (Fig. 2C). The identified variants were grouped into four categories according to their expected impact on the gene function, ranging from modifier (likely to modify the gene expression, from UTR variants to more distant variants within a 5 kb region upstream or downstream of the gene), to low (synonymous variant, intron variant, splice region variant), moderate (missense variant, conservative or disruptive in-frame deletion) and high impact (frameshift variant, stop gained or start loss).

Interestingly, no high-impact variants were detected in annotated genes encoding drug efflux transporters. Only low-impact variants (a synonymous SNP and a 3' UTR insertion) were found in two isolates, affecting the MFS transporters ZtIPO323_037160 and ZtIPO323_093740. However, higher impact variants were identified in genes encoding other transport proteins, like a missense variant in a putative mitochondrial carrier (ZtIPO323_026900) and a transport-associated stomatin family protein (ZtIPO323_071870) encoding gene.

Strongest effect variants occurred principally in genes encoding enzymes likely to be involved in various metabolic processes: Copper amine oxidase 1 (ZtIPO323_089370), Sorbitol dehydrogenase-

like protein (ZtIPO323_013440), Long chain alcohol oxidase FAO1 (ZtIPO323_034990), Urease (ZtIPO323_053030), Metallo dependent phosphatase (ZtIPO323_100400), Ubiquitin carboxyl-terminal hydrolase 10 B (ZtIPO323_124850), intermembrane space aaa iap-1 like protease (ZtIPO323_047150). They may be involved in the observed phenotype, although their role is not obvious neither their link to efflux phenotypes.

While not directly associated with increased efflux functions, transcriptional regulators are key candidates for deciphering MDR regulatory networks. Several putative transcription regulators carried moderate to high-effect variants across the isolates. Notably, ZtIPO323_132950 was the only mutated gene (*via* in-frame insertion or deletion) in isolates from both experimental evolutions (Mixt_CPB_I_A, Mixt_CPB_I_C and 3BC_I_12_C_a). It encodes an uncharacterized protein containing a zinc finger CCHC domain (InterProScan), a domain found in proteins affecting DNA transcription (Ohira et al., 2019). Of higher impact, a frameshift variant in the mediator of RNA polymerase II transcription subunit 12 (Med12) encoding gene also occurred in one mutant (Alt_CPB_III_A). Interestingly, three genes impacted by missense variants in different isolates, encode putative zinc cluster ($Zn(II)_2Cys_6$) transcription factors (ZtIPO323_012590, ZtIPO323_001150 and ZtIPO323_058010). This family of transcription factors is exclusive to fungi and required in various cellular processes as well as in drug resistance (Balzi et al., 1987; Delaveau et al., 1994; Hagiwara et al., 2017; Kusuya et al., 2022; Lee et al., 2005; MacPherson et al., 2006; Sasse et al., 2023). ZtIPO323_012590 and ZtIPO323_001150 belong to the same orthogroup as the Odr1/Mdu2 (Afu1g03800) and NscR (Afu7g00130) transcription factors of *Aspergillus fumigatus* respectively involved in oxidative stress and drug resistance (Sasse et al., 2023) and in secondary metabolism (Chooi et al., 2013). ZtIPO323_058010 was annotated as an ABC-transporter-regulating transcription factor-like (PTHR46910). The C>T substitution (chromosome 4, position 2178723) in the gene ZtIPO323_058010, leading to the amino acid substitution C740Y in the protein, was found in six mutants from two different treatments (1BCP, 3BP) and three independent lines (1BCP_I, 3BP_III, 3BP_IV) also sharing similar efflux phenotypes. In that sense, it was identified as a strong candidate in explaining both MDR and efflux phenotype and was retained for further investigations.

ZtIPO323_058010 encodes an AtrR-like transcription factor

The ZtIPO323_058010 protein was scanned for known protein domains with InterPro scan. Besides its zinc-dependent DNA binding domain at the N-terminus of the protein it contains a fungal Middle Homology Region (MHR, cd12148 domain in CDD data base) involved in transcription regulation (Kolaczkowska and Goffeau, 1999; Schjerling and Holmberg, 1996). The ZtIPO323_058010 protein organization is schematized in Fig.4A.

In order to identify the homologs of ZtIPO323_058010 and to estimate its conservation among

fungi, we retrieved 40 sequences of the orthogroup 9SI6P (EggNog) at the *Ascomycota* level, containing ZtIPO323_058010 as well as the well described transcription factor AtrR from *Aspergillus fumigatus* (Hagiwara *et al.*, 2017). These sequences were aligned and used for phylogenetic analysis (Fig. 4A). It appeared that ZtIPO323_058010's homologs and especially the two described fungal-type transcription factor domains are highly conserved across ascomycetes, suggesting a functional conservation. In particular, the *Aspergillus fumigatus* AtrR transcription factor has been functionally dissected (Hagiwara *et al.*, 2017; Paul *et al.*, 2019; Ror *et al.*, 2024) and shown to be involved in azole resistance. This information supported a role of ZtIPO323_058010, later on referred to as ZtAtrR, in adaptive responses and resistance to antifungal associated stress.

Moreover, the cysteine C740 in ZtAtrR (fig 4B) appeared to be highly conserved among the homologous sequences suggesting this amino acid to be important for the protein function. In the six mutants displaying a variation of this protein sequence, C740 replacement by a tyrosine may cause potential modification of the protein function or even loss of function. The C740 residue is located at the C-terminus of the MHR domain, at the start of a 58-residue-long alpha helix Fig.4C. D modeling of the protein showed that the substitution C740Y did not changed the helical conformation but it lowered the confidence score of the structure and the lateral chain of the tyrosine caused an increase of the steric hindrance at this position (Fig.4.D).

ZtAtrR*^{C740Y} is a gain of function mutation leading to multidrug resistance in *Zymoseptoria tritici

In order to decipher the role of *ZtAtrR*^{C740Y} in the MDR phenotype and its impact on the protein function, we carried out functional analysis relying on resistance profiling of deletion and ectopic complementation transformants.

Fig. 5 presents the vegetative growth of the different strains on medium supplemented with fungicides of different MoAs. For each transformation, at least three transformants were phenotyped. The results from one representative strain are shown in Fig. 5. The *ZtatrR*^{C740Y} experimentally evolved strain 1BCP_I_12_C_b showed similar growth on all tested fungicides as the MDR reference strain 6.18 (Fouché *et al.*, 2022; Omrane *et al.*, 2017) overexpressing *MFS1*.

Deleting *ZtAtrR* in the sensitive ancestor IPO323 did not change the sensitivity to the tested fungicides concentrations. This revealed that a loss of function of this gene is not responsible for the MDR phenotype.

However, deletion of *ZtAtrR* in the 1BCP_I_12_C_b strain carrying the *ZtatrR*^{C740Y} allele (1BCP_I_12_C_b Δ *zatrR*) restored the wild-type sensitive phenotype. This result indicated that *ZtatrR*^{C740Y} is responsible for the MDR phenotype of 1BCP_I_12_C_b (verified in two additional experimentally

evolved mutants; Fig.S3). This result was corroborated by complemented transformants, since ectopic integration of *ZtAtrR^{C740Y}*, but not of *ZtAtrR^{WT}*, in IPO323 $\Delta ztatrR$ background was sufficient, but necessary to obtain the MDR phenotype.

Taken together, these results indicate that *ZtAtrR^{C740Y}* alleles is associated with a gain of function of the ZtAtrR transcription factor, causing MDR phenotype in *Z. tritici*.

Deletion or C740Y mutation of ZtAtrR does not significantly affect fitness *in vitro*

Despite the selective advantage conferred by *ZtAtrR^{C740Y}* under *in vitro* fungicide exposure, this mutation was not detected in the 2020-2021 collection of a hundred of MDR field strains (Patry-Leclaire et al., 2023; data unpublished), indicating that this mutation has not been selected in the field yet. It may be associated with fitness cost. Notably, in *Aspergillus fumigatus*, the AtrR transcription factor has been implicated in hypoxic stress resistance and virulence in this opportunistic pathogenic fungus (Hagiwara et al., 2017). It was therefore important to measure the potential fitness cost in order to better estimate its likelihood of emerging *in natura*.

Fitness of *ztatrR* mutants was assessed *in vitro*, by measuring the vegetative growth of the different strains in YPD liquid medium, without fungicide selection, under normal and O₂-limiting conditions. Growth curves of individual tested mutants, of IPO323 genetic background, are presented in Fig.6A. Three parameters extracted from a logistic model were compared for each genotype: *ZtatrR^{WT}* (both IPO323 ancestral strain and complemented mutants), *ZtatrR* knock out (KO) and *ZtatrR^{C740Y}* (Fig6.B). They correspond to the maximal biomass production, expressed as spore concentration (asymptote, asym), the time to reach half of the maximal spore concentration (xmid), and the growth rate (scal), which together served as indicators of fitness.

The ANOVA of the model revealed significant effect of oxygen availability, genotype and interaction of both on the three tested fitness parameters. They did not differ significantly between wild type (IPO323 ancestor and $\Delta ztatrR/ecto-WT$ mutants pooled) and deletion mutants, under either normal or O₂-limiting conditions (Fig. 6, Fig. S4). Unlike in *AfAtrR*, *ZtAtrR* does not seem to be crucial for hypoxic stress adaptation, in our experimental conditions. Similarly, xmid, scal of *ZtAtrR^{C740Y}* genotype did not differ significantly from those of *ZtAtrR^{WT}*, indicating that *ZtAtrR^{C740Y}* allele does not impair fitness, neither under normo- nor hypoxic conditions. Interestingly, the maximal biomass production associated with *ZtatrR^{C740Y}* genotype is significantly higher compared to both *ZtAtrR^{WT}* and $\Delta ztatrR$ groups under hypoxic conditions only. This indicates that, even if ZtAtrR is not directly involved in hypoxic-stress response in its wild-type form, ZtAtrR^{C740Y} improves the hypoxic-stress adaptation.

When used as a proxy for fitness, *in vitro* growth results suggest that ZtAtrR^{C740Y} does not impose a fitness cost on *Z. tritici*.

Discussion

In this work, we characterized 42 experimentally evolved strains selected under fungicide alternation and mixture regimes identified as displaying generalist resistance profile by Ballu *et al.*, 2023 and 2024.

Until now the mechanism behind resistance to a wide range of unrelated compounds relied on increased efflux, major MDR mechanism described in fungi (reviewed by (Hu and Chen, 2021; Paul and Moye-Rowley, 2014)) and the only one in field isolates of *Zymoseptoria tritici* identified so far (Omrane *et al.*, 2017, 2015). Approximately half of the MDR isolates we phenotyped in this study through two efflux assays, relying on Nile red efflux and synergy assays between transport modulators and terbinafine, showed increased efflux. Transporter specificity of both Nile Red efflux and Chlorpromazine inhibition is not well characterized. In *Candida albicans*, Nile red is a substrate of both MFS and ABC transporters (Ivnitski-Steele *et al.*, 2009); Chlorpromazine has also been described as a modulator of various ABC and MFS transporters (Leroux and Walker, 2013; Omrane *et al.*, 2015; Roohparvar *et al.*, 2007). Increased efflux potentially involves Mfs1 activity (as suggested by its overexpression in several isolates) as well as the activity of other transporters among the 229 MFS and 44 ABC transporters predicted in the *Z. tritici* genome (Goodwin *et al.*, 2011). Some of these proteins have been reported to transport various chemicals and may contribute to multidrug resistance acquisition (Stergiopoulos *et al.*, 2003, 2002). Interestingly, in some MDR isolates increased efflux was not detected. All together these results highlight a diversity of mechanisms at play, yet to be discovered, in *in vitro* selected MDR isolates.

Resequencing data provides insights into molecular actors underlying these mechanisms. Notably, none of the 42 isolates displays mutations in fungicide target genes, highlighting the importance of non-target-site resistance in enabling individuals to survive and multiply under heterogeneous selection pressure exerted by selective doses of three MoAs applied either in combination or in alternation regime. Some selected isolates even displayed full growth on AIs at twice the selection dose (Ballu *et al.*, 2023) indicating that NTSR can lead to substantial resistance factors while often considered as marginal. A detailed review (Piddock, 2006) of clinically relevant MDR bacteria underpins that overexpression of membrane efflux pump can lead to MIC increase above clinical breakpoint values in some cases, emphasizing the threat it represents for treatment efficacy.

Not all MDR isolates display SNPs in gene-containing regions, indicating that their phenotype is explained by other drivers. Indeed, analysis of sequencing-depth and coverage revealed events of genomic rearrangements and aneuploidy (partial and complete losses or gains of accessory and core chromosomes) in most of MDR evolved isolates when compared to their ancestors. This is consistent with the intense genome instability and widespread chromosome rearrangements during *Z. tritici*

vegetative growth described by Möller and collaborators (Möller et al., 2018). Moreover, aneuploidy may have eventually been promoted by antifungal treatment. Fluconazole for example was suggested to stimulate the formation of aneuploids in *Cryptococcus neoformans* (Altamirano et al., 2017). More obviously, MBCs, including carbendazim, disrupt microtubule assembly by binding β -tubulin and are therefore known to cause inaccurate chromatid distribution during mitosis *in vitro* and *in vivo* (Bentley et al., 2000; Jeffay et al., 1996; Sarrif et al., 1994). Habig and collaborators notably used carbendazim to generate aneuploid strains of *Zymoseptoria tritici* (Habig et al., 2017). Whole-chromosome aneuploidy and shorter-range amplifications have been described in pathogenic fungi as genome changes that can cause resistance. This phenomenon is well documented in *Candida albicans* in which two extra copies of the left arm of chromosome 5 harboring genes involved in resistance, namely the azole target Erg11 and the transcription factor Tac1 involved in regulating drug efflux (Selmecki et al., 2008). To our knowledge, while this phenomenon has also been documented in other clinical fungi like *Aspergillus fumigatus* (Khateb et al., 2023), it has never been described as driver for fungicide resistance in phytopathogenic fungi so far. Our results revealing a strong association between chromosome 7 duplication and prothioconazole-desthio resistance in *Zymoseptoria tritici* suggest that aneuploidy may also contribute to resistance acquisition to agriculture fungicides. Chromosome 7 contains the genes encoding the target of prothioconazole-desthio, *CYP51*, but also *MFS1* as well as 5 other MFS and 2 ABC transporter encoding genes, its duplication may result in increased dosage of these genes leading to resistance. It is notable that all isolates displaying the chromosome 7 duplication were selected under regime including carbendazim. This emphasizes that AIs can act both as driving force, generating genetic diversity, and selection agents in resistance emergence, similarly to the proposed mechanism in *C. albicans* and as hypothesized in *C. neoformans* against fluconazole (Harrison et al., 2014; Sionov et al., 2010).

Regarding the mutations detected through resequencing and variant calling, the first observation is that SNPs were identified in a wide range of genes, with various molecular functions as candidates for MDR actors. However, we want to highlight a detail of the experimental design by Ballu *et al.* that limits the interpretation of causal link between genetic variation and MDR phenotype. Indeed, to prevent extinction, immigration from the untreated control line was used to reach the inoculum size at each growth cycle (Ballu *et al.*, 2023, 2024). Both for SNPs and larger structural variants, whether they occurred randomly in control lines, were subsequently introduced during the transfer processes and maintained by genetic drift, or actively selected by alternation and mixture selection regime is unclear.

Still, the likeliness of candidate genes to be involved in MDR phenotype can be inferred from the effect of the mutation (the stronger the effect, the more likely the candidate), the association between efflux phenotype and the gene function from literature. For example, the missense variant of Ubiquitin L terminal hydrolase 10b or frameshift variant in Mediator of RNA pol II med 12 are two good candidates. The first may be associated with a post translational modification involved in drug resistance

as described in cancer cells (Miao 2025,) while the mediators of RNA pol II are involved in drug resistance in cancer cells and in *Saccharomyces cerevisiae* (through activation of *PDR5* transcription) (Meng et al., 2024; Shahi et al., 2010). As another example, indels were detected in the CDS of ZtIPO323_132950 in isolates from both experimental evolutions, derived from two different ancestor. This strong candidate gene encodes a protein containing a ZCCHC10 domain. While the function of this domain is not known, silencing of the human ZCCHC10 gene promotes venetoclax resistance of acute myeloid leukemia indicating a role of this protein in negatively regulating drug resistance (Zhou et al., 2023).

Unexpectedly, few genes encoding putative drug efflux transporters were affected by mutations. This indicates that the mechanisms leading to increased efflux in evolved isolates does not involve alterations of transporter coding sequences neither *cis*-regulating elements but possibly mutations in transcriptional regulators, as described in other fungi like *Botrytis cinerea*, *C. albicans* and *Claviceps homoeocarpa* (Coste et al., 2004; Kretschmer et al., 2009; Sang et al., 2018). In that sense, fungal specific Cys₆ Zn₂ transcription factors displaying missense variants in this work were also considered as reasonable candidates. Three of them are homologs of oxidative stress and drug resistance genes. ZtIPO323_058010, mutated in six experimentally evolved isolates, was identified as homologous of the *A. fumigatus* transcription factor AfAtrR and renamed ZtAtrR. This suggests that several members of a global transcription regulation pathway have been selected through experimental evolution.

As to state an example, we dissected the involvement of this ZtAtrR^{C740Y} mutation in MDR. By a reverse genetic approach, we identified the C740Y in the middle homology region (MHR) of ZtAtrR as a gain of function substitution that confers resistance to DMI, SBI class IV, QoI and QiI. Other examples of mutation in the MHR domain of a Cys₆ Zn₂ transcription factor associated with drug resistance were reported in fungal plant pathogens *Magnaporthe oryzae* and *Botrytis cinerea*, in the last one a gain of function mutation led to overexpression of the ABC transporters AtrB (Kretschmer et al., 2009; Wang et al., 2018).

While the mechanisms explaining how ZtAtrR^{C740Y} leads to MDR in *Z. tritici* remains to be elucidated, studies of its well described homolog AfAtrR provide some clues. AfAtrR has been identified as a transcription factor involved in hypoxic stress response as well as azole-mediated stress adaptation through co-regulation of ergosterol biosynthesis (including *Cyp51* and *Erg24*) and of the ABC efflux pump Cdr1B (Hagiwara et al., 2017). Similar targets could potentially be regulated by ZtAtrR and contribute to the C740Y-associated MDR. Deletion of *ZtatrR* did not affect growth under either normal or hypoxic conditions in our experimental settings, suggesting that its function may differ from that of AfAtrR. These findings suggest that C740Y-associated MDR involves either (i) non-constitutive mechanisms in which activation of efflux pumps and sterol biosynthesis genes occurs only under specific conditions, such as fungicide treatment, or (ii) mechanisms different from the AfAtrR regulatory pathway.

Ror and collaborators (2024) demonstrated the importance of the C-terminal region of AfAtrR for transcriptional activation of specific target genes, supporting the possibility that C740Y substitution located at a conserved position at the C-terminus of the MHR of ZtAtrR, may alter the transcription regulation specificity of the protein or its interaction with co-activator or co-repressor, such as with homologs of Ffma and SrbA, transcription regulation partners of AfAtrR (Paul et al., 2023, 2022; Song et al., 2025). This hypothesis represents a potential direction for future analyses.

Since the *ZtAtrR*^{C740Y} mutation was not detected in field strains, one may suppose that it is associated with a fitness cost as often observed with mutations selected in laboratory that do not emerge *in natura* (Hawkins and Fraaije, 2018). Such fitness cost was not observed *in vitro*, consistently with what is reported for loss of function mutations of *MolRR* in *Magnaporthe oryzae* (Wang et al., 2018), highlighting the potential risk of emergence of this mutation in the field under heterogeneous selection pressures.

Experimental procedures

Biological material and culture conditions

All *Z. tritici* strains used in this study are listed in Table 1. The nomenclature of isolates' name from Ballu *et al.* 2023 indicates the rhythm of fungicide alternation (first number), the fungicides used (B: Benzovindiflupyr (SDHI), C: Carbendazim (MBC), P: Prothioconazole-destio (DMI)), the identifier of the individual line (Roman numeral), the number of growth cycles performed before isolation (12) and identifiers of the isolated clone (letters). Isolates from Ballu *et al.*, 2024 are named according to the regime (Alt: alternation, Mixt: mixture) of fungicide treatment, the fungicides used: Benzovindiflupyr (B), Chlorothalonil (C), Fluxapyroxad (F), Mefentrifluconazole (M) and Prothioconazole-desthio (P), the identifier of the individual line and isolated clone (Roman numeral and letters). This informative nomenclature was kept in the current study; more information about selection regime are provided in Fig.S1.

Strains were stored in 20% glycerol at -80°C. Unless otherwise indicated, they were spread on solid YPD medium (20 g.L⁻¹ bacteriological peptone, 10 g.L⁻¹ yeast extract, 20 g.L⁻¹ glucose, 15 g.L⁻¹ agar) and cultivated for 4 to 7 days at 18°C in the dark before being grown for 6-day in 10 mL of liquid YPD medium in 50 mL erlenmeyer flasks under 150 rpm agitation at 18°C in the dark.

Table 1: *Zymoseptoria tritici* strains used in this study

Strain name	MDR phenotype	<i>P_{MFS1}</i> genotype	<i>ZtAtrR</i> genotype	Mutant complementary informations	Reference
IPO323_Ancestor_2023	Susceptible	WT	C740		

1BC_I_12_B_b	MDR	WT	C740	Ballu <i>et al.</i> , 2023	
1BC_I_12_C_a	MDR	WT	C740		
1BC_III_12_A_b	MDR	WT	C740		
1BC_III_12_C_b	MDR	WT	C740		
1BC_IV_12_A_a	MDR	WT	C740		
1BC_IV_12_B_b	MDR	WT	C740		
1BC_IV_12_D_a	MDR	WT	C740		
2BC_I_12_B_a	MDR	WT	C740		
2BC_II_12_A_a	MDR	WT	C740		
2BC_III_12_B_a	MDR	WT	C740		
2BC_III_12_C_b	MDR	WT	C740		
2BC_IV_12_A_a	MDR	WT	C740		
2BC_IV_12_C_a	MDR	WT	C740		
2CP_I_12_B_a	MDR	WT	C740		
2CP_II_12_C_b	MDR	WT	C740		
3BC_I_12_C_a	MDR	WT	C740		
3BP_III_12_A_b	MDR	WT	Y740		
3BP_III_12_B_b	MDR	WT	Y740		
3BP_III_12_C_a	MDR	WT	Y740		
3BP_IV_12_A_b	MDR	WT	Y740		
3BP_IV_12_B_a	MDR	WT	Y740		
3CP_I_12_A_b	MDR	WT	C740		
3CP_I_12_C_a	MDR	WT	C740		
3CP_III_12_A_a	MDR	WT	C740		
3CP_III_12_C_b	MDR	WT	C740		
1BCP_I_12_A_b	MDR	WT	C740		
1BCP_I_12_C_b	MDR	WT	Y740		
1BCP_II_12_C_a	MDR	WT	C740		
1BCP_III_12_B_a	MDR	WT	C740		
IPO323_Ancestor_2024	Susceptible	WT	C740		
Alt_CPB_I_A	MDR	WT	C740		Ballu <i>et al.</i> , 2024
Alt_CPB_III_A	MDR	WT	C740		
Mixt_CPB_I_A	MDR	WT	C740		
Mixt_CPB_I_C	MDR	WT	C740		
Mixt_MF_I_B	MDR	WT	C740		
Mixt_MF_I_C	MDR	WT	C740		
Mixt_MF_II_A	MDR	WT	C740		
Mixt_MF_II_B	MDR	WT	C740		
Mixt_MF_II_C	MDR	WT	C740		
Mixt_MF_III_A	MDR	WT	C740		
Mixt_MF_III_B	MDR	WT	C740		
Mixt_MF_III_C	MDR	WT	C740		
Mixt_PB_II_C	MDR	WT	C740		
09-ASA-3apz	MDR	Type I insert	C740	Leroux and Walker, 2011 Omrane et al, 2015	

<i>mfs1</i> ^{MDR-Type I} #6.18	MDR	Type I insert	C740		Fouché et al., 2022
Δ <i>mfs1</i> #T1	Susceptible	KO	C740		Fouché et al., 2022
3BP_III_12_C_a Δ <i>zatrR</i> #21	Susceptible	WT	<i>ΔzatrR::Sur^R</i>	DBD	this study
3BP_IV_12_B_a Δ <i>zatrR</i> #6	Susceptible	WT	<i>ΔzatrR::Sur^R</i>	DBD	
1BCP_I_12_C_b Δ <i>zatrR</i> #33	Susceptible	WT	<i>ΔzatrR::Sur^R</i>	DBD	
IPO323 Δ <i>zatrR</i> #11	Susceptible	WT	<i>ΔzatrR::Sur^R</i>	DBD	
IPO323 Δ <i>ZtAtrR</i> #13	Susceptible	WT	<i>ΔzatrR::Sur^R</i>	no DBD	
IPO323 Δ <i>zatrR</i> #11 / <i>ecto</i> -WT #3.1	Susceptible	WT	<i>ΔzatrR::Sur^R #11</i> <i>ZtatrR^{wt}</i> (<i>ecto</i> -topic)	DBD, no RFP, out Ku70	
IPO323 Δ <i>zatrR</i> #11 / <i>ecto</i> -C740Y #5.3	Susceptible	WT	<i>ΔzatrR::Sur^R #11</i> <i>ZtatrR^{C740Y}</i> (<i>ecto</i> -topic)	DBD, no RFP, out Ku70	
IPO323 Δ <i>zatrR</i> #13 / <i>ecto</i> -WT #8.8	Susceptible	WT	<i>ΔzatrR::Sur^R #13</i> <i>ZtatrR^{wt}</i> (<i>ecto</i> -topic)	No DBD, RFP, in Ku70	
IPO323 Δ <i>zatrR</i> #13 / <i>ecto</i> -C740Y #10.13	MDR	WT	<i>ΔzatrR::Sur^R #13</i> <i>ZtatrR^{C740Y}</i> (<i>ecto</i> -topic)	No DBD, RFP, in Ku70	

DBD/no DBD: indicates if DNA binding domain of *ZtAtrR* is deleted or conserved in the deletion mutant. No RFP/RFP: indicates if the complemented *ZtAtrR* allele was introduced as fusion protein with mRFP1 or not.

In Ku70/out Ku70: indicates if the complemented *ZtAtrR* allele was introduced in the targeted *ecto* locus *Ku70* or not (unknown genomic location).

Fungicide preparation

Fungicides were used as technical products. Prothioconazole-desthio (Sigma Aldrich), mefenfentrifluconazole (Sigma Aldrich), terbinafine (Sandoz SA, BALE/Switzerland), tolnaftate (Sigma), benzovindiflupyr (Syngenta), fenpicoxamid (Corteva), azoxystrobin (Syngenta) and metyltetraprole (Medchem) were solubilized in 80% ethanol (v/v). A concentrated solution was prepared at 4000 $\mu\text{g}\cdot\text{mL}^{-1}$ for each fungicide except for Prothioconazole desthio (400 $\mu\text{g}\cdot\text{mL}^{-1}$) and stored at 4°C. The concentration of each fungicide solution was adjusted for the volume of the solvent (ethanol) introduced in the culture medium to do not exceed 0.5% of the final volume, preventing ethanol-induced toxicity on *Zymoseptoria tritici* growth (Ballu *et al.*, 2021, 2022, 2023).

Qualitative fungicide sensitivity assays

Sensitivity to fungicides was assessed for all strains listed in Table 1 according to the following procedure, described by Ballu *et al.*, 2021. Spore concentrations of 6-day liquid cultures were estimated from the OD_{405nm} with a mathematical model calibrated by serial dilutions and hemocytometer-based spore quantification, and cross-validated ($R^2 = 0.91$) (Ballu, 2021). Approximately 5 μL drops of 10^7 , 10^6 , 10^5 and 10^4 spores.mL⁻¹ water suspensions were spotted onto 12 cm square Petri dishes containing YPD supplemented with discriminatory doses of fungicides or with 0.5% solvent (control). Fungicides discriminatory doses used were as follows: benzovindiflupyr 0.5 $\mu\text{g.mL}^{-1}$, prothioconazole-desthio 0.005 $\mu\text{g.mL}^{-1}$ and mefentrifluconazole 0.004 $\mu\text{g.mL}^{-1}$, terbinafine 0.03 $\mu\text{g.mL}^{-1}$, tolnaftate 2.5 $\mu\text{g.mL}^{-1}$, azoxystrobin 0.5 $\mu\text{g.mL}^{-1}$, metyltetraprol 0.0335 $\mu\text{g.mL}^{-1}$ and fenpicoxamid 0.3 $\mu\text{g.mL}^{-1}$. Resistance was scored after 7-day incubation, using a scoring scale (Fig.1A) and final resistance scores were computed as the ratio of growth on fungicide-supplemented medium by the growth on control medium in order to correct by basal growth rate.

A total of 128 isolates from 13 different treatment modalities and 47 independent lines identified as tolnaftate-resistant in the studies of Ballu *et al.* (2023, 2024) were screened using this sensitivity assay. From these, 53 mutants displaying the highest resistance scores across the widest range of fungicides were selected for resequencing. Sequencing data confirmed that the 29 isolates selected in Ballu *et al.* (2023) derived from IPO323_Ancessor_2023, as expected. In contrast, 11 isolates from Ballu *et al.* (2024) were excluded because they had evolved from the ancestral H152R strain. In total, 42 mutants (listed in Table 1) derived from either IPO323_Ancessor_2023 or IPO323_Ancessor_2024 were retained for further analysis.

Characterization of efflux phenotype

Nile red efflux assay

For the assessment of efflux phenotypes, an efflux assay previously established in our laboratory was utilized, based on the fluorescence of the neutral lipid probe Nile Red. To this end, 10 mL aliquots of 7-day YPD liquid cultures from each of the 42 MDR isolates were centrifuged at 3000 rpm for 10 minutes. The resulting spore pellets were resuspended in deionized sterile water, and the OD_{405 nm} was adjusted to approximately 1.0. Subsequently, 10 mL of each culture or isolate were incubated for one hour in 50 mL Erlenmeyer flasks at 18°C, 150 rpm, in the dark. A 10 μL aliquot of a 3.5 mM Nile Red solution in DMSO was added to each spore suspension and to 10 mL of sterile water for the blank, to achieve a final concentration of 7.5 μM . Samples were then incubated for an additional

hour under the same conditions. Subsequently, a 200 μL samples of each Nile Red-treated spore suspension were transferred into a black, clear-bottom 96-well microplate (Greiner) in at least four technical replicates. Fluorescence was recorded at 600 nm following excitation at 547 nm. Fluorescence values were normalized to the corresponding $OD_{405\text{ nm}}$ values to account for cell density, and efflux was expressed in arbitrary units as the inverse of this ratio.

$$Efflux = \frac{OD_{405} - blankOD_{405}}{(Fluo_{547} - blankFluo_{547})}$$

This experiment was repeated at least three times for each isolate. IPO323_Ancessor_2024 and strains displaying differential *MFS1* expression, IPO323 $\Delta mfs1\#T1$ and IPO323 *mfs1*^{MDR-Type1} #6.18 (Fouché et al., 2022) used for setting up the assay were tested in every biological replicate as positive and negative control, respectively.

Raw fluorescence and OD data were processed and analyzed using R software (version 4.5.0) and the RStudio interface (2025.05.0) (R Core Team, 2025). For each strain, individual efflux measurements were summarized as the mean of at least three biological replicates, each integrating at least four technical replicates and standard deviations were calculated. Efflux measurements were modeled using a linear mixed-effects model, with strain as a fixed effect and experimental date as a random effect (lmer function from lme4 package (Bates et al., 2015)). Model assumptions of normality and homoscedasticity were evaluated using Shapiro–Wilk and Levene tests. Residual inspection, QQ-plot, and Kolmogorov–Smirnov test on standardized residuals indicated that the normality assumption was sufficiently met. Estimated marginal means (emmeans package (Lenth, 2025)) were calculated for each strain, and pairwise contrasts against the corresponding ancestral strain were performed. P-values were adjusted for multiple testing using the Bonferroni correction.

Double disk synergy tests (DDST)

For assessing the interaction type between terbinafine and transport modulators, 5-mm diameter disks were prepared from Whatman filter paper using a paper punch, and subsequently autoclaved. They were saturated with 20 μL of either an 80% ethanol solution of terbinafine (10 $\mu\text{g}\cdot\text{mL}^{-1}$), or modulators amitriptyline hydrochloride, chlorpromazine hydrochloride, and verapamil hydrochloride (all purchased from Sigma Aldrich) at 4000 $\mu\text{g}\cdot\text{mL}^{-1}$, or solvent alone (80% ethanol). After complete drying, they were placed on 9-cm diameter Petri dishes containing solid YPD medium inoculated with 10^7 spores of *Zyloseptoria tritici*. The terbinafine disk was placed in the center while solvent and modulator-filled disks were disposed equidistantly around, at diametrically opposed positions on a 2-cm diameter circle (cross-shaped arrangement). The shape of the inhibition halo between terbinafine and modulators' disks

(Laishram *et al.*, 2018), was assessed visually indicating interaction type (neutral, synergistic or antagonistic interaction) after 7-day incubation, 18°C, in the dark.

Two technical replicates and at least two biological replicates were performed for the 42 evolved isolates tested. In each biological replicate IPO323_Ancestors_2023 and 09-ASA-3apz (Leroux and Walker, 2011; Omrane *et al.*, 2015), a MDR field isolate overexpressing *MFS1* (Table 1), were also tested as negative and positive control, respectively, for synergy between chlorpromazine or amitriptyline, and terbinafine.

DNA extraction and genome resequencing

Fresh fungal material was harvested from a 7 days old culture on YPD solid medium (6-cm diameter Petri dish) for each 42 selected experimentally evolved isolates, IPO323_Ancestors_2023 and IPO323_Ancestors_2024 for genomic DNA extraction with DNeasy Plant mini kit (Qiagen) following the supplier's instructions. The Integrity of DNA was assessed by electrophoresis, the quantity and quality were determined spectrophotometrically (NanoDrop, Thermofisher). Samples were stored at -20°C prior to sequencing.

150 base paired-end 25 X Illumina sequencing was performed by the sequencing provider (Novogene, U.K., Cambridge Sequencing Center). Raw reads from released fastq files were trimmed with Trimmomatic (0.39) (Bolger *et al.*, 2014) to remove adapters, low-quality bases (leading and sliding window trimming with $Q \geq 28$), and short reads (<50 bp). Unpaired reads were discarded. Trimmed reads were mapped to the reference genome of *Z. tritici* IPO323 (Goodwin *et al.*, 2011) using bwa mem (bwa v0.7.17) (Li and Durbin, 2009). Resulting SAM files were converted to BAM, sorted, and indexed with samtools (v1.20) (Danecek *et al.*, 2021). PCR duplicates were marked and removed using Picard (v2.21.6) MarkDuplicates (Broad Institute, 2019). Read groups were added to the BAM files for downstream GATK processing. Variants were called per sample using GATK (v4.4.0.0) HaplotypeCaller in GVCF mode (ploidy = 1) (Poplin *et al.*, 2017) and gvcf files were gathered in a common gvcf. Variant quality filters were applied using GATK VariantFiltration based on common thresholds of FS (>10), MQ (<20), QD (<20) and DP (<3). Additional filters included ReadPosRankSum, MQRankSum, and BaseQRankSum values outside the range [-2, 2], which identify potential biases in read positioning, mapping quality, or base quality between reference and alternative alleles. Variants present in ancestral strains IPO323_Ancestors_2023 and IPO323_Ancestors_2024 were identified and filtered out using Bcftools (v1.21) (Danecek *et al.*, 2021), to focus on variant selected under experimental evolution conditions. Variants were annotated with predicted functional effects using SnpEff v5.1 (Cingolani *et al.*, 2012), using custom database for *Z. tritici* IPO323. The highest effect (based on the effect score computed by SnpEff) of each variant was extracted

for downstream analysis using a custom Python script. Variant positions were intersected with gene annotations from Lapalu et al. (2025).

Read depth analysis for CNV identification

Genome-wide sequencing coverage was calculated for each sample BAM file using samtools (v1.20) (Danecek et al., 2021). For each BAM per-base coverage was computed across the genome including positions with zero coverage. The genome was partitioned into 5 kb non-overlapping windows, and the mean coverage per window was calculated. The resulting coverage matrix (isolate x windows) was imported into R for downstream analysis. Coverage values were normalized per sample by the median depth across core chromosomes (chromosomes 1–13, excluding chromosome 7 that was found to be variable in copy numbers between strains). Heatmaps were generated using pheatmap package (Kolde, 2025).

MFS1 expression analysis

For RNA extraction, IPO323_Ancessor_2023, IPO323_Ancessor_2024 and a subset of mutants displaying contrasted efflux phenotypes were pre-cultivated in YPD media, solid and then liquid (10 mL), for two subsequent 4-day precultures (dark, 150 rpm, 18°C). Spore concentration was inferred from OD_{405nm} of 200 µL of the liquid preculture, and Erlenmeyer flasks containing 25-mL YPD were inoculated at final concentration of 5×10^4 spores.ml⁻¹. After a minimum of 72h of culture (in the dark, 18°C, 150 rpm) or until reaching OD_{405nm}=2, 2 mL of culture containing roughly 1.10^8 spores were centrifuged at 5,000 g, 4°C during 15 min and the pellets were immediately frozen in liquid nitrogen and kept at -80°C before RNA extraction. Total RNA was extracted by mixing the frozen pellets with 500 µL of NucleoZOL (Macherey-Nagel™). After homogenization 30 s by vortexing, RNA isolation was carried out following NucleoZOL supplied instructions.

RNA integrity was assessed by electrophoresis; absence of residual genomic DNA was checked by PCR amplification of *UBC*. In the case of DNA amplification 10 µg of the contaminated RNA sample was treated with 5 units of DNase I from Thermo Scientific (89836) in the provided buffer for 30 min at 37°C before inactivation with 50 mM EDTA for 10 min at 65°C. After precipitation, the RNA samples were resuspended in RNase/DNase-free water. Concentration was assessed by Nanodrop. RNA samples were stored at -20°C. 750 ng of RNA was retrotranscribed using ProtoScript® II First Strand cDNA Synthesis Kit (New England BioLabs) following the supplier's instructions. cDNAs were diluted 5 times before quantitative PCR (qPCR) analysis with the Takyon® No ROX SYBR 2X MasterMix blue dTTP Kit (Eurogentec). The *mfs1* cDNA was amplified with primers 110044_Fw and 110044_RV (Omrane et al, 2015). Relative expression levels in comparison to IPO323_Ancessor_2023 and to the housekeeping

genes *EF1α* and *UBC* were determined according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Differences in *MFS1* expression levels among strains were analyzed on the means of at least two biological replicates, each of which integrated three technical replicates. A linear model including strain as a fixed effect was fitted to the relative expression data and model assumptions of normality and homoscedasticity were verified by inspection of residuals, Shapiro–Wilk test, and Levene’s test. Analysis of variance (ANOVA) was applied, followed by post hoc pairwise comparisons using estimated marginal means (emmeans package in R) with IPO323_Ancestors_2023 set as the reference strain. P-values were adjusted for multiple testing with Bonferroni correction.

Primers and RT-qPCR conditions were those published by Omrane et al., 2015.

ZtIPO323_058010 phylogenetic analysis and modeling

To explore ZtIPO323_058010 orthologs, 433 sequences from orthogroup 9SI6P (EggNOG v6.0 (Hernández-Plaza et al., 2023)), containing ZtIPO323_058010 (UniProt ID: A0A1X7RS07), were retrieved from the UniProt database (The UniProt Consortium, 2025). To select the most likely ortholog of each species, BLAST search (NCBI BLAST+ v2.15.0 (Camacho et al., 2009)) of ZtIPO323_058010 was performed against this 433-sequence database, and only the top hit per species (highest bitscore) was retained. From the remaining 297 sequences, forty sequences were selected from ascomycete species differentially related to *Zymoseptoria tritici*, including the well-characterized AtrR protein from *Aspergillus fumigatus*. These sequences were aligned using MAFFT (v7.505 (Kato and Standley, 2013)), and the multiple sequence alignment was inspected in Geneious Prime (v2025.2.1). A phylogenetic consensus tree was then inferred with IQ-TREE (v2.2.2.6, (Minh et al., 2020)) using the best-fit model according to BIC (Q.insect+F+I+G4). Branch support was assessed with 1000 ultrafast bootstrap replicates, and the resulting consensus tree was visualized with iTOL (v7, Letunic and Bork, 2024).

For assessing the putative effect of the C740Y mutation on ZtIPO323_058010, 3D modeling of the ZtIPO323_058010 protein in its C740 wild-type and Y740 allele variant was performed with ColabFold AlphaFold2 notebook in version 1.5.5 for structure prediction (github.com/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb) (Mirdita et al., 2022). Protein structures were predicted from the protein sequence annotated by Lapalu et al., (2025). For each variant, five models were generated and the highest-ranked PDB structure (based on pLDDT confidence scores) was visualized with Mol* viewer (Sehnal et al., 2021) (Plugin 4.18).

Functional analysis of ZtIPO323_058010-C740Y

For functional analysis of ZtIPO323_058010, we generated knockout mutants in the ancestral

strain IPO323_Ancessor_2023 and experimentally evolved strains (3BP_III_12_C_a, 3BP_IV_12_B_a and 1BCP_I_12_C_b) as well as complemented ectopic transformants with integration of the wild type or the C740Y allele of this gene.

For the knock out approach, two plasmids were constructed. pCAMBIA_0380_Sulf_KO-Zt058010-DBD was designed to remove most of the ZtIPO323_058010 coding sequence while leaving the predicted DNA-binding domain intact, without altering the 1 kb 5' flanking region of the putative gene ZtritIPO323_04g09172. In contrast, pCAMBIA_0380_Sulf_KO-Zt058010-noDBD deletes the entire ZtIPO323_058010 coding sequence, retaining only 790 bp of the upstream region of ZtritIPO323_04g09172. This strategy was expected to verify, if transformants have the same profile, that phenotypic change was neither driven by the remaining of the DNA binding domain nor the potential disturbance of ZtritIPO323_04g09172. They were constructed by amplifying flanking regions (1000 pb/790 pb kb before start and 1kb after stop codons) of ZtIPO323_058010 from IPO323_Ancessor_2023 gDNA along with the sulfonyleurea resistance gene cassette from the plasmid pNOV_3Gate_SUL by High fidelity PCR using primers listed in Supplementary Table S3. The three inserts were cloned into a pCambia_0380_HPH (Amezrou et al., 2023) vector after linearization by EcoRI/XhoI double digestion. PCR fragments were cloned using Gibson Assembly cloning kit (New England Biolabs) according to the manufacturer's instructions.

Escherichia coli NEB 5- α strain carrying the pCAMBIA_0380_Sulf_KO-Zt058010-DBD or pCAMBIA_0380_Sulf_KO-Zt058010-noDB were selected on kanamycine (50 $\mu\text{g.mL}^{-1}$) supplemented LB-agar and screened by PCR. Plasmids were extracted with the Nucleospin Plasmid QuickPure Kit (Macherey-Nagel) following the manufacturer's instructions and verified by Sanger sequencing (eurofins, primers listed in Table S3). *Agrobacterium tumefaciens* strain AGL1 was transformed by heat shock with the verified plasmids. Resistant colonies grown onto YEB medium supplemented with rifampicin (50 $\mu\text{g.mL}^{-1}$), kanamycin (50 $\mu\text{g.mL}^{-1}$), and ampicillin (100 $\mu\text{g.mL}^{-1}$) were also validated by PCR using the same primers.

IPO323_Ancessor_2023 and experimentally evolved strains (3BP_III_12_C_a, 3BP_IV_12_B_a and 1BCP_I_12_C_b) were transformed with the two plasmids *via A. tumefaciens*-mediated transformation (ATMT) following a procedure adapted from Bowler et al. (2010) with modifications described in Marchegiani et al., 2015. *A. tumefaciens* strains carrying the two plasmids were grown overnight at 28°C, 200 rpm in liquid YEB medium under antibiotic selection described for *Agrobacterium* transformant selection. Cultures were diluted in IM (induction medium containing 50 $\mu\text{g.mL}^{-1}$ kanamycin and 40 $\mu\text{g.mL}^{-1}$ acetosyringone) to an OD_{660nm} of 0.4 and mixed v/v with IM suspension of *Z. tritici* blastospores, harvested from 7-day solid YPD cultures, adjusted to 10⁷ spores.mL⁻¹. *A. tumefaciens*-*Z. tritici* mixtures were plated onto cellophane membranes (Cellophane Membrane Backing 165-0963, Biorad) placed on

IM-agar plates for 48h at 18 °C and transferred onto MM-Zt plates containing 250 µg.mL⁻¹ cefotaxime (Kalys SA) and 10 µg.mL⁻¹ Chlorimuron-ethyl (Sulfonylurea, Sigma) for 15–20 days at 18 °C. 50 transformant colonies were picked and spread onto YDP-agar unselective medium before purification by single colony propagation twice. Successful deletion of ZtIPO323_058010 and replacement by the resistance marker at the locus was confirmed by colony PCR (primers listed in Supplementary Table S3). For this, spores were picked, resuspended in 20 µL of sterile water, incubated at 95 °C for 10 min, and then centrifuged for 2 min at 11,000g and 4 °C. Supernatant, kept on ice, was used as template in the PCR reaction. Validated *Z. tritici* clones amplify a fragment overlapping the 5' genomic region and the *SulfR* resistance gene, as well as a *SulfR*-3'overlapping fragment, and do not amplify the deleted sequence of ZtIPO323_058010 (see Fig.S4). IM and MM-Zt recipes are provided in Table S4.

For the complementation approach with the ZtIPO323_058010-C740 (WT) and ZtIPO323_058010-Y740 (mutated) alleles, both IPO323 Δ ZtAtrR_DBD and IPO323 Δ ZtAtrR_noDBD genetic backgrounds were transformed, even though phenotypically indistinguishable. The locus *Ku70* was targeted for ectopic insertion (Sidhu et al., 2015). Four constructs were generated, using plasmid pKu70_Hygro_PmeI_RFP as a backbone for integrating wild-type or C740Y alleles with the mRFP1 reporter, depending on whether the ZtIPO323_058010 fragment preserved the native ORF with RFP. Two wild-type fragments were amplified by high-fidelity PCR from IPO323_AncestOr_2023 genomic DNA: one including the stop codon and terminator of ZtIPO323_058010, which is not in frame with RFP ("C740_noRFP"), and one without the stop codon and terminator, which is in frame with RFP ("C740_RFP"). The same design was applied to the C740Y mutant fragments, yielding a total of four constructs. Using the same primers, two mutated fragments were obtained from 3BP_IV_12_A_b gDNA ("Y740_noRFP" and "Y740_RFP"). The plasmid was digested with PmeI (ThermoFisher) prior to fragment cloning. All subsequent steps were performed as described for the knockout transformations, with selection of *Z. tritici* transformants on hygromycin (100 µg.mL⁻¹), according to the plasmid backbone's resistance marker.

Due to the low number of transformants obtained (only 75 transformants across the eight complementation transformations), we retained all transformants that amplified the replacement cassette. Doing so we ensured integration of the gene into the genome without controlling the exact insertion locus. The information of ectopic insertion or at the *ku70* locus is recorded in Table 1. Sanger sequencing was also performed on some transformants to confirm the C>T mutation resulting in the C740Y amino acid change in ZtIPO323_058010. Their fungicide resistance phenotype was assessed through droplet tests described before.

Fitness assays in different O₂ availability conditions

Fitness was inferred for ancestral strain IPO323_Ancestral_2023 and several transformants from their growth in YPD liquid culture without fungicide under different oxygen availability conditions. Spore concentrations of 7-day YPD cultures were estimated from OD_{405nm} as previously described (Ballu *et al.*, 2021). Suspensions were adjusted to 5.10^4 spores.mL⁻¹ in YPD and 200 μ L were distributed into six wells of 96-well clear microplates (Sarstedt) per isolate. Wells were sealed with breathable film (Breathe-easy®; Diversified Biotech). For normoxic condition, the upper plastic film was removed, while it was left in place for hypoxic condition. Microplates were incubated at 18°C; normoxic plates were additionally agitated at 150 rpm in a microplate shaker (Innova S44i, Eppendorf). OD_{405nm} was measured daily over 7 days, after gently vortexing the plates.

Corrected optical density (OD) values, obtained after blank subtraction, were converted into spore concentrations. Growth curves were modeled using non-linear mixed-effects logistic models (nlme package, (Pineiro et al., 2025)). Two separate non-linear mixed-effects logistic models were fitted: one with strain and another with genotype as fixed effects, both including the interaction with oxygen condition (normoxia vs. hypoxia) and biological replicate as a random effect. Variance structures (weights) were applied to account for heteroscedasticity across time points. First model was used to estimate growth curves for each strain, and the second one for assessing the contribution of the genetic background. Model parameters, final population size (Asym), time to half-maximal growth (xmid), and growth rate (scal), were extracted from the second model, and pairwise contrasts of marginal means were tested with multiple-comparison correction (emmeans package, CLD method). The same method was applied to the first model for testing parameters of individual strains (Fig.S5).

Data Availability

Supplementary figures are provided in this paper following core figures, supplementary tables as well as maps of plasmids used in this study are publicly available on:

<https://doi.org/10.5281/zenodo.17970213>

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Figures

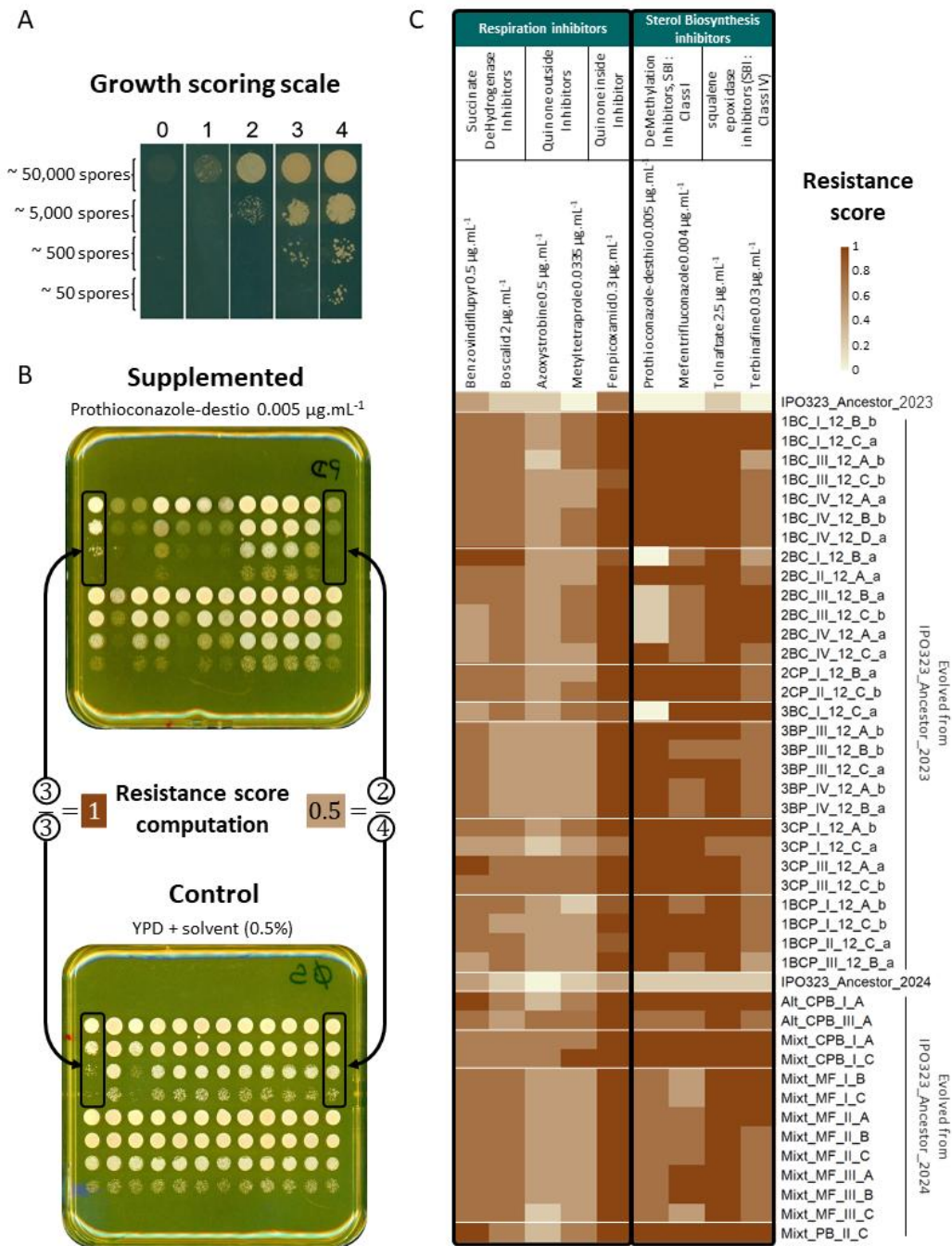


Fig.1: Selected experimentally evolved isolates from Ballu *et al.* (2023, 2024) display multi-drug resistance profiles. A: Scoring scale (droplet tests) used to evaluate growth of isolates on YPD solid medium supplemented with discriminatory doses of fungicides, adapted from Ballu *et al.*, 2024. B: Resistance score assessment, calculated as the ratio of the growth score in fungicide-supplemented medium to that in control medium after 7 days of culture. C: Heatmap of resistance scores of 42 selected individual isolates evolved from two IPO323 ancestral strains and displaying the widest and strongest MDR profile. The names of isolates contain information on the experimental evolution procedure they were selected from, as described in Ballu *et al.* (2023, 2024) and detailed in experimental procedures.

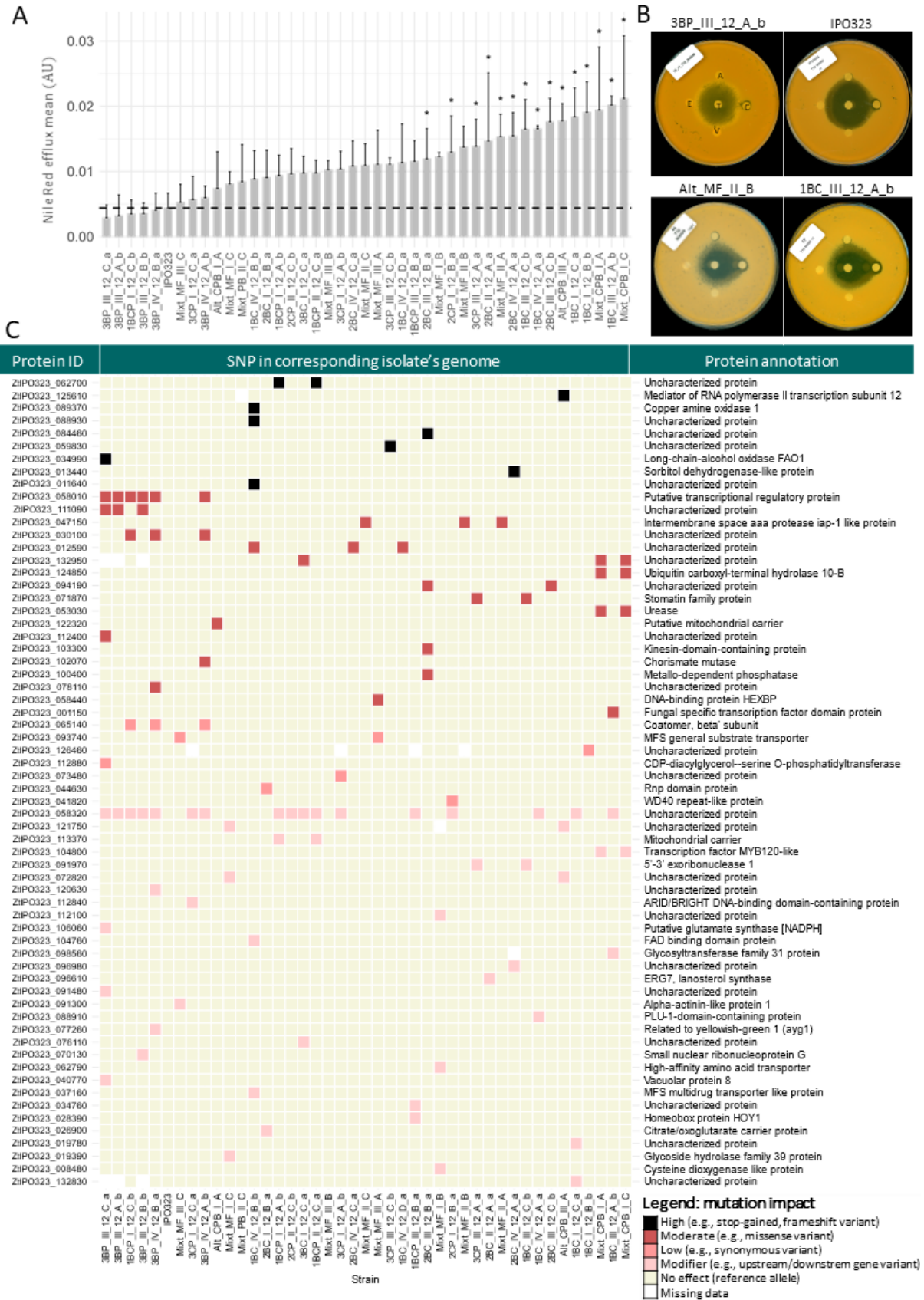


Fig.2: Evolved MDR isolates show contrasted efflux phenotypes associated with mutations in wide range of gene functions. A: Efflux capacity of selected isolates assessed by a Nile Red efflux assay. Spore suspensions were incubated for one hour with the fluorescent dye Nile red (final concentration of 7.5 μ M) before measuring the

fluorescence (547nm_{ex}, 600nm_{em}) and the OD_{405nm}. The bar represents the mean efflux per strain, ± standard deviation (n≥3 per strain), expressed as the inverse of Fluorescence_{600nm}/OD_{405nm}. The black line indicates the efflux mean of wild-type ancestor strains IPO323 (pooled IPO323_Ancestral_2023 and 2024). *: Bonferroni corrected p-values < 0.05 of pairwise contrasts against the corresponding ancestral IPO323 strain, extracted from a linear mixed model. B: Representative results of double disk synergy test (DDST) assays between terbinafine (T, central disk) and transport modulators (lateral disks, A: Amytriptiline, V: Verapamil, C: Chlorpromazine) on growth of IPO323 and some evolved mutants. Interactions between terbinafine and modulators were inferred from the shape of the inhibition halo (Laishram *et al.*, 2018). C: Overview of the genomic variations identified in evolved isolates (ordered as in panel A) within gene regions (gene plus 5 kb upstream and downstream region) and predicted effects on encoded proteins and associated functions. Variations present in ancestral strains and/or intergenic regions were filtered out (see exp. methods). The presence, absence and effect of remaining variations are indicated by the cell colors. Beige: reference sequence; white: absence of sequencing information. Other colors (from lighter to darker): modifier variant (upstream or downstream gene, light pink), low effect variant (splice region, intron or synonymous, darker pink), moderate effect variant (missense or in-frame indel, red), and high effect variant (start loss, stop gained or frameshift, black). Variant effect in coding sequences correspond to the highest effect score retrieved from SNPeff output. Protein IDs and annotations were retrieved from Lapalu *et al.* (2025).

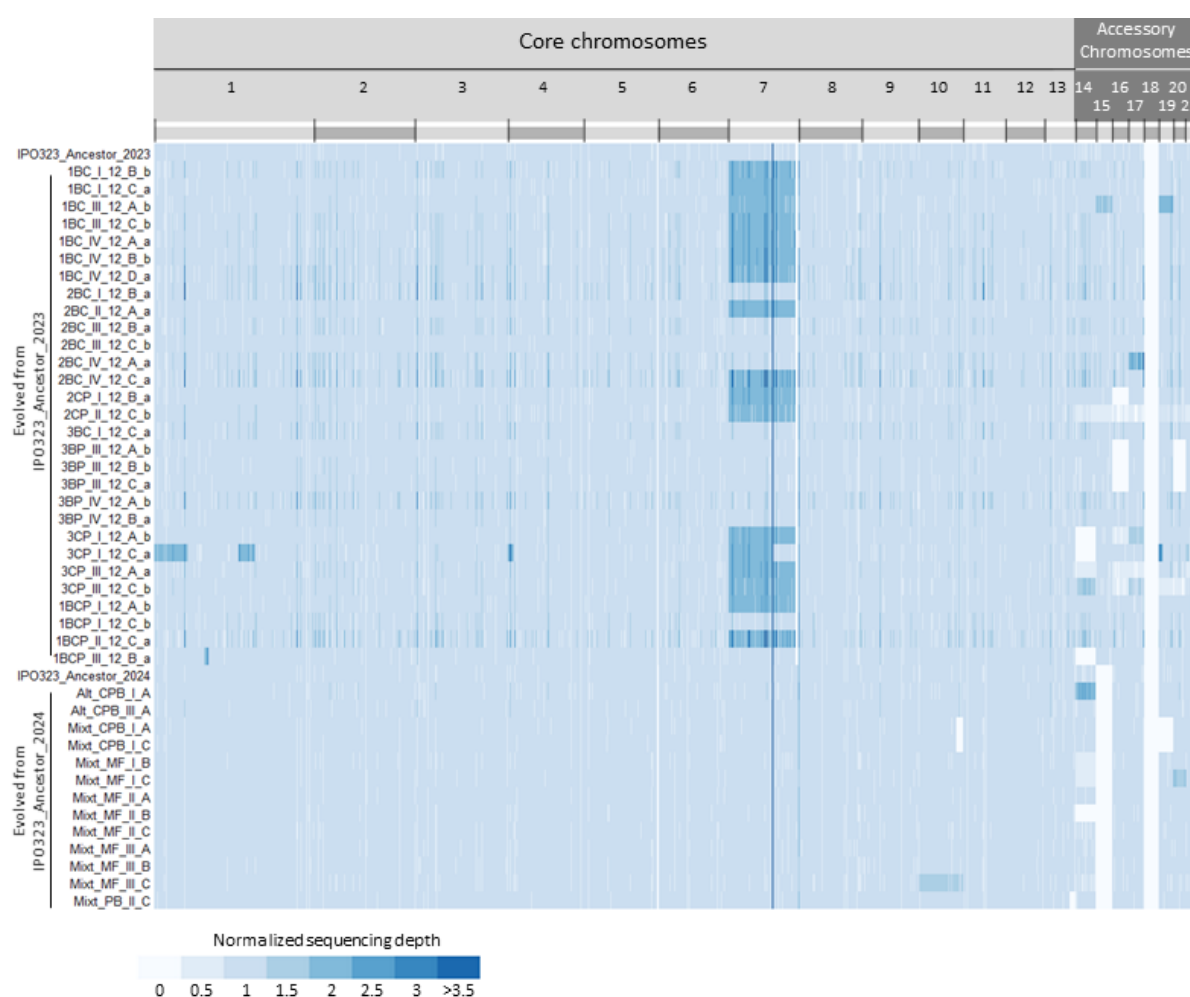
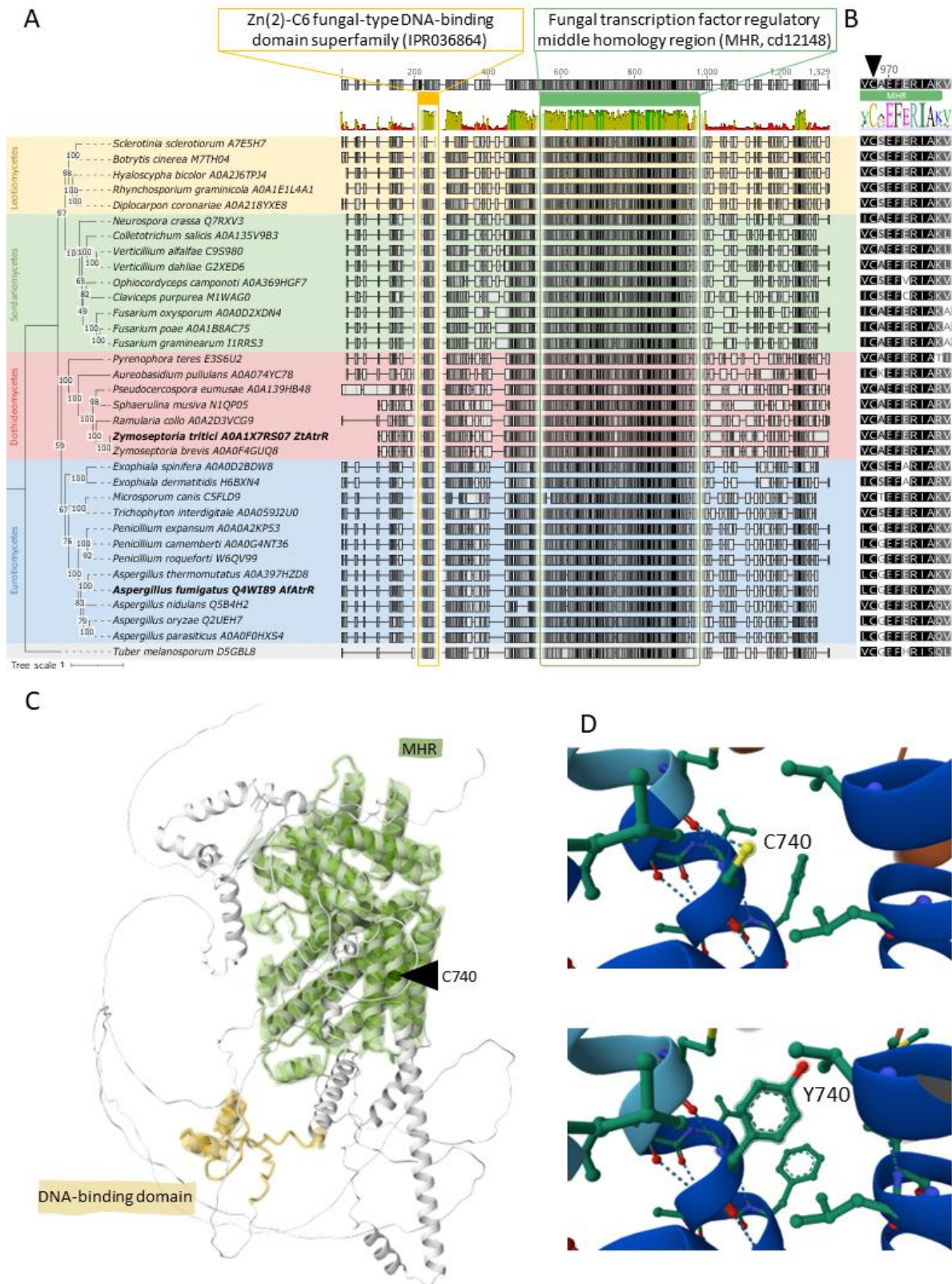


Fig.3: Chromosome losses and gains occurred during experimental evolution. Sequencing depth of IPO323 ancestral strains and evolved isolates was mapped onto the IPO323 reference genome. Mean depth was calculated in consecutive 5 kb windows across all 21 chromosomes and normalized to the median depth of core chromosomes (excluding chromosome 7) for each isolate. A normalized depth near 0 across a chromosome indicates chromosome loss, whereas values approaching 2 indicate duplication. Intermediate values reflect heterogeneity in chromosome content within the sequenced cell population of a given isolate.



number of substitutions per site, with the tree scale indicating length for one. Sequence alignment is presented in the right part. B: Zoom in multiple sequence alignment at position C740 in ZtIPO323_058010. C: Predicted structure of wild type ZtIPO323_058010. Models were built with ColabFold, functional domains are colored as in panel A, the black arrow indicates the C740 residue located in the terminal position of middle homology region. D: Focus on the proximal environment of the 740 position in the WT and the C740Y variant protein ZtIPO323_058010, colored by per-residue measure of local confidence (pLDDT) scores (from red to blue, low to high confidence score).

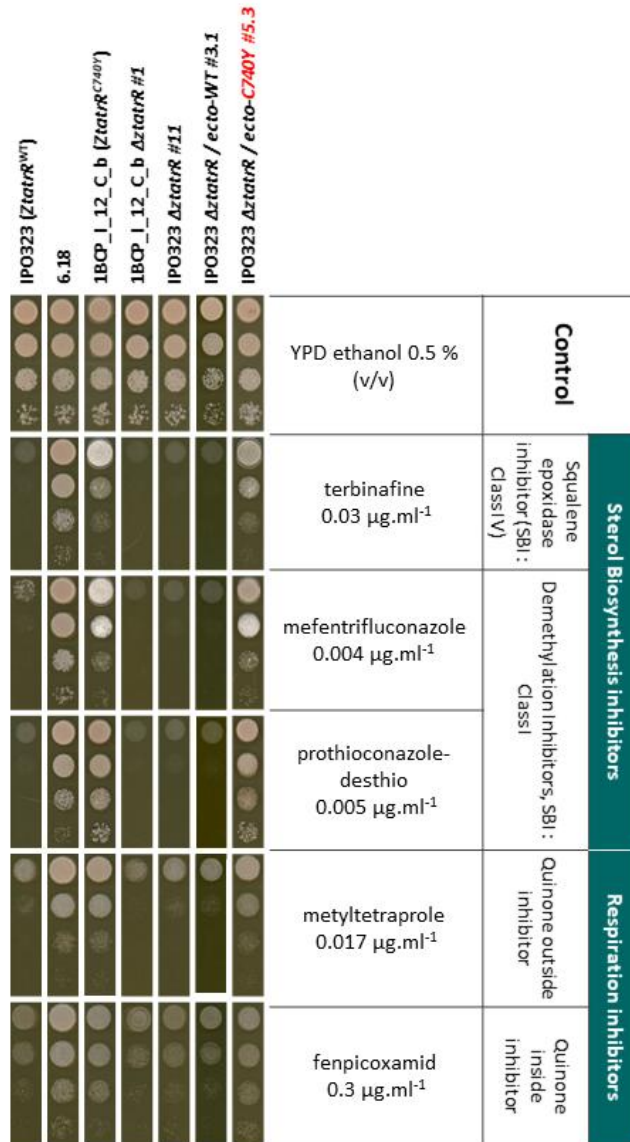


Fig.5: ZtatrR-C740Y is a gain of function mutation leading to multidrug resistance in *Zymoseptoria tritici*.

Growth of ZtatrR mutants (deletion mutants, complemented with the WT, or the C740Y allele) was assessed on different fungicides. For droplet tests, strains were precultured for 7 days and approximately 5 μL of serial spore dilutions (10^7 , 10^6 , 10^5 and 10^4 spores. mL^{-1}) were spotted on YPD solid medium supplemented with discriminatory doses of fungicides. Growth was assessed after 7-day culture. Strains: IPO323: ancestral strain with *ZtatrR*^{WT} allele (Ancestor_2023), 1BCP_I_12_C_b: experimentally evolved with acquired *ZtatrR*^{C740Y} allele, 6.18: positive MDR control. Mutants: $\Delta zatrR$ deletion mutants in both IPO323_Ancestral_2023 (IPO323 $\Delta zatrR$ #11) and 1BCP_I_12_C_b (1BCP_I_12_C_b $\Delta zatrR$ #13) backgrounds. $\Delta zatrR$ / *ecto*-WT and $\Delta zatrR$ / *ecto*-C740Y: complementation mutants in IPO323 $\Delta zatrR$ background, carrying ectopic insertion(s) of the wild-type (*ecto*-WT) or mutated (*ecto*-C740Y) *ZtatrR* allele.

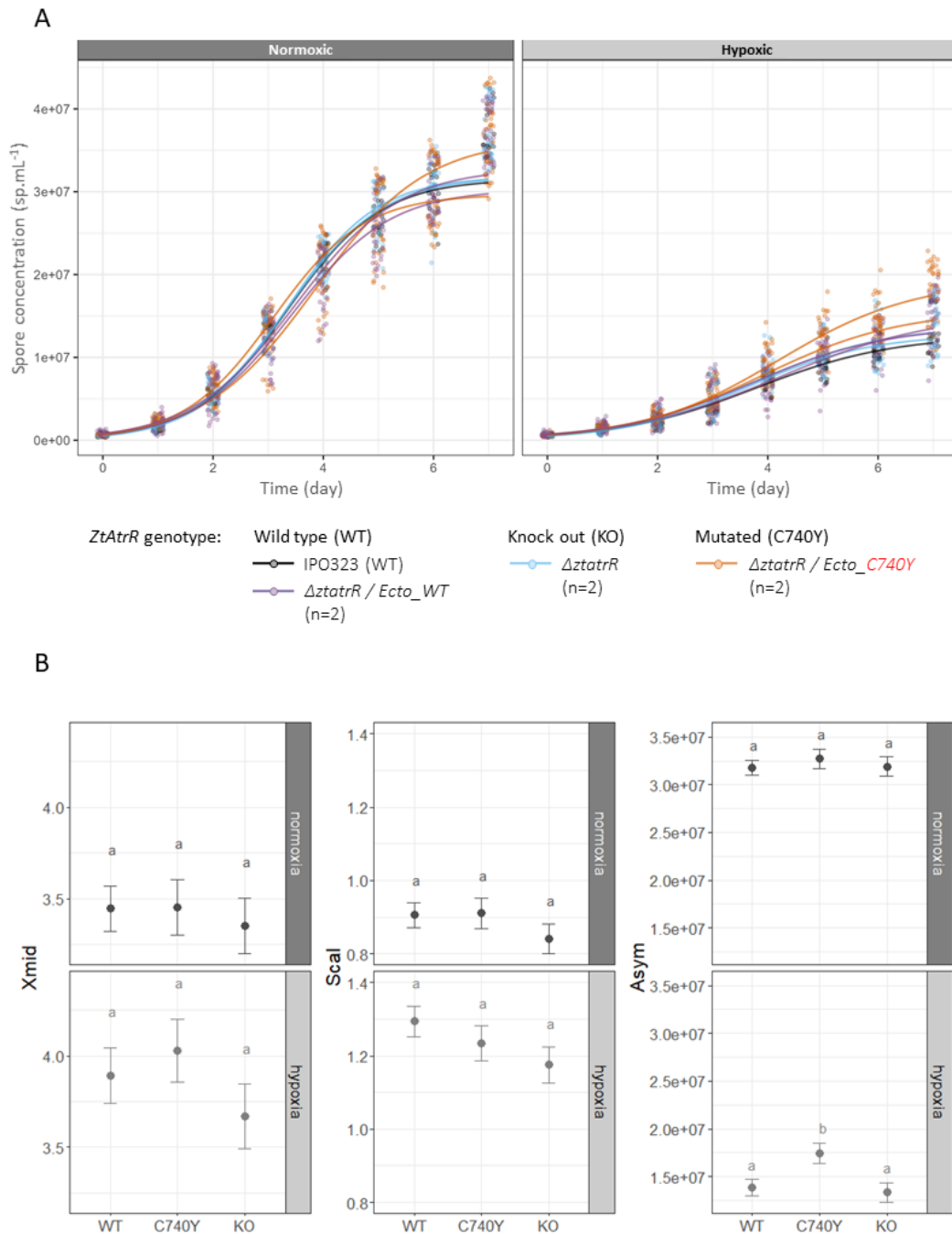


Fig.6: Neither *ZtatrR* deletion nor *ZtatrR*^{C740Y} allele impair fitness *in vitro*. Growth curves of the ancestral strain IPO323 (Ancestor_2023) and *ZtatrR* mutants under normal or O₂-limiting conditions. Vegetative growth in YPD medium was assessed by monitoring optical density at 405 nm for 7 days under normoxic or hypoxic condition at 18 °C in the dark. Each strain was tested in three biological replicates, with six technical replicates each. A: growth curves fitted for individual strains, colored by *ZtatrR* genotypes: WT strains (IPO323 in black and ectopic complemented mutants $\Delta ztatrR$ / *ecto*-WT displaying *ZtatrR*^{WT} allele in purple), Knock out (KO) mutants ($\Delta ztatrR$ deletion mutants in IPO323_Ancestor_2023 background in blue), C740Y mutants (complemented mutants $\Delta ztatrR$ / *ecto*-C740Y: ectopic complemented mutants displaying *ZtatrR*^{C740Y} allele in orange). B: Estimated parameters of the logistic growth curves obtained from a mixed non-linear regression model with *ZtatrR* genotype as a fixed effect. 'asym' = asymptote (maximum growth), 'xmid' = time to reach 50% of the asymptote, and 'scal' = inverse slope (growth rate). Error bars represent standard error. Pairwise comparisons of estimated marginal means for each parameter were performed within each growth condition, with Tukey adjustment for multiple testing. Different letters indicate significant differences ($p < 0.05$).

Supplementary Figures

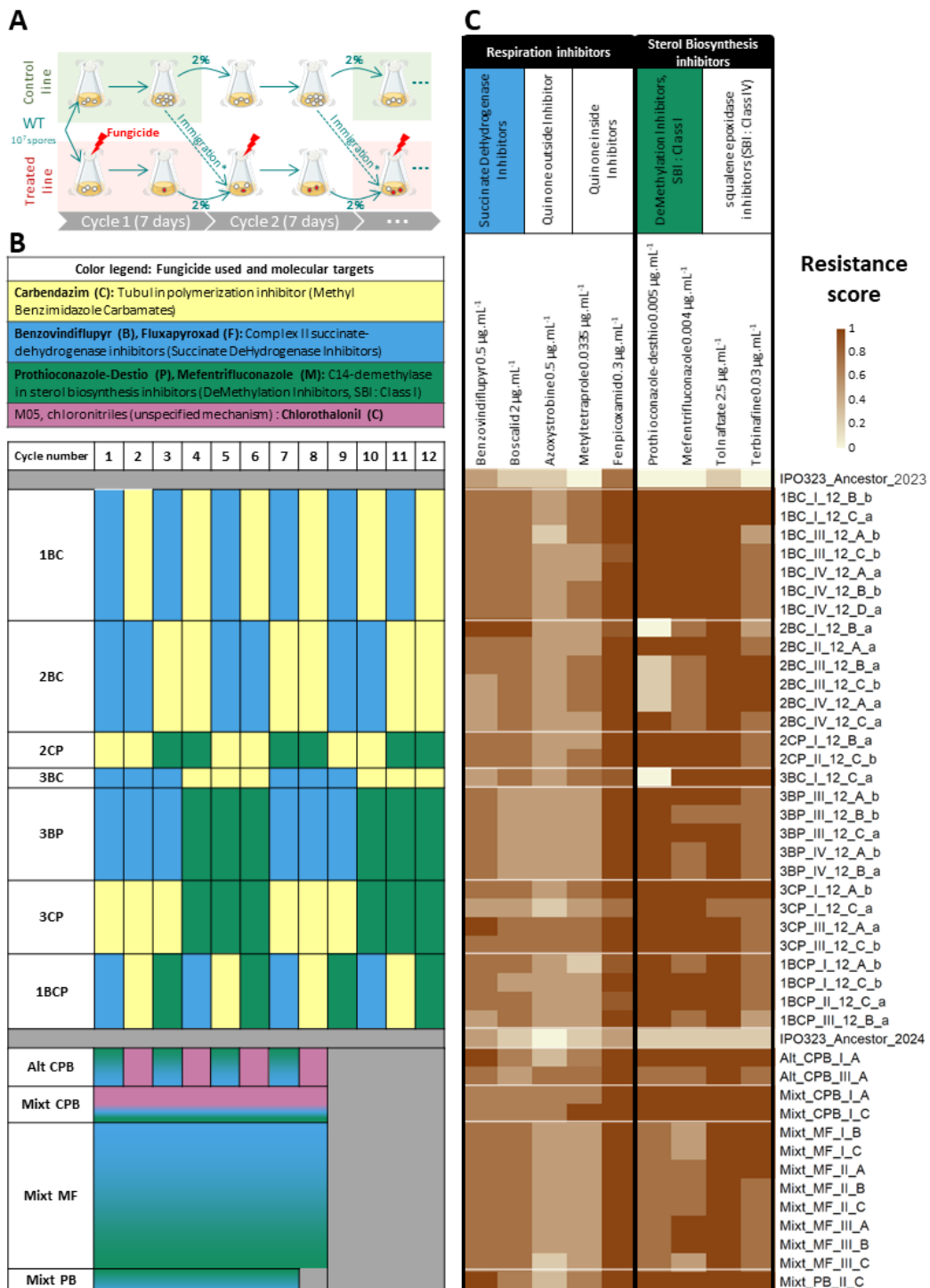


Fig.S1: Graphical summary of Ballu *et al.*, 2023 and 2024 experimental evolution procedure. A. workflow of the experimental evolution protocol indicating starting inoculum size, selection pressure application and transfers between cycles, adapted from Ballu *et al.*, 2023, Created in Biorender.com. B. Fungicide regime used in the different experimental evolutions. Gradient indicate a mixture of fungicides at 1:1 ratio or 1:0.5:0.5 when mixed with Chlorothalonil. C. Heatmap of resistance scores of 42 selected individual isolates studied in this work to fungicides with different MoAs. These isolates were evolved through corresponding treatment illustrated in B.

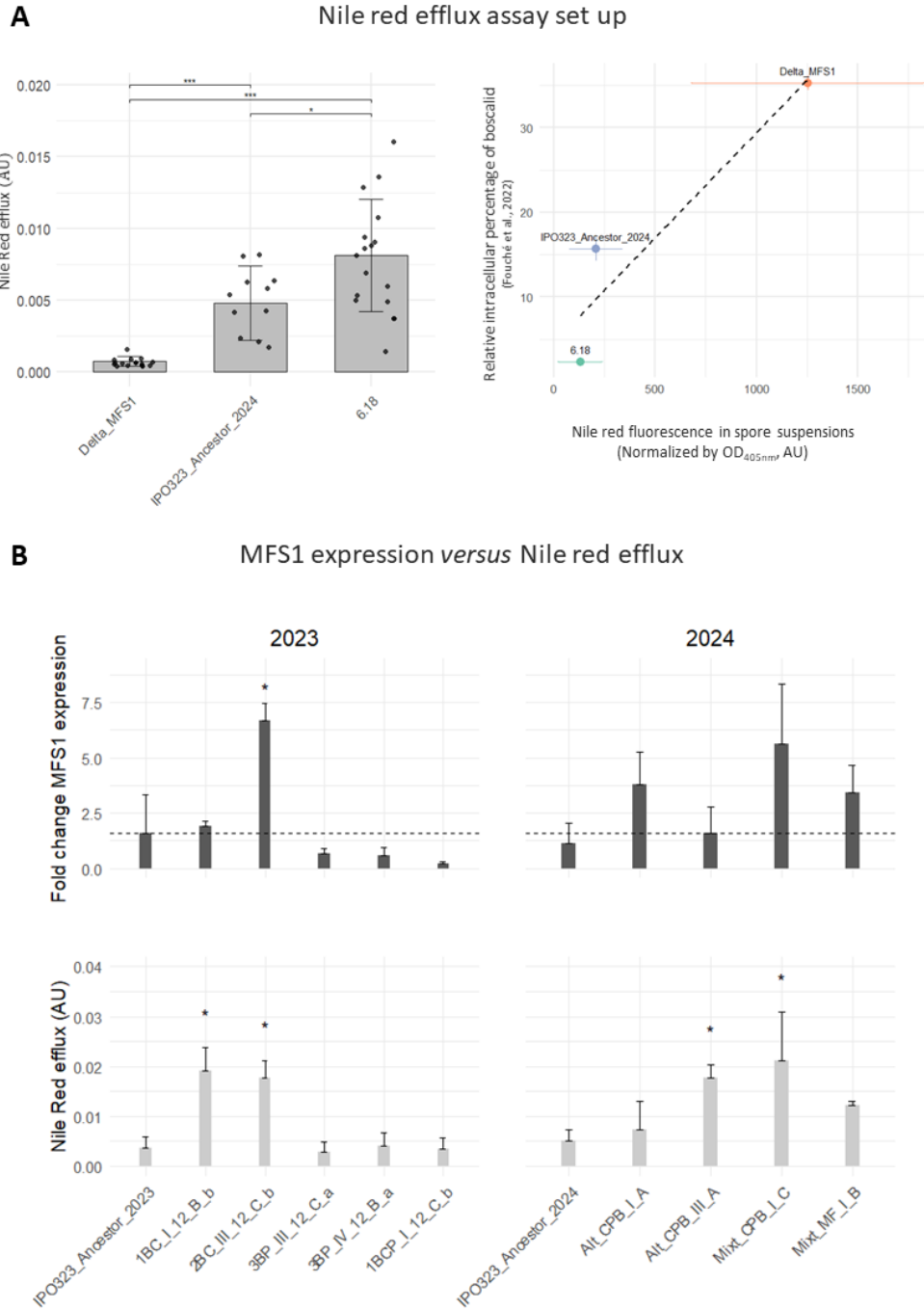


Fig.S2: A. Nile red efflux assay as a proxy for MFS1 activity. Left: bar plots show Nile red efflux in IPO323 isogenic control strains differing in *mfs1* expression (Fouché et al., 2022). Bars represent mean efflux per strain \pm SD ($n \geq 11$), expressed as the inverse of Fluorescence600nm/OD405nm. Strain differences were tested on log-transformed values using a linear mixed-effects model; estimated marginal means were compared with Bonferroni-corrected post hoc tests ($* p < 0.05$). Right: correlation between boscalid accumulation (LC-MS, 7 days, Fouché et al., 2022) and Nile red fluorescence in spore suspensions after 1 h. Points show mean of two biological replicates for boscalid and ≥ 11 for Nile red; error bars indicate SD. Strong correlation observed (Pearson $r = 0.93$; Spearman $\rho = 1$). **B. MFS1 expression and Nile red efflux in evolved isolates.** Top: relative *mfs1* expression by qRT-PCR, normalized to UBC and *Ef1 α* , expressed relative to IPO323_Ancest_2023. Mean fold-change ($2^{-\Delta\Delta C_t}$) \pm SD ($n = 2$). ANOVA of the linear model followed by Bonferroni-corrected pairwise comparisons using emmeans ($* p < 0.05$). Bottom: Nile red efflux for the same isolates. Bars show mean \pm SD ($n \geq 3$), expressed as inverse Fluorescence600nm/OD405nm; $*$ indicates Bonferroni-corrected $p < 0.05$ versus IPO323 ancestor from linear mixed-effects model.

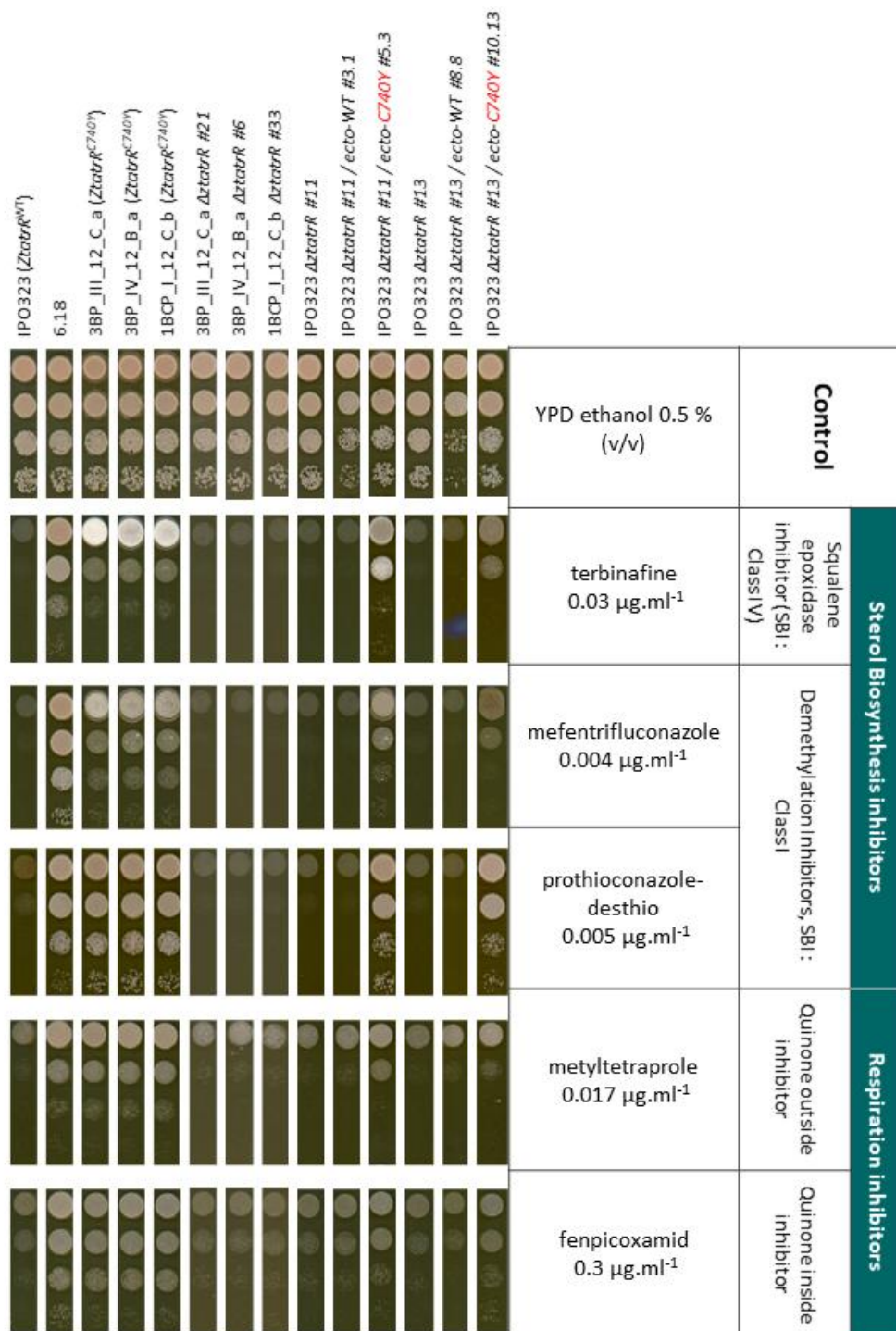


Fig.S3: Growth of all individual *ZtatrR* mutants analyzed in this study on different fungicides. For droplet tests, strains were precultured for 7 days and approximately 5 μ L of serial spore dilutions (10^7 , 10^6 , 10^5 and 10^4 spores.mL⁻¹) were spotted on YPD solid medium supplemented with discriminatory doses of fungicides. Growth was assessed after 7-day culture.

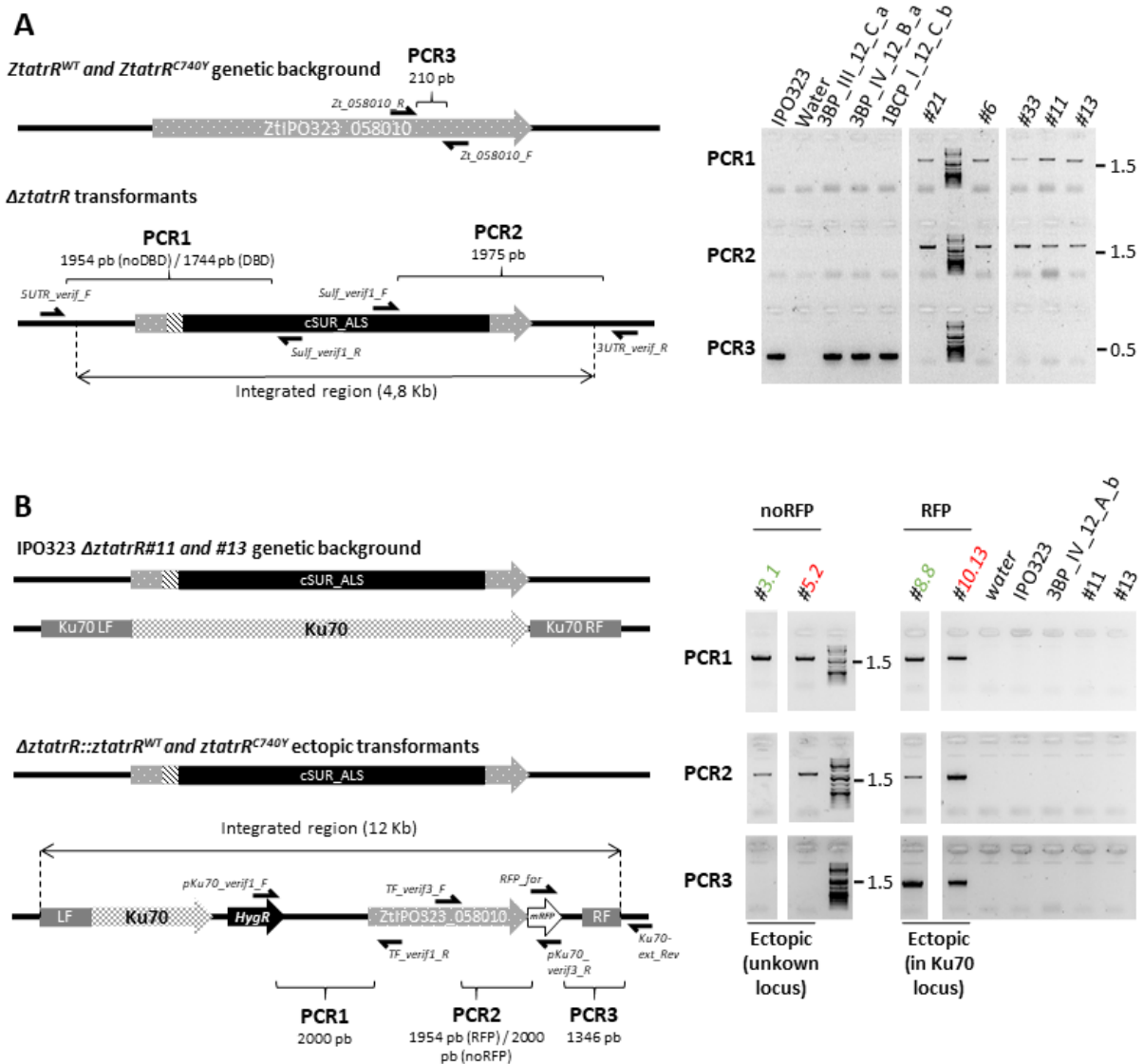
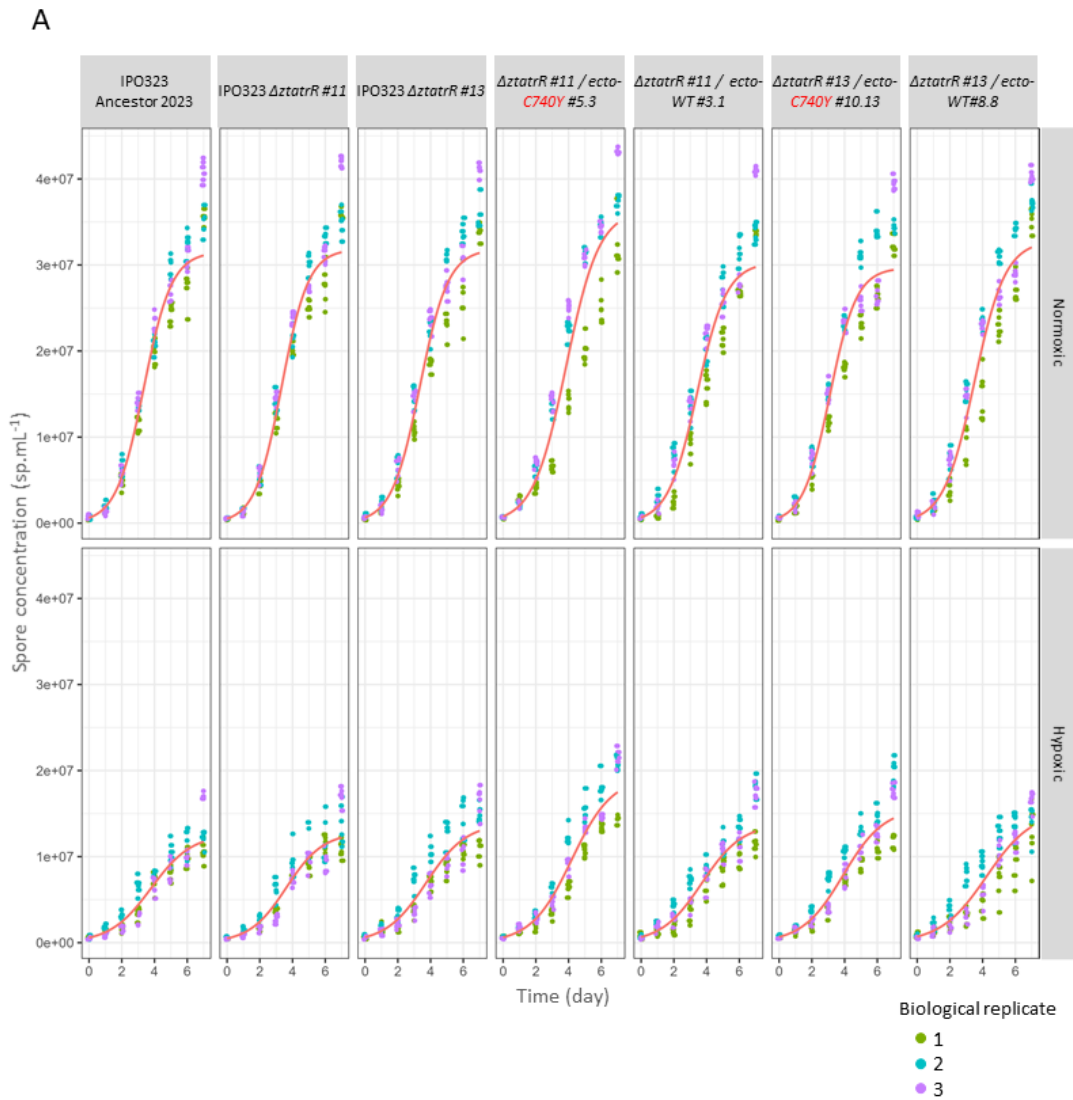


Fig.S4: Validation of transformants analyzed in this study by PCR on genomic DNA. A: Validation of *ΔztatrR* mutants in *Ztatr*^{WT} and *Ztatr*^{C740Y} background. Left: Schematic of the *ZtatrR* gene and deleted *ΔztatrR::SUR_ALS* locus showing validation primer position. Right: PCR product migration. The SUR_ALS resistance gene is detected at the expected position in mutants (#21, #6, #33 from 3BP_III_12_C_a, 3BP_IV_12_B_a, 1BCP_I_12_C_b backgrounds, and #11, #12 from IPO323), while primers internal to *ZtatrR* amplify only in parental strains, confirming successful deletions. B. Validation of ectopic complementation in *ΔztatrR* / *ecto-WT* and *ΔztatrR* / *ecto-C740Y* mutants in IPO323 *ΔztatrR*. **Left:** Schematic of *ΔztatrR::SUR_ALS* and *Ku70* locus before and after ectopic *ZtatrR* integration, with primer positions. **Right:** PCR product migration. PCR1 and PCR2 confirm all transformants carry the construct (*hygR*, *ZtatrR*, *mRFP1*), absent in *ΔztatrR* parental strains. PCR3 shows *Ku70*-targeted integration for #8.8 and #10.13 but no #3.1 and #5.2, indicating ectopic insertion. Red: *Ztatr*^{C740Y}, green: *Ztatr*^{WT}.



B

Strain	Normoxic			Hypoxic		
	Asym	xmid	scal	Asym	xmid	scal
<i>IPO323 Ancestor 2023</i>	3,15E+07 (1,14E+06) ab	3,35 (0,20) a	0,85 (0,05) a	1,38E+07 (1,19E+06) a	1,23 (0,06) a	3,73 (0,24) a
<i>IPO323 ΔzatrR #13</i>	3,19E+07 (1,14E+06) ab	3,43 (0,20) a	0,90 (0,05) a	1,53E+07 (1,30E+06) ab	1,25 (0,07) a	3,91 (0,23) a
<i>IPO323 ΔzatrR #11</i>	3,18E+07 (1,13E+06) ab	3,35 (0,20) a	0,87 (0,05) a	1,39E+07 (1,18E+06) a	1,24 (0,07) a	3,79 (0,25) a
<i>ΔzatrR / ecto-C740Y #5.3</i>	3,64E+07 (1,23E+06) b	3,85 (0,20) a	1,01 (0,05) a	1,93E+07 (1,27E+06) b	1,40 (0,07) a	4,23 (0,26) a
<i>ΔzatrR / ecto-C740Y #10.13</i>	2,97E+07 (1,12E+06) a	3,10 (0,20) a	0,82 (0,05) a	1,26E+07 (1,19E+06) a	1,13 (0,06) a	3,62 (0,23) a
<i>ΔzatrR / ecto_WT #3.1</i>	3,03E+07 (1,15E+06) a	3,34 (0,20) a	0,82 (0,05) a	1,28E+07 (1,15E+06) a	1,23 (0,07) a	3,71 (0,24) a
<i>ΔzatrR / ecto_WT #8.8</i>	3,28E+07 (1,17E+06) ab	3,50 (0,20) a	0,95 (0,05) a	1,57E+07 (1,22E+06) ab	1,25 (0,06) a	4,19 (0,22) a

Fig.S5: growth dynamics and model-derived parameters for *ZtatrR* individual mutants. A. Time-course of spore concentrations in different mutants under normoxia and hypoxia. Concentrations were corrected for blank and transformed from OD values as described in Materials and Methods. Points are colored by individual biological replicates ($n = 3$) and several points from the same color are technical replicates. Lines show predictions from a non-linear mixed-effects logistic model including strain, oxygen condition, and their interaction as fixed effects, and biological replicate as a random effect. B. Estimated model parameters extracted from the model for each strain: asymptote (Asym), half-maximal growth (xmid), and growth rate (scal). Letters indicate groups with significantly different marginal means for each parameter, determined using pairwise comparisons of estimated marginal means (emmeans package, Bonferroni-adjusted $p < 0.05$).

CHAPTER II: IDENTIFICATION OF MDR MECHANISMS IN FIELD POPULATIONS

Existing material, objectives and approaches

This second chapter focuses on the analysis of recent MDR field populations collected between 2020 and 2021 in Northwestern Europe. This work was initiated by Patry Leclaire (2024), who established and conducted miniaturized dose-response assays enabling determination of terbinafine EC_{50} in these strains as a quantitative MDR phenotyping. This approach was combined with PCR-Sanger based genotyping of the proximal promoter region of P_{MFS1} (i.e., -500 pb). The resulting data revealed a quantitative shift in MDR levels *in natura* among strains sharing the same proximal P_{MFS1} genotype, providing the foundation for the present PhD work. We aimed to further characterize the extent of this phenomenon and to decipher the mechanistic basis underlying this quantitative shift by expanding the original study through several complementary approaches:

- Investigating the transposon insertion polymorphism (TIP) previously detected in P_{MFS1} , functionally validating the role of a novel P_{MFS1} allele through forward genetics, and extending the analysis of the promoter region to its full length to better assess its plasticity;
- Characterizing the quantitative and polygenic nature of MDR by studying its heritability in a segregating progeny obtained through sexual crosses and phenotyped through dose-response methodology;
- Elucidating this polygenic basis using genome-wide association study (GWAS), combining the existing terbinafine EC_{50} dataset with newly generated whole-genome sequencing data, enabling the identification of candidate genes.

In the following section, we present the second article of this PhD, which integrates results from both Patry Leclaire's work (2024) and the present study, with the aim of providing a comprehensive overview of P_{MFS1} promoter plasticity and its contribution to MDR, together with additional genetic drivers shaping a complex MDR landscape *in natura*.

Article 2: The complex landscape of Multidrug Resistance in *Zymoseptoria tritici*: MFS1 promoter plasticity and beyond

Authors: Elza Neau, Simon Patry-Leclaire, Cécile Lorrain, Anaïs Pitarch, Matéa Vencatasamy, Anne-Sophie Walker, Nicolas Lapalu, Anaïs Lalève and Sabine Fillinger

Abstract

Multidrug resistance (MDR) in fungal pathogens poses a growing threat to fungicide efficacy and sustainable agriculture. In the wheat pathogen *Zymoseptoria tritici*, MDR is primarily linked to overexpression of the *MFS1* transporter gene, driven by transposable element (TE) insertions in its promoter or their remnants. Here, we provide a comprehensive analysis of *MFS1* promoter polymorphism and its phenotypic impact on MDR across 374 field isolates collected in Europe between 2020 and 2021. We identify six novel structural variants derived from diverse TE types, confirming that the *MFS1* promoter undergoes recurrent and independent insertion events. While previously characterized inserts consistently confer resistance, other variants show limited or variable phenotypic effects. Genetic crosses and quantitative phenotyping further reveal that MDR behaves as a quantitative trait and genome-wide association study confirmed *MFS1* as the major resistance locus but also identified additional candidate genes involved in xenobiotic detoxification and membrane transport, supporting a polygenic basis for MDR. Our findings highlight the interplay between TE-driven structural variation and background polygenic architecture in shaping resistance phenotypes. In parallel, this study highlights the need to improve MDR monitoring beyond single-locus genotyping and re-evaluate current resistance management strategies that may inadvertently select for broad-spectrum resistance.

Introduction

Major advances in public health and agriculture over the past century were largely driven by the development and widespread use of pharmaceutical active ingredients (AIs). These innovations have significantly reduced disease-related mortality and increased crop yields (OECD, 2018; Oerke, 2006). However, the extensive use of AIs imposes strong selective pressure on microbial populations, inevitably leading to resistance development, referred to as anti-microbial resistance (AMR). This growing concern is not limited to bacterial pathogens. In recent years, fungal resistance to AIs has emerged as a parallel threat, both in the clinics and in agriculture where its impact on global food security is underestimated (Fisher and Denning, 2023; Stukenbrock and Gurr, 2023). Fungicide resistance has historically been dominated by target-site resistance, arising from mutations or overexpression of the target protein. Such mechanisms, often associated with high resistance factors, have been central to resistance management strategies for decades (Lucas et al., 2015). To slow down their selection, growers are encouraged to alternate or mix fungicides of different modes of action (MoAs) (Rex Consortium, 2013). Despite the

widespread application of fungicides and the deployment of resistant crop varieties, global agricultural losses due to fungal pathogens remain significant, estimated between 10% and 23% annually (Fisher et al., 2012). This persistence of losses, despite control efforts, is increasingly linked to the rise of fungicide-resistant fungal strains that undermine the effectiveness of current management strategies. This highlights that advancing our understanding of fungicide resistance, including both target-site and emerging non-target-site mechanisms, is essential to sustain crop productivity and addressing future food security challenges.

Multidrug resistance (MDR) refers to non-target site mutation(s) that confer broad-spectrum resistance to multiple fungicides with unrelated MoAs ("Glossary – R4P," n.d.; R4P Network, 2016). The most commonly described mechanism underlying MDR in fungal plant pathogens involves increased efflux activity typically mediated by membrane transporters of the ATP-binding cassette (ABC) family and major facilitator superfamily (MFS) (Hu and Chen, 2021). MDR has been well documented in human health including in cancer (Krishna and Mayer, 2000), and since the early 2000s, mounting evidence has revealed its presence in phytopathogenic fungi such as *Botrytis cinerea*, *Oculimacula* spp., *Penicillium digitatum*, *P. expansum*, *Claviceps homoeocarpa* (formerly *Sclerotinia homoeocarpa*), and *Zymoseptoria tritici* (Kretschmer et al., 2009; Leroux et al., 2013; Sang and Hulvey, 2018; Sun et al., 2013). In the necrotrophic grey mold agent *B. cinerea*, MDR results from two different mutational events. The gain-of-function mutation in the transcription factor BcMrr1 leads to constitutive overexpression of the ABC transporter BcAtrB, while overexpression of the MFS transporter BcMfsM2 is driven by retrotransposon insertion in its promoter (Kretschmer et al., 2009). In the turfgrass dollar spot agent *C. homoeocarpa*, MDR is linked to the upregulation of three cytochrome P450 enzymes and two ABC transporters, controlled by a gain-of-function mutation in the transcription factor ShXDR1 (Sang and Hulvey, 2018). These examples highlight the complex genetic architecture of MDR, which can arise from diverse and independent events and may be combined in populations via sexual recombination. When efflux-based MDR co-occurs with target-site resistance in the same isolate, resistance levels can be significantly amplified (Hu and Chen, 2021; Mitchell et al., 2014; Vázquez-García et al., 2020), posing a serious threat to the efficacy of both registered and novel fungicides. Given the global European objectives to reduce fungicide use and the slow pace of AI discovery (Renwick and Mossialos, 2020; Sparks and Bryant, 2021), MDR surveillance should be a key priority for sustainable resistance management, particularly through the development of field-adapted monitoring tools.

Zymoseptoria tritici the causal agent of the Septoria Leaf Blotch (STB) disease, is the most damaging wheat leaf pathogen in temperate regions (Orton et al., 2011; Dean et al., 2012, Klink et al., 2021). Yield losses attributed to this ascomycete fungus vary annually, ranging from 5% to 50% (Fones and Gurr, 2015). In Europe, the control of STB relies heavily on fungicide applications, with an estimated market size of \$1.2 billion (Torriani et al. 2015; Figueroa, Hammond-Kosack, and Solomon 2018)

accounting for approximately 70% of annual fungicide usage dedicated to wheat. Several single-site fungicides with different MoAs are used to manage STB in the field, including 14-demethylation inhibitors (DMIs), succinate dehydrogenase inhibitors (SDHIs), and more recently the quinone inside inhibitor (Qil) fenpicoxamid (Garnault et al., 2019). In the field, a typical fungicide program includes azole-SDHI mixtures, particularly for the critical second treatment targeting the emergence of the first leaf (ARVALIS, 2024, 2016). However, *in vitro* experimental evolution studies have revealed that diversifying selection pressure through such mixtures or alternations may inadvertently favor the emergence of MDR in *Z. tritici*, but also in other fungal pathogens and pests (Ballu et al., 2024, 2023, 2021; Barbosa et al., 2021; Comont et al., 2020; Lagator et al., 2013; Zhou et al., 2022).

MDR in *Z. tritici* was first identified in DMI-resistant field isolates from France, displaying moderate cross-resistance to DMIs, SDHIs, Qols, and even to squalene epoxidase inhibitors (terbinafine, tolnaftate) a MoA not used in agriculture (Leroux and Walker, 2011). This phenotype was linked to the constitutive overexpression of the *MFS1* gene, encoding an MFS transporter (Omrane et al., 2015, 2017). In most cases, overexpression was driven by a 519 bp long-terminal repeat (LTR) insertion in the *MFS1* promoter region (P_{MFS1}), designated as the type I insert (Omrane et al., 2017). Additional insertions, types II and III, have also been identified, each contributing to varying levels of *MFS1* expression and MDR phenotypes (Omrane et al., 2017). Such transposable elements (TE)-derived insertion polymorphisms exemplify an important role of TEs in driving genome evolution and adaptive responses in *Z. tritici* (Baril et al., 2025; Lorrain et al., 2021; Möller and Stukenbrock, 2017; Oggenfuss and Croll, 2023; Singh et al., 2021)

Field monitoring from 2008 to 2017 in France showed a rise and stabilization of MDR frequency at approximately 25%, with ~75% of MDR isolates carrying the P_{MFS1} type I insert (Garnault et al., 2019b). More recently, new insertions, referred to as types IV and V, have been described (Lavrukaitė et al., 2023; Mäe et al., 2020), but no clear phenotypic association with MDR has been demonstrated. A genotyping approach based on PCR amplicon size upstream of *MFS1* is widely used to diagnose MDR in *Z. tritici*, its accuracy is limited by the high polymorphism of the locus (Huf et al., 2018; Lavrukaitė et al., 2023; Mäe et al., 2020; Omrane et al., 2015, 2017), since not all insertions are functionally linked to resistance, such as the type V insert, and MDR phenotypes can arise in the absence of any P_{MFS1} insertion, as shown in recent experimental evolution studies. Therefore, a diagnostic approach solely based on P_{MFS1} insertion polymorphism is insufficient for reliably monitoring MDR evolution *in natura*.

This study provides an in-depth study of P_{MFS1} insertion polymorphisms, and associated MDR phenotypic variation in a collection of 374 *Z. tritici* isolates sampled between 2020 and 2021. By combining PCR-based screening with whole-genome sequencing, we uncovered an unprecedented level of promoter diversity in the P_{MFS1} region, significantly expanding the known allelic landscape and

revealing where current MDR genotyping protocols may lack resolution. Specifically, we identified six new structural variants within the proximal P_{MFS1} promoter, not consistently associated with the MDR phenotype. We also showed that some strains with identical P_{MFS1} genotypes exhibited quantitative variation in MDR intensity. These results indicate that P_{MFS1} polymorphisms are not solely responsible for the MDR phenotype, as revealed by a genome-wide association study (GWAS) that uncovered a polygenic basis and identified several candidate resistance-related genes.

Results

The *MFS1* promoter is subject to TE-Mediated structural variation in multidrug-resistant populations of *Z. tritici*

To unravel the P_{MFS1} evolution and its correlation with MDR in *Z. tritici*, we assembled a collection of 374 field strains collected from Northern Europe between 2020 and 2021 (Table S1). Potential MDR strains were discriminated using minimal media supplemented with tolnaftate or terbinafine two squalene epoxidase inhibitors commonly employed as proxies for detecting MDR in field populations (Leroux & Walker, 2011). We obtained 283 potential MDR isolates among the roughly 600 isolates selected from the French field populations in these two years. Additional potential MDR isolates were provided by collaborators including 25 from Germany, 26 from the United Kingdom, 22 from Ireland, 12 from Lithuania, 4 from Belgium, 1 from Estonia and from Denmark (Table S1).

We first genotyped the promoter of the *MFS1* gene (P_{MFS1}) of all 374 isolates using a PCR-based assay targeting the proximal promoter upstream of the gene. Based on amplicon size and Sanger sequencing of selected isolates, we identified several P_{MFS1} genotypes including: type I (1005 bp), type II (750-855 bp), type III (636 bp), and $P_{MFS1}^{no-insert}$ (486 bp, \pm 50 bp), as well as multiple amplicons of unexpected sizes (Fig.1A). Insert types were assigned based on size and sequence similarity to previously characterized elements (Omrane et al., 2017). The genotyping results revealed a high level of P_{MFS1} promoter diversity. As only the French isolates were randomly selected, the relative insert proportion was analyzed among those (n=283). The already described insertion $P_{MFS1}^{type I}$ was the most frequent genotype (~70%, n=195), followed by $P_{MFS1}^{type II}$ (~12%, n=34 for type IIA and IIB), and $P_{MFS1}^{type III}$ (~5%, n=15) (Table S1, Fig.1A). Notably, approximately 10% of isolates selected on terbinafine from the French collection displayed no detectable insertion, $P_{MFS1}^{no-insert}$ genotype (n=28) (Table S1, Fig.1A). We detected seven new insertions at the P_{MFS1} locus. Four new insertions were identified at the $P_{MFS1}^{type II}$ insertion locus (471 bp upstream of *MFS1* start codon) including types $P_{MFS1}^{type IIC}$, $P_{MFS1}^{type IID}$, $P_{MFS1}^{type IIE}$, and $P_{MFS1}^{type IIF}$ (sizes 267 bp to 339 bp), which shared conserved domains with the known $P_{MFS1}^{type IIA}$ and $P_{MFS1}^{type IIB}$ variants (Fig.1A and B). The fifth insert at the $P_{MFS1}^{type II}$ site, lacking sequence similarity to the others, was designated $P_{MFS1}^{type VII}$. We identified a sixth novel insert, $P_{MFS1}^{type VI}$ found 215 bp upstream to *MFS1* start codon and with a 360

bp size. Finally, one structural rearrangement consisting of a 787 bp deletion from the proximal promoter (108 bp upstream of *MFS1* start codon) combined with a 159 bp insertion. This new structural variation was named $P_{MFS1}^{type\ VIII}$. All these novel inserts were found in French isolates, except $P_{MFS1}^{type\ IIC}$ and $P_{MFS1}^{type\ IIE}$ that were also detected in strains from Lithuania (n=3) and Germany (n=3). These findings expand the known repertoire of P_{MFS1} insert polymorphisms and suggest that the *MFS1* promoter region is particularly prone to insertion events.

To explore the origin of these insertions, we aligned their sequences against the IPO323 reference genome, and performed similarity searches to IPO323 annotated transposable elements (Baril & Croll, 2023). This analysis confirmed that nearly all novel and known insertions are derived from or share homology with known TEs, including LTR retrotransposon ($P_{MFS1}^{type\ I}$), Long interspersed nuclear element (LINE) retrotransposons ($P_{MFS1}^{type\ IIA}$, $P_{MFS1}^{type\ IIB}$, $P_{MFS1}^{type\ IIC}$, $P_{MFS1}^{type\ IID}$, $P_{MFS1}^{type\ IIE}$, $P_{MFS1}^{type\ IIF}$), unclassified TE ($P_{MFS1}^{type\ III}$) and miniature inverted-repeat transposable elements (MITEs) ($P_{MFS1}^{type\ VI}$, $P_{MFS1}^{type\ VII}$), Fig.1B. Finally, the $P_{MFS1}^{type\ VIII}$ variant did not match any annotated TE in the IPO323 genome. However, the inserted fragment displayed partial homology (40-55%) to repetitive sequences on chromosomes 3, 5, and 11, suggesting it originates from a high copy element (data not shown) These results indicate that recurrent transposition events shaped the structural polymorphism of the *MFS1* promoter region (Fig. 1C-1), establishing this locus as a potential hotspot for TE insertions among strains able to grow on SBI-class IV fungicides.

To further investigate the evolutionary history and origin of P_{MFS1} insertions, we conducted a phylogenetic analysis of the *MFS1* promoter region based on *de novo* assemblies of 131 strains. We first aligned the 3 kb intergenic region between *ZtIPO323_079130* and *ZtIPO323_079140* (the *MFS1* gene) to examine large-scale structural variation beyond the proximal promoter. This revealed previously undetected structural variations even in three strains initially genotyped as $P_{MFS1}^{no-insert}$, as well as three strains we failed to amplify by PCR (Fig.1C-2). Strain 21-VU20 carries a large ~5 kb insertion, while strains 21-VO12 and 21-VX2 exhibit large deletions of 757 bp and 322 bp. These structural variants, often located around the 401 bp upstream relative to the *MFS1* start codon, underline the structural plasticity of the *MFS1* promoter and confirm that current genotyping approaches may overlook substantial structural variants diversity.

To better understand the evolutionary dynamics underlying this structural diversity, we next examined the sequence conservation and divergence of the P_{MFS1} region through phylogenetic analysis. We constructed a maximum likelihood tree based on a multiple sequence alignment (MSA) of 2,074 positions shared in P_{MFS1} across the 131 strains, excluding insert sequences. This revealed that promoter sequences carrying the same insert, particularly $P_{MFS1}^{type\ I}$ and $P_{MFS1}^{type\ III}$, formed well-supported monophyletic clades, indicating strong conservation of surrounding promoter regions. Similarly, P_{MFS1}

sequences carrying the same $P_{MFS1}^{type II}$ insert variants (IIA, IIB, IIC, IID, IIE and IIF) showed high internal sequence similarity, but did not cluster together across subtypes (Fig.1D-1). These findings argue against the hypothesis of a single ancestral $P_{MFS1}^{type II}$ insertion that diversified through mutation and recombination. Instead, they indicate that the different $P_{MFS1}^{type II}$ variants likely arose from multiple, independent transposition events. One exception may be the $P_{MFS1}^{type IID}$ variant, which clustered closely with $P_{MFS1}^{type IIA}$, possibly reflecting a shared origin. To assess whether these patterns were driven by population structure, we compared the P_{MFS1} -based tree to a whole-genome SNP phylogeny (Fig.1D-2). While some clonal groups were apparent (e.g., strains 21-VCD and 21-JM), the broader patterns of P_{MFS1} conservation could not be explained by population structure alone. Finally, we quantified nucleotide diversity (π) across P_{MFS1} haplotypes. Strains lacking insertions ($P_{MFS1}^{no-insert}$) showed tenfold higher diversity ($\pi = 0.0197$) than insert-carrying haplotypes such as $P_{MFS1}^{type I}$ ($\pi = 0.0004$), $P_{MFS1}^{type IIA}$ ($\pi = 0.0017$), $P_{MFS1}^{type IIB}$ ($\pi = 0.001$), and $P_{MFS1}^{type III}$ ($\pi = 0$). These patterns are consistent with strong selection maintaining specific insertion haplotypes, further pointing to their potential functional relevance in multidrug resistance.

P_{MFS1}* insertion variants quantitatively impact multidrug resistance in *Z. tritici

To dissect the contribution of structural polymorphisms at the *MFS1* promoter to MDR in *Z. tritici*, we evaluated terbinafine resistance across field isolates grouped by P_{MFS1} genotype. Both qualitative resistance profiling (Fig.2) and quantitative EC₅₀ measurements were performed (Fig.3), allowing a high-resolution assessment of phenotypic outcomes associated with individual insertions (Fig.3A-1).

Strains carrying the $P_{MFS1}^{type I}$ insertion consistently displayed high levels of terbinafine resistance (Fig.2B). EC₅₀-based phenotyping of 19 randomly selected strains confirmed strong resistance, with field isolates displaying resistance factors (RFs) around 8 compared to the sensitive IPO323 reference (Fig.3A-3). The $P_{MFS1}^{type I}$ reference strain Ref_I, which carries the $P_{MFS1}^{type I}$ insertion in the sensitive IPO323 background (Omrane et al., 2017), was included in all replicates as a positive control. Most $P_{MFS1}^{type I}$ field isolates showed EC₅₀ values comparable to or exceeding Ref_I, with some strains such as 21-JM1 reaching an RF of 15 (Fig.3A-3). These results confirm that $P_{MFS1}^{type I}$ insertions robustly contribute to the MDR phenotype in *Z. tritici*.

Similar trends were observed for $P_{MFS1}^{type II}$ insertions. Qualitatively, both $P_{MFS1}^{type IIA}$ and $P_{MFS1}^{type IIB}$ were strongly associated with MDR, with 88% (22/25) of $P_{MFS1}^{type IIA}$ and 97% (44/45) of $P_{MFS1}^{type IIB}$ strains of HR phenotype (Fig2B). EC₅₀ quantification supported this association. Field strains carrying $P_{MFS1}^{type IIA}$ or $P_{MFS1}^{type IIB}$ showed significantly elevated terbinafine EC₅₀ values compared to IPO323 (Fig.3A-4). The $P_{MFS1}^{type II}$ reference strain Ref_IIA, carrying a type IIA insert in the IPO323 background, served as the positive control for this group. Most field strains were comparable to Ref_IIA, although several displayed

even higher EC₅₀ values. As with $P_{MFS1}^{type I}$, this demonstrates that $P_{MFS1}^{type IIA}$ and $P_{MFS1}^{type IIB}$ insertions are functionally linked to elevated MDR, although some inter-strain variability was observed.

$P_{MFS1}^{type III}$ was also consistently associated with terbinafine resistance. In the qualitative screen, 90% (19/21) of strains displayed an HR profile (Fig.2B). EC₅₀ measurements revealed RF values ranging from 3 to 7, generally exceeding those of the Ref_III strain, which served as the internal control for this genotype (Fig.3A-5). This variability suggests additional background effects. To test this, we examined the progeny of a sexual cross between two $P_{MFS1}^{type III}$ strains (20-LQ2 with low EC₅₀ and 20-WK1 with high EC₅₀). The distribution of EC₅₀ values among the progeny revealed a continuous, quantitative pattern (Fig.3B), with a broad-sense heritability (H^2) of ~40%. This indicates that, beyond the $P_{MFS1}^{type III}$ insert, other genetic factors contribute to the observed resistance. These results support a polygenic model of MDR even among strains sharing the same insert.

The newly identified P_{MFS1} variants showed variable MDR associations. Among the five strains harboring $P_{MFS1}^{type IIC}$, qualitative resistance profiles ranged from sensitive (S) to HR (Fig.2C). Only one of these strains exhibited a significantly elevated EC₅₀, suggesting an inconsistent link to resistance (Fig. 4A). The $P_{MFS1}^{type IID}$ strain (20-TY14) displayed low resistance in qualitative screening and no significant EC₅₀ increase, indicating no effect of this insert on MDR. In contrast, the single $P_{MFS1}^{type IIE}$ strain (20-UX2) showed high resistance qualitatively and a high EC₅₀, but with large variability across replicates. The $P_{MFS1}^{type IIF}$ variant was observed in two strains with contrasting qualitative resistance profiles (one LR and one HR). However, EC₅₀ measurements showed no significant differences compared to the sensitive control, indicating that $P_{MFS1}^{type IIF}$ is not consistently associated with increased resistance. These results suggest that, unlike canonical $P_{MFS1}^{type II}$ inserts (IIA and IIB), the newly identified $P_{MFS1}^{type II}$ variants may not be sufficient on their own to confer MDR, or that additional epistatic genetic factors modulate its effect.

Strains with $P_{MFS1}^{type VI}$, $P_{MFS1}^{type VII}$, and $P_{MFS1}^{type V}$ insertions also showed little or no association with terbinafine resistance. EC₅₀ measurements for these variants were not significantly different from the sensitive reference strain IPO323 (Fig. 4A). For $P_{MFS1}^{type VI}$, this lack of association was further confirmed by functional validation using a gene replacement approach. $P_{MFS1}^{type VI}$ insert was introduced into IPO323, and the resulting transformants failed to grow on discriminatory concentrations of terbinafine or tolnaftate (Fig.S1). In contrast, one $P_{MFS1}^{type VIII}$ variant (strain 21-UW1) showed a strong MDR phenotype in both qualitative and EC₅₀ assays. The isolate displayed an eightfold increase in terbinafine EC₅₀ relative to IPO323. Genetic validation through sexual crossing between 21-UW1 and the sensitive IPO94269 strain demonstrated complete co-segregation between the $P_{MFS1}^{type VIII}$ genotype and resistance among 79 progeny isolates (Fisher's exact test, $p < 2.2 \times 10^{-16}$), confirming the causative role of this structural variant (Fig.4B). Finally, strains genotyped as $P_{MFS1}^{no-insert}$ displayed a broad range of resistance

profiles, from sensitive to HR (Fig.2B). EC₅₀ analysis identified two $P_{MFS1}^{no-insert}$ strains with significantly elevated resistance levels, including strain 21-VY4, which had an RF of ~3 (Fig.3A-2). Additionally, none of the strains carrying uncharacterized variants detected with whole-genome sequencing showed increased EC₅₀ values (Fig.4A), suggesting these variants do not contribute to resistance under tested conditions. This suggests that resistance in some strains can arise independently of detectable promoter insertions, highlighting the possibility of distal or alternative genetic mechanisms contributing to MDR. Altogether, our phenotyping results show that structural variants at the P_{MFS1} promoter have differential impacts on terbinafine resistance in *Z. tritici*. While canonical insertions like types I, IIA, IIB, and III are strongly associated with MDR, other variants show limited or inconsistent effects, indicating that additional genetic factors should be involved in MDR.

GWAS confirms P_{MFS1} as the main contributor to MDR and supports polygenic determinisms of MDR in the field

To uncover the genetic determinants contributing to MDR in *Z. tritici*, we performed a genome-wide association study (GWAS) on 128 strains of our collection. As phenotype, we used the log-transformed EC₅₀ values for terbinafine resistance, adjusted for experimental variation using a linear mixed model (Fig.S2 and S3; Table S1).

In total, 68 SNPs were significantly associated with variation in terbinafine EC₅₀ values using a 10% false discovery rate (FDR) threshold (Fig.5A, Table S2). The GWAS including all strains (Fig.5A-1 and B-1) revealed 49 significant SNP in total. As expected, the most significant associations clustered at the telomeric end of chromosome 7, between positions chr7_22767 and chr7_36953, encompassing the P_{MFS1} locus. Among these, the top-associated SNP (chr7_36375) is located within the $MFS1$ coding sequence, and the second (chr7_35421) lies 12 bp upstream of the known 401 bp upstream insertion site. Both SNPs passed the Bonferroni-corrected threshold for significance, and likely reflect linkage with structural variant polymorphisms at P_{MFS1} , rather than direct causality. These results confirm P_{MFS1} as a major resistance *locus* in the population. Beyond chromosome 7, several other *loci* were significantly associated with EC₅₀ values at 10% FDR. SNPs on chromosomes 1, 9, and 10 mapped near genes with potential roles in xenobiotic metabolism or transport (Fig.5B-1). For example, 5 significant SNPs on chromosome 1 lie between *ZtIPO323_005000*, encoding a short-chain dehydrogenase/reductase (SDR), and *ZtIPO323_005010*, a predicted sugar transporter. The first one is plausible contributor to MDR, given known roles of SDRs in detoxification (De Rouck et al., 2023; Persson et al., 2009; Xue et al., 2020). Another significant SNP on chromosome 9 is close to *ZtIPO323_099690*, annotated as a homolog of the fluconazole resistance protein FLU1. Finally, we found 4 significantly associated SNPs on chromosome 10, in the 3'UTR of *ZtIPO323_104670*, a gene of unknown function. While promising, these candidates will require functional validation, especially considering genotype imputation was applied during

association testing, and some SNPs (e.g., chr1_1471141) had missing genotypes. The GWAS results indicate that the P_{MFS1} locus explains the high proportion of phenotypic variance (15–21%), while other loci also contribute substantially (14–17%), indicating that terbinafine resistance is a quantitative trait influenced by multiple loci.

To uncover loci associated with MDR independently of P_{MFS1} , we also performed two nested GWAS on subsets of strains carrying either the reference (REF; $n = 68$) or alternate (ALT; $n = 60$) allele at the top P_{MFS1} -linked SNP (chr7_36375). The first one revealed 12 significant SNPs and the second one 7. As expected, no significant SNPs were detected on chromosome 7 in either subset, confirming that the P_{MFS1} signal was effectively masked. Several loci reached the 10% FDR threshold in these nested scans (Fig.5A-2,3, 5B-2,3), supporting the existence of additional MDR-related loci beyond the canonical P_{MFS1} insertions. In the REF-GWAS, we identified 9 SNPs significantly associated (10% FDR threshold) overlapping the coding region of ZtIPO323_064220 encoding a tetratricopeptide repeat (TPR)-like protein and 2 significant SNPs overlapping ZtIPO323_068540, a gene of unknown function (Fig.5A-2, 5B-2). The ALT-GWAS revealed three loci with 2 significant SNPs each, on chromosome 1, and 3, overlapping with ZtIPO323_00630 (nucleotidase-like protein), ZtIPO323_041970 (unknown function) and ZtIPO323_041990 (mannosyltransferase), respectively (Fig.5A-3, 5B-3). These genes, despite no obvious association with non-target site resistance mechanisms may still participate to a global regulation of MDR. Altogether, our GWAS results corroborate our phenotypic analyses, confirming that while the P_{MFS1} locus is a major determinant of MDR in *Z. tritici*, multiple additional loci also contribute to resistance. This strengthens the conclusion that multidrug resistance behaves as a quantitative trait underpinned by a polygenic architecture.

Discussion

In this study, we provide an extensive analysis of structural variation at the *MFS1* promoter in *Z. tritici*, revealing the complex spectrum of MDR genetic basis. By combining high-resolution phenotyping with genetic crossing and genome-wide association analyses across a large, recently sampled European population, we demonstrate that MDR is not only driven by major-effect transposable elements insertions (e.g., P_{MFS1} types I, IIA, IIB, and III), but is also shaped by a polygenic background. Our quantitative phenotyping confirms that even strains with identical P_{MFS1} genotypes can differ markedly in resistance levels, and our GWAS further supports this view by identifying additional loci that may contribute to MDR. Together, our findings revise the current understanding of resistance evolution in *Z. tritici* by highlighting the interplay between transposon-driven structural changes and polygenic architecture in shaping MDR phenotypes in field populations.

The promoter region of *MFS1* is subject to diverse and recurrent transposon-mediated structural variation, in particular in this MDR targeted collection. We confirmed that nearly all the inserts described

in previous studies (Mäe et al., 2020; Omrane et al., 2017, 2015) as well as the newly identified variants in the present work, exhibit derive from various TEs, including LTR/copia retrotransposon (type I), LINES (type II), and MITEs (type VI and VII). These insertions are not randomly scattered across the promoter region but are concentrated within a narrow locus, often at shared or nearby insertion sites. Despite sharing insertion sites, our phylogenetic analysis of flanking promoter regions suggests that most of the insertions arose independently. For instance, although all type II variants occupy the same insertion site 471 bp upstream of the *MFS1* start codon, they potentially derive from multiple, independent insertion events and implies ongoing TE activity in the P_{MFS1} region. These findings are consistent with recent work showing reduced TE silencing and active TE transcription in European *Z. tritici* populations (Abraham et al., 2024; Feurtey et al., 2023; Möller et al., 2021; Oggenfuss and Croll, 2023), particularly in subtelomeric and gene-rich regions such as the *MFS1* locus. Furthermore, we observed strong sequence conservation in the promoter sequences flanking insertions associated with MDR, specifically types I, IIA, IIB, and III, which suggests that these TE insertion polymorphisms (TIPs) may be under positive selection. Although the evolutionary forces shaping MDR-associated TIP remain unclear, TE insertions are known to contribute to standing genetic variation and may facilitate rapid adaptation under strong selection (Baril et al., 2025). Environmental stresses were shown to trigger TE de-repression (Fouché et al., 2020), and fungicide exposure may similarly act as both a selective pressure and as *stimulus* for TE mobilization, as proposed by (Baril and Croll, 2025). These findings underscore the dynamic interplay between TE activity, genome architecture, and adaptation in shaping the evolution of resistance in fungal pathogen populations.

Among newly identified inserts in P_{MFS1} , only the indel VIII was validated as conferring MDR. Although detected in a single isolate, this new P_{MFS1} allele is expected to increase in frequency due to its adaptive advantage under fungicide selection. It represents a novel MDR marker that should be monitored in field surveys. However, the current genotyping method (Omrane et al., 2017) may not be sufficiently general to detect new P_{MFS1} alleles associated with MDR. As illustrated by the polymorphism found in both proximal and distal *MFS1* promoter regions, designing PCR primers in conserved regions is highly challenging for large-scale genotyping. A promising alternative would be to amplify the entire region using primers hybridizing within the coding sequences of both flanking genes, followed by amplicon-based nanopore sequencing (R4P Network, 2016).

Other newly identified TIPs were not associated with increased terbinafine resistance under our experimental conditions. The discrepancy, given that isolates were initially selected by germination on terbinafine-supplemented media, could stem from differential resistance expression across developmental stages, morphotypes, or fungicide availability in the medium. These hypotheses may also explain inconsistencies between qualitative resistance scores obtained on solid media and EC_{50} measurements in liquid culture. The latter, based on optical density, is strongly influenced by

morphological transitions towards filamentous growth. In their study, (Puccetti et al., 2025b, 2025a) found distinct genetic *loci* associated with fungicide resistance depending on the phenotyping method, highlighting the challenge of selecting the ecologically most relevant assay to describe resistance dynamics *in natura*. In addition, germination of apparently sensitive strains on terbinafine-supplemented media might reflect fungicide tolerance, an underrated concept in phytopathology despite major clinical implications (Berman and Krysan, 2020; Levin-Reisman et al., 2017).

Our results show that while the P_{MFS1} insertion polymorphism is the major determinant of multidrug resistance (MDR) in *Z. tritici*, it is not the sole driver. We observed considerable variation in terbinafine resistance among strains carrying the same P_{MFS1} genotype and in progeny from crosses between such strains, indicating that MDR behaves as a quantitative trait shaped by additional genetic factors, which was confirmed by our GWAS analyses that identified candidate *loci* beyond P_{MFS1} . These candidates include genes potentially involved in xenobiotic detoxification and membrane transport, consistent with resistance such as a short-chain dehydrogenase/reductase (SDR) and a homolog of the FLU1 transporter known to mediate MDR in *C. albicans* (Calabrese et al., 2000). Such polygenic architectures have been observed in other fungi such as *Botrytis cinerea* (Kretschmer et al., 2009; Leroux and Walker, 2013) and *Candida glabrata* (Huang et al., 2025). These findings are in alignment with those made by Puccetti and colleagues (Puccetti et al., 2025a) about the complex genetic nature of azole resistance in *Z. tritici*. The polygenic basis of fungicide resistance raises important implications for the evolution and management of resistance in pathogen populations.

Even if individual genetic effects are small, cumulative resistance increases remain a major threat to treatment durability. This is especially critical for MDR, which compromises both current and future active ingredients, including biocontrol compounds. Our results highlight the urgent need for improved MDR prevention strategies. Indeed, while current recommendations promote the diversification of selection pressure through fungicide mixtures or alternations to delay target-site resistance (Rex Consortium, 2013), such approaches may paradoxically create selective conditions that promote multidrug resistance instead (Ballu et al., 2024, 2023, 2021; Barbosa et al., 2021; Comont et al., 2020; Lagator et al., 2013; Zhou et al., 2022, Neau et al., *in prep*).

Experimental procedures

Biological material

- *Z. tritici* 2020-2021 field strain collection

French field strains used in this study were obtained from the "Performance Network" during the years 2020 and 2021. Both French and British strains in our panel were isolated from infected wheat leaves and suspended in water following the protocol described by Garnault *et al.*, (2019). The spore

suspensions were plated on PDA media supplemented with 0.015 µg/mL discriminatory concentration of terbinafine (Leroux and Walker, 2011) and incubated for 24 hours. After incubation, colonies with elongated germ tubes were picked using a sterile toothpick and isolated three to five times on PDA media supplemented with antibiotics (Kanamycin, Streptomycin, and Penicillin (Sigma-Aldrich) at 37.5 µg/mL). Strains from Belgium, Denmark, Estonia, Germany, Ireland, and Lithuania were provided by collaborators as pure strains.

- *Progeny from sexual crosses*

Parental strains for crosses 21-UW1 x IPO94269 and 20-LQ2 x 20-WK1 were preliminary checked for mating type compatibility as described by (Waalwijk et al., 2002). Then, crosses were performed following the *in planta* procedure developed by Kema and collaborators (Kema et al., 1996) with adaptations described by Orellana-Torrejon and collaborators (Orellana-Torrejon et al., 2022). For inoculation, performed on 12/06/2023, blastospores of parental strains were harvested from 5-days solid precultures, resuspended in sterile water to a final volume of 20 mL and a concentration of 2.10^5 spores.mL⁻¹ (adjusted with hemacytometer). The two suspensions of parental strains were mixed in a 1:1 ratio, with final concentration of each strain reaching 1.10^5 spores.mL⁻¹ and a final volume of 40 mL, supplemented with two drops of surfactant (Tween 20; Sigma, France). These suspensions were sprayed with an atomiser (Ecospray, VWR, France) onto three adult plants (nine stems) of wheat Apache cultivar. We promoted infection by enclosing plants immediately after inoculation for 72 h in a transparent polyethylene bag previously sprayed with distilled water. Plants were maintained greenhouse environment for about three months, until complete drying (June to August 2023). Then, they were placed outdoors in August 2023 in Grignon (France, 73200) for five months in order to promote ascosporeogenesis. In December 2023, the residues of leaves and stems from plants of each cross were cut into 2 cm pieces dried at room conditions for one week. Progeny was harvested through experimental set up for ascospore ejection and catching. For this purpose, residues were soaked in water for 30 min and spread on dry filter paper in a crystal-clear polystyrene box (24 × 36 cm) leaving the lid half-open. Eight Petri dishes (90 mm in diameter) containing PDA medium were placed opened upside down 1 cm above the residues, for receiving the ascospores ejected from asci during residues drying process. Boxes were left at room conditions for 18 h before closing Petri dishes and incubating them under usual culture conditions. Between three to five days post-ejection, we regularly picked ascospore-derived colonies using sterile toothpicks and purified them through two successive single spore subcultures on PDA solid medium before long term storage. We harvested 76 individual progenies from 21-UW1 x IPO94269 and 151 from 20-LQ2 x 20-WK1 crosses, lately genotyped for their *P_{MES1}* insertion polymorphism as described hereafter.

All field strains, progenies and reference strains used in this study are listed in Table S1.

General growth conditions

Z. tritici strains were preserved at -80°C in 25 % glycerol suspensions and precultured on solid YPD medium (20 g.L^{-1} dextrose, 20 g.L^{-1} peptone, 10 g.L^{-1} yeast extract, 10 g.L^{-1} agar) in the dark, 18°C , 60% humidity. For resistance phenotyping, strains were grown in 10 mL liquid YPD medium in 50 mL sterilized Erlenmeyer flasks plugged with cotton wool for 7 days at 150 rpm. Cell concentrations were determined by OD measurements at $\lambda=405$ nm in a Spectramax M2 microtiterplate reader (Molecular Devices, USA) according to the formula established by Ballu (Ballu, 2021).

Fungicide preparation

Terbinafine (Sandoz SA, BALE/Switzerland) was solubilized either in DMSO or 80% ethanol (v/v). 4000 $\mu\text{g.mL}^{-1}$ concentrated solution were prepared and stored at 4°C . For fungicide supplemented media preparation, serial dilutions in solvent were performed and concentration adjusted for the amount of solvent introduced to not exceed 0.5% of the final volume, preventing solvent-induced toxicity (Ballu et al., 2021, 2022, 2023).

Qualitative resistance assay to fungicides (droplet growth test)

The qualitative assessment of terbinafine resistance as proxy for the MDR phenotype was performed using a droplet test according to the following conditions described by Ballu *et al.* (2021). To this end, serial water dilutions of spores of 10^7 , 10^6 , 10^5 , and 10^4 spores. mL^{-1} were deposited as approximately 5 μL drops on 12 cm square Petri dishes containing solid YPD medium supplemented with antibiotics (Kanamycin, Streptomycin, and Penicillin (Sigma-Aldrich) at 37.5 $\mu\text{g.mL}^{-1}$), fungicide (terbinafine 0.03 $\mu\text{g.mL}^{-1}$) or solvent alone (see fungicide preparation). Fungal growth was scored after 7 days of culture. Each strain was scored with a number representing growth intensity, ranging from 0 (no growth) to 4 (growth at the lowest spore concentration). According to this score, strains were categorized as highly resistant (3 to 4, HR), moderately resistant (2, MR), low resistant (1, LR), or sensitive (0, S). Specifically for terbinafine resistance screening in field strain collection and 21-UW1 x IPO94269 progeny, Fisher's exact test was used to assess the association between indel VIII and terbinafine resistance (sensitive versus resistant, with low, moderate, and high resistance grouped together) among the 21 UW1 x IPO94269 progenies (Table S3).

Quantitative terbinafine resistance assessment (EC_{50} determination through dose response curve)

We performed quantitative terbinafine resistance assessment for a 126-strain subset of the 2020-2021 strain collection and 151 progenies from 20-LQ2 x 20-WK1 cross (Table S4). To the first set we added four $P_{MFS1}^{\text{no-insert}}$ field strains obtained through the Performance network between 2012 and 2013 (Table S1, Garnault et al. 2019), sensitive to terbinafine (not germinating on PDA supplemented

with 0.015 $\mu\text{g}\cdot\text{mL}^{-1}$) for establishing the basal field terbinafine EC_{50} .

We computed terbinafine EC_{50} , corresponding to the concentration inducing its half-maximum inhibitory effect on fungal growth. For doing so, spore concentration of 7-days liquid precultures were adjusted to 1.25×10^5 spores. mL^{-1} in 96-well microplates (Sarstedt) in a final volume of 200 μL of YPD medium supplemented with antibiotics (37.5 $\mu\text{g}\cdot\text{mL}^{-1}$ Kanamycin and Streptomycin (Sigma-Aldrich)) and terbinafine at a range of different concentrations or solvent alone (control). Different terbinafine ranges were tested for high resistance (HR) and low resistance (LR) strains (Table S5). A breathable film (Breathe-easy®; Diversified Biotech) was applied on microplates in order to limit evaporation and contaminations before 7-day incubation at 18°C and 150 rpm in a microplate shaker (Innova S44i, Eppendorf). Each strain was tested in three technical replicates (three wells per fungicide condition in the same plate) and at least 2 independent biological replicates (independent days). As internal control, we systematically add IPO323 reference strain as negative control to every biological replicate. As positive controls for the 2020-2021 MDR collection we tested as well IPO323 isogenic transformants carrying either the $P_{MFS1}^{\text{type I}}$ allele, $P_{MFS1}^{\text{type II}}$ allele, or $P_{MFS1}^{\text{type III}}$ allele integrated at the *MFS1 locus* of IPO323 (Omrane et al., 2017), renamed Ref-I, Ref-II, and Ref-III, respectively (Table S1). Among biological replicates including progenies from 20-LQ2 x 20-WK1 cross, we also systematically tested both parental strains.

After 7 days of growth, spore concentrations were determined from OD405 nm values (Ballu et al., 2021) and expressed as percent of growth of the control condition (solvent alone). EC_{50} were computed from dose-response curves fitted to each technical replicate, using the R software (R Core Team, 2025) and drc package. After filtering data for quality, several dose-response models (LL.5, LL.4, LL.3, LL.2, W2.2, and W1.2) were fitted and the model with the lowest Bayesian Information Criterion (BIC) value was selected. Log-transformed EC_{50} mean per strain and biological replicate (mean of technical replicates) were modeled using a linear mixed-effects model (lmer function from lme4 package (Bates et al., 2015), with strain as a fixed effect and biological replicate (experiment date) as random one. Model assumptions were verified using Levene tests for homoscedasticity and residual inspection, QQ-plot, and Kolmogorov-Smirnov test on standardized residuals for normality assumption (Fig.S2). We calculated estimated marginal means (emmeans package (Lenth, 2025)) for each strain, and performed statistical comparisons with contrast function. P-values were adjusted for multiple testing using the Bonferroni correction for each set of comparisons and are presented in Table S1. Raw data for both MDR field strains and 20-LQ2 x 20-WK1 progeny are available (Tables S6 and S7).

Broad-sense heritability (H^2) of log-transformed terbinafine EC_{50} among progenies of the 20-LQ2 x 20-WK1 cross was estimated fitting a linear mixed model including Strain and Date as random effects as: $\text{Log}(\text{EC}_{50})_{ijk} = \mu + S_i + D_j + \varepsilon_{ikj}$ with μ the overall mean (the intercept), S_i the random effect of strain, D_j the random effect of experimental date and ε the residual error. H^2 was calculated as the

proportion of total phenotypic variance explained by the strain effect as $H^2 = \frac{\sigma_S^2}{\sigma_S^2 + \sigma_D^2 + \sigma_\varepsilon^2}$.

***P_{MFS1}* PCR-based genotyping**

For DNA extraction, 50 – 100 mg of fresh spores were harvested per strain from 7-day old cultures on YPD agar media, placed in a microtiter plate and immediately frozen at -80°C. Cell lysis was performed mechanically twice in AP1 buffer supplemented in Rnase A and reagent DX (Qiagen) using tungsten beads in MIXER MILL MM 400 homogenizer (RETSCH®) at 20 Hz for 20 seconds. DNA was precipitated with sodium acetate pH 5 at 0.5 M final concentration and the addition of 0.7 vol of isopropanol. After centrifugation, dry DNA was resuspended in AE buffer (Qiagen). Genotyping of the *MFS1* promotor was performed on all 398 strains by PCR using the primer pair *P_{MFS1}_2F* and *P_{MFS1}_4R* and PCR condition described by (Omrane et al., 2017). All amplicon displaying length compatible with *P_{MFS1}^{type II}* and *P_{MFS1}^{type III}* alleles were sequenced with the same primers using Sanger protocol (Eurofins Genomics, Köln, Germany). Among others, 21 isolates with *P_{MFS1}^{type I}* and 16 isolates with *P_{MFS1}^{no insert}* length compatible amplification were also sequenced. Facing amplification failures for some strains (Table S1) we designed another pair of primers, ZtIPO323_079130-3F and ZtIPO323_079140-bR in coding sequences of both *MFS1* (ZtIPO323_079130) gene and upstream gene ZtIPO323_079130 (Table S8). We performed this last PCR using Taq polymerase Phusion® (Thermo Fisher Scientific Inc., Waltham, MA, USA) and adapted PCR conditions (Table S8) before sequencing with the same primers using Sanger protocol (Eurofins Genomics, Köln, Germany).

Whole genome sequencing and variant calling

Genomic DNA was isolated from approximately 100 mg fresh fungal material collected from 7-day-old cultures grown on YPD agar (one 60 mm diameter Petri dish) for each of the 132 sequenced isolates (Table S1). DNA extraction was carried out using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. DNA integrity was verified by agarose gel electrophoresis, and its concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Extracted DNA was stored at -20 °C until sequencing.

Paired-end Illumina sequencing (150 bp, 25× coverage) was performed by BMKgene (Biomarker Technologies GmbH, Germany). Adapter sequences and low-quality regions were removed from raw reads with Trimmomatic v0.39 (Bolger et al., 2014), applying leading and sliding-window trimming at Q ≥ 28 and discarding reads shorter than 50 bp. The cleaned paired reads were aligned to the *Zyloseptoria tritici* IPO323 reference genome (Goodwin et al., 2011) using BWA-MEM v0.7.17 (Li and Durbin, 2009) and resulting SAM files were converted to sorted and indexed BAM files with samtools v1.20 (Danecek et al., 2021). PCR duplicates were identified and removed using Picard v2.21.6 (Broad Institute, 2019).

Variant discovery was performed for each sample using GATK v4.4.0.0 HaplotypeCaller in GVCF mode (ploidy = 1) (Poplin et al., 2017), and individual GVCFs were combined into a single dataset. Variants were filtered with GATK VariantFiltration using the following thresholds: FS > 10, MQ < 20, QD < 20, and DP < 3. Additional filters excluded variants with ReadPosRankSum, MQRankSum, or BaseQRankSum values outside [-2, 2], to remove potential biases in mapping position, base quality, or alignment. Functional annotation of variants was performed using SnpEff v5.1 (Cingolani et al., 2012) with a custom database built for *Z. tritici* IPO323. For downstream analyses, the variant effect with the highest predicted impact (according to the SNPEFF effect score) was extracted using a custom Python script. Variant positions were then intersected with gene annotations from (Lapalu et al., 2025).

Genome Wide Association analysis and population structure

Prior to GWAS analysis, per-sample sequencing quality metrics were computed using VCFtools (v0.1.17) (Danecek et al., 2011) to estimate both the proportion of missing genotypes per individual and the mean sequencing depth across core chromosomes. Isolates were filtered based on missing data and coverage thresholds: individuals showing $\geq 20\%$ missing genotypes or an average sequencing depth $\leq 6X$ were excluded from downstream GWAS analyses (Fig S2). Relatedness between isolated was assessed using a square identity-by-state matrix computed with PLINK (Chang et al., 2015) to identify clonal strains (Fig.S4).

As tested phenotype, we input emmeans of $\text{Log}(EC_{50})$ extracted from mixed linear model described in previous sections fitted on a set of strains excluding ones with >40% deviation between predicted and observed values (Fig.S2, 20-UX2 and 20-UX5). The final set of 128 strains tested in GWAS and associated emmeans is presented in Table S1.

Association tests were performed with GEMMA (v0.98.3; (Zhou and Stephens, 2012)) included in vcf2GWAS wrapper (Vogt et al., 2022). Genotypes were provided as a vcf input filtered as described before, including all chromosomes. A linear mixed model (LMM) was fitted, adding the first three principal components as fixed-effect covariates to account for population structure, setting parameters --cfile PCA and --covar 3 in vcf2gwas function. A kinship matrix, calculated from the genotype matrix by vcf2gwas, was automatically included as a random effect in the LMM to correct for relatedness among isolates. 758,800 biallelic SNPs with a minor allele frequency ≥ 0.05 were retained through vcf2GWAS per-preprocess as input for GEMMA. Nested GWAS were performed on two subsets of strains carrying either the reference or alternative allele at the most significant SNP from the initial GWAS (68 and 60 strains, respectively), with 774,740 and 756,655 SNPs retained for analysis. SNPs with p-value lower than FDR < 10 % were considered candidate variants (Table S2). Percent variance explained by each SNP was computed as in (Kumar et al., 2021) (Table S2).

We reproduced principal component analysis (PCA) upon 14806 retained biallelic SNPs (100 %

call rate, minor allele frequency ≥ 0.05 , thinned to one variant per 1 kb, Linkage disequilibrium-pruned markers ($r^2 < 0.2$)), either including all strains or after retaining a single representative per clonal group. The first two principal components were visualized in R and provided in Fig.S4.

Sequence analysis

For sequence analysis of proximal and distal P_{MFS1} promoter, we combined sequences obtained by Sanger sequencing of PCR amplicons, and sequences obtained from WGS illumina sequences reassembled genomes (described in previous P_{MFS1} genotyping and Whole genome sequencing sections). Individual genome assemblies were generated using SPAdes v3.15.5 (Bankevich et al., 2012) from paired-end reads (R1 and R2) for each isolate. Sequences were designed as hooks (conserved sequences in all isolates) in CDS of ZtIPO323_079130 (5' hook, Chr7_33925-33950 "GAGAGGAGAATTCGCTGAGGAGGGAT") and $MFS1$ (3' hook, Chr7_37216-37259 "CAACCTGGCGCTCCAAACTCCAACAATTCGACATCTTCGGCAC") and used to locate and extract P_{MFS1} from each assembly (10 kb downstream to 5' and upstream of 3' hooks) using seqkit (Shen et al., 2016) and SAMtools (Danecek et al., 2021). Sequences extracted with both hooks were aligned with Kalign algorithm (Lassmann, 2020) and manually curated for obtaining full locus. Because repetitive sequences of inserts can compromise assembly from short reads, Sanger sequences were integrated to confirm and complete P_{MFS1} sequences for defining a consensus sequence per isolate. When insertion polymorphisms were detected only in the reassembled genome upstream of the Sanger-genotyped region, they were retained but flagged as potentially unreliable. Only their insertion sites within the promoter were considered informative. Multiple sequence alignments (MSAs) of all P_{MFS1} sequences were generated with MUSCLE (Edgar, 2004) and manually adjusted (see data availability).

For investigation of insertion origin, known and novel insert sequences were blasted against the annotated IPO323 reference genome annotated for transposable elements (Baril and Croll, 2023). Conservation of the insert sequences among recent field isolates was assessed *via* blastn against *Z. tritici* NCBI experimental databases.

MSA of P_{MFS1} was transformed into VCF format with custom Python scripts, capturing variant positions and indels. Haplotypes were defined as sequences with distinct combinations of indels >100 bp, and these haplotypes were used to generate synteny maps with gggenome in R (Hackl et al., 2024). The P_{MFS1} MSA filtered for positions present in all isolates (2074 positions) was used for phylogenetic analysis of conserved regions. Consensus tree was inferred with IQ-TREE v2.2.2.6 using the HKY+F+I+R2 model (Minh et al., 2020). For whole-genome phylogeny, MSA were generated from previously described whole-genome VCFs. Variants were filtered to retain biallelic SNPs with minor allele frequency >5%, thinned to one SNP per 1 kb, and LD pruning was performed with a 50-SNP sliding window moving 5 SNPs at a time at $r^2 > 0.2$ using PLINK. Remaining 14,833 variants were converted to PHYLIP format with

vcf2phylip.py (Ortiz, 2019) and used to build consensus trees in IQ-TREE (GTR+F+ASC+R5 model). For both analysis, branch support was assessed with 1,000 ultrafast bootstrap replicates, and trees were visualized with iTOL v7 (Letunic and Bork, 2024). Nucleotide diversity (π) was computed with pegas in R (Paradis, 2010) from both P_{MFS1} MSA and the whole-genome SNP set, among strains sharing the same P_{MFS1} insertion polymorphism haplotype using only one representative per clonal group.

P_{MFS1}^{typeVI} gene replacement strategy

To introduce the P_{MFS1}^{typeVI} allele into the sensitive IPO323 strain, a replacement cassette was built using the same strategy described in Omrane *et al.* (2017) with minor changes. 5' promotor and 3' terminator sequence of *MFS1* in 20-TY3 *Z. tritici* strain were obtain by two PCR with primer seq_prom_for/seq_prom_rev and seq_term_for/seq_term_rev (Table SX) and sanger sequencing (Eurofins, Luxembourg) with same primers. For all PCR performed to obtain cloning fragments, the Taq polymerase Phusion® (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used under adapted PCR conditions using primers referenced in Table S8. Region ranging from 1,273 bp upstream of *MFS1* until 510 bp downstream of the open reading frame (ORF), was amplified from 20-TY3 strain genomic DNA with the primer pair MDR6-7_pKr_F/ Gibs_MDRIV_R. A 737-bp 3' flank of the *MFS1* gene to facilitate homologous recombination was amplified from IPO323 genomic DNA with primers Ipo323_hyg_F and Ipo323_pKr_R. Finally, the hygromycin resistance marker gene *hph* was amplified from plasmid pCAMB-HPT-Hind (Kramer *et al.*, 2009) with the primer pair Gibs_Hygro_for/Hygro_ipo323_R. The three fragments (0.05 pmol each) were assembled with XhoI-EcoRI-digested pCAMB-HPT-Hind (0.05 pmol) using the Gibson Assembly Cloning kit (New England Biolabs, Ipswich, MA, USA) according to the supplier's instructions. NEB 5-alpha competent *E. coli* (High Efficiency) (New England Biolabs, Ipswich, MA, USA) were transformed by heat shock with 2 μ l of the assembly reaction mixture. Successfully transformed colonies were selected on medium added with kanamycin (50 mg.L⁻¹) then validated by PCR on colonies with 3 pairs of primers pCAMBIA_rev/*MFS1*_rev, *MFS1*_for/pHygro_rev and pHygro_for/pCAMBIA_for. Positive clones were picked for plasmid extractions and mini-prepped plasmid construct was validated by Sanger sequencing (Eurofins, Luxembourg) with primers listed in Table S8.

Agrobacterium tumefaciens strain AGL1 was transformed by heat shock with the generated plasmid pCAMBIA0380_RH_ P_{MFS1} _V_*MFS1*_hygro (map in supplementary data X). Positive colonies were selected on YEB broth (beef extract 5 g.L⁻¹, yeast extract 1 g.L⁻¹, peptone 5 g.L⁻¹, saccharose 5 g.L⁻¹, MgCl₂ 0,5 g.L⁻¹) with rifampicin (50 mg.L⁻¹), kanamycin (50 mg.L⁻¹), and ampicillin (100 mg.L⁻¹) then screened by same colonies PCR as on *E.coli*.

IPO323 reference strain was transformed with this construction *via A. tumefaciens*-mediated transformation (ATMT) following a procedure adapted from Bowler *et al.* (2010) with modifications described in (Marchegiani *et al.*, 2015). *A. tumefaciens* strains carrying the

pCAMBIA0380_RH_*P_{MFS1}*_V_*MFS1*_hygro plasmid were grown overnight at 28°C, 200 rpm in liquid YEB medium with antibiotics as described before. Cells were diluted in induction medium (IM, recipe table S9) containing 50 µg.mL⁻¹ kanamycin and 40 µg.mL⁻¹ acetosyringone, to a final OD_{660nm} of 0.4 and mixed v/v with 10⁷ spores.mL⁻¹ IM suspension of IPO323 blastospores harvested from 7-day solid YPD cultures. Mixtures were plated onto IM-agar plates covered with cellophane membranes (Cellophane Membrane Backing 165-0963, Biorad) and incubated for 48h at 18 °C before transferred onto MM-Zt plates supplemented with 250 µg.mL⁻¹ cefotaxime (Kalys SA) and 10 µg.mL⁻¹ Chlorimuron-ethyl (Sulfonylurea, Sigma) for 15 to 20 days at 18 °C. transformant colonies were picked and spread onto YDP-agar unselective medium before purification by single colony propagation twice. Successful allele replacement at the locus was confirmed by colony PCR (primers listed in Supplementary Table X).

Data availability

Supplementary figures are provided in this paper following core figures. Supplementary tables as well as maps of plasmids used in this study and multiple sequence alignments are publicly available on:

<https://doi.org/10.5281/zenodo.17982864>

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Figures

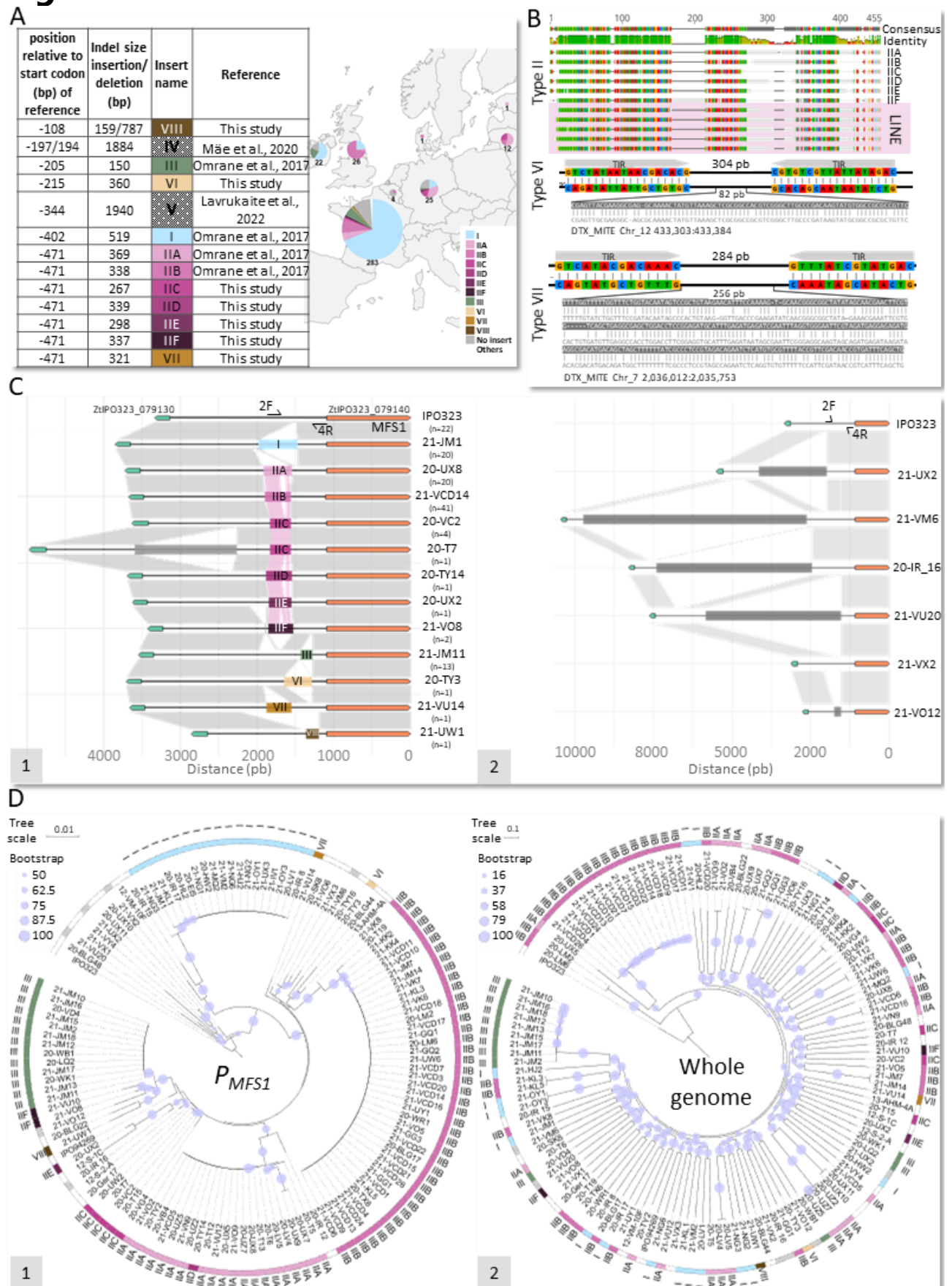


Fig.1: *MFS1* promoter (P_{MFS1}) of *Zyoseptoria tritici* European field strains shows an extended transposon

insertion polymorphism. A. Summary of inserts detected by PCR amplicon sequencing of the proximal P_{MFS1} and geographic origin of detected polymorphisms from 2020-2021 survey. Inserts are listed according to their localization relative to the start codon of *MFS1* in the IPO323 reference genome. Insert numbering is according to chronological discovery. Frequencies shown for French isolates reflect proportions of each P_{MFS1} genotype in multidrug-resistant (MDR) field populations (isolated on terbinafine and tolnaftate prior to genotyping). For other European countries, strains were provided by partners because they displayed particular P_{MFS1} promoter inserts. Inserts indicated with a dotted pattern were reported in previous studies but not detected in this survey. "Others" refers to genotypes not characterized by PCR amplicon sequencing due to amplification failure. **B. New P_{MFS1} inserts found in *Z. tritici* field isolates contain transposable element signatures.** Type II insert variants were aligned with the end of LINE retrotransposons of the IPO323 genome (pink highlighting). Sequence identity is shown by colored bases. Type VI and VII inserts contain terminal inverted repeats (TIRs, grey boxes) and internal sequences aligned to known MITEs from IPO323 as indicated by colored bases. **C. P_{MFS1} sequence reassembly from whole genome sequencing reads reveals insertion polymorphism upstream of the routinely genotyped proximal promoter by amplification with 2F/4R primer pair.** A subset of 130 isolates from the 2020–2021 MDR survey were sequenced (Illumina, 25× coverage, 150 bp reads). P_{MFS1} sequences were extracted from genome assemblies, aligned with MUSCLE, and manually curated. Haplotypes (based on indels >100 bp) are shown with a representative strain's name and the number of strains sharing that haplotype (n). 2F/4R primer hybridization sites (Omrane et al., 2015) on the IPO323 reference are indicated. C1: haplotypes with insertions detected within the proximal P_{MFS1} via 2F/4R PCR amplification. C2: haplotypes harboring insertions (dark grey boxes) not captured by 2F/4R PCR genotyping. Lengths of uncharacterized indels are illustrative, as genome reassembly is unreliable for repetitive elements. Conserved sequences relative to IPO323 are shown as grey polygons, shared sequences among type II inserts are shown in pink. **D. Phylogenetic analysis indicate local conservation of P_{MFS1} .** Maximum likelihood phylogenies were inferred with IQ-TREE from aligned P_{MFS1} 2,074 positions shared by all strains (D1) or synthetic sequences made of variants distributed evenly across the genome. Node supports were assessed with 1000 bootstrap replicates (percentages shown). Colors indicate insert types, consistent with panel A. Branch lengths are proportional to the expected number of substitutions per site, indicated by the tree scale.

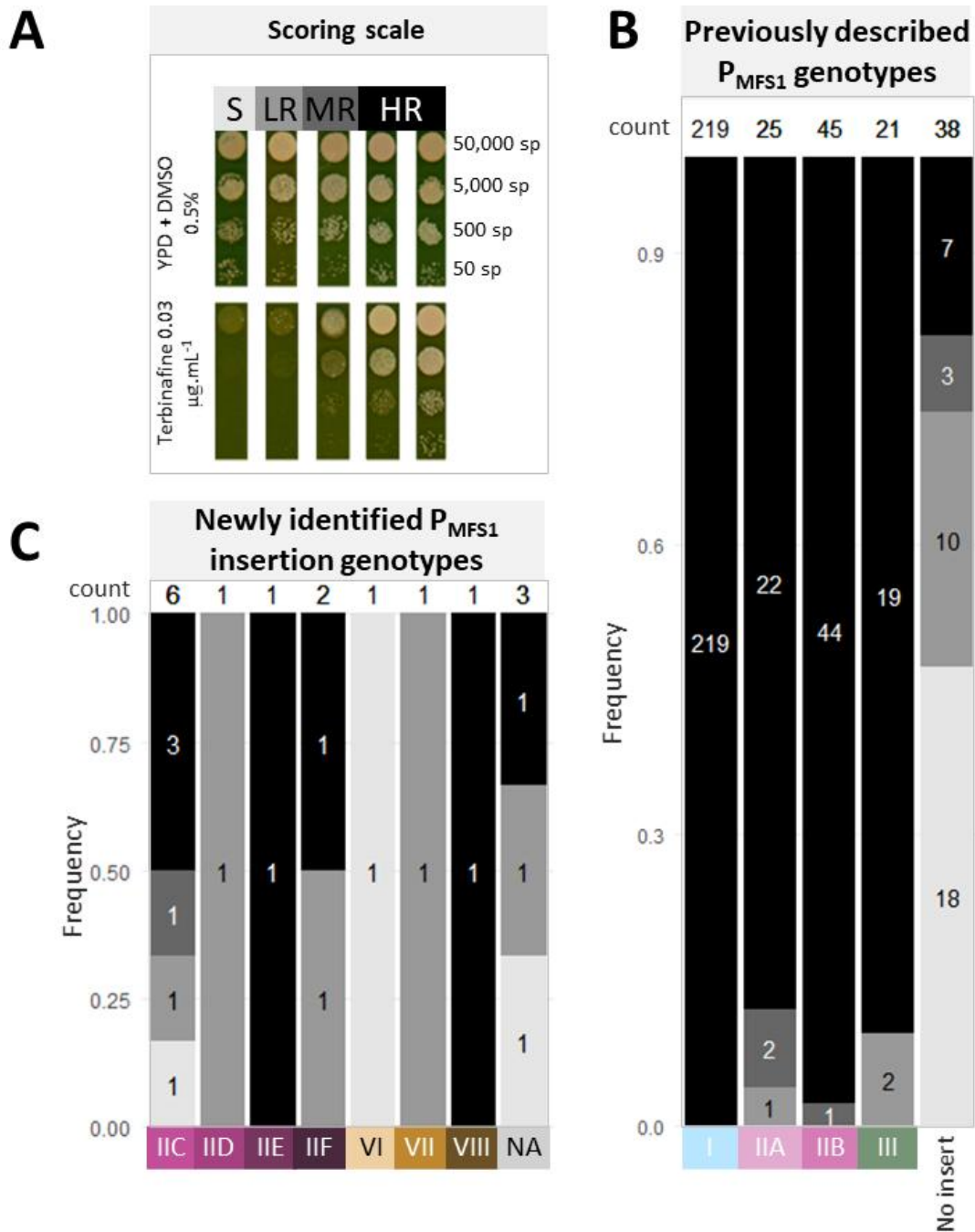


Fig.2: Qualitative terbinafine resistance assessment reveals intra and inter P_{MFS1} genotype phenotype variations. A total of 374 strains selected on terbinafine or tolnaftate from the 2020–2021 collection was genotyped for insertions in the 500-pb proximal P_{MFS1} sequence *via* PCR amplicon sequencing. The strains were also screened for terbinafine resistance through growth tests on YPD solid medium supplemented with discriminatory terbinafine dose (scoring scale in A). Strains are grouped by insertions: **(B) previously associated with MDR (I, IIA, IIB, IIC)** and **(C) newly identified insertions (IID, IIE, IIF, VI, VII, VIII) as well as NA for uncharacterized genotypes** due to PCR amplification failure. Resistance classes: HR = high resistance, MR = medium resistance, LR = low resistance, S = sensitive.

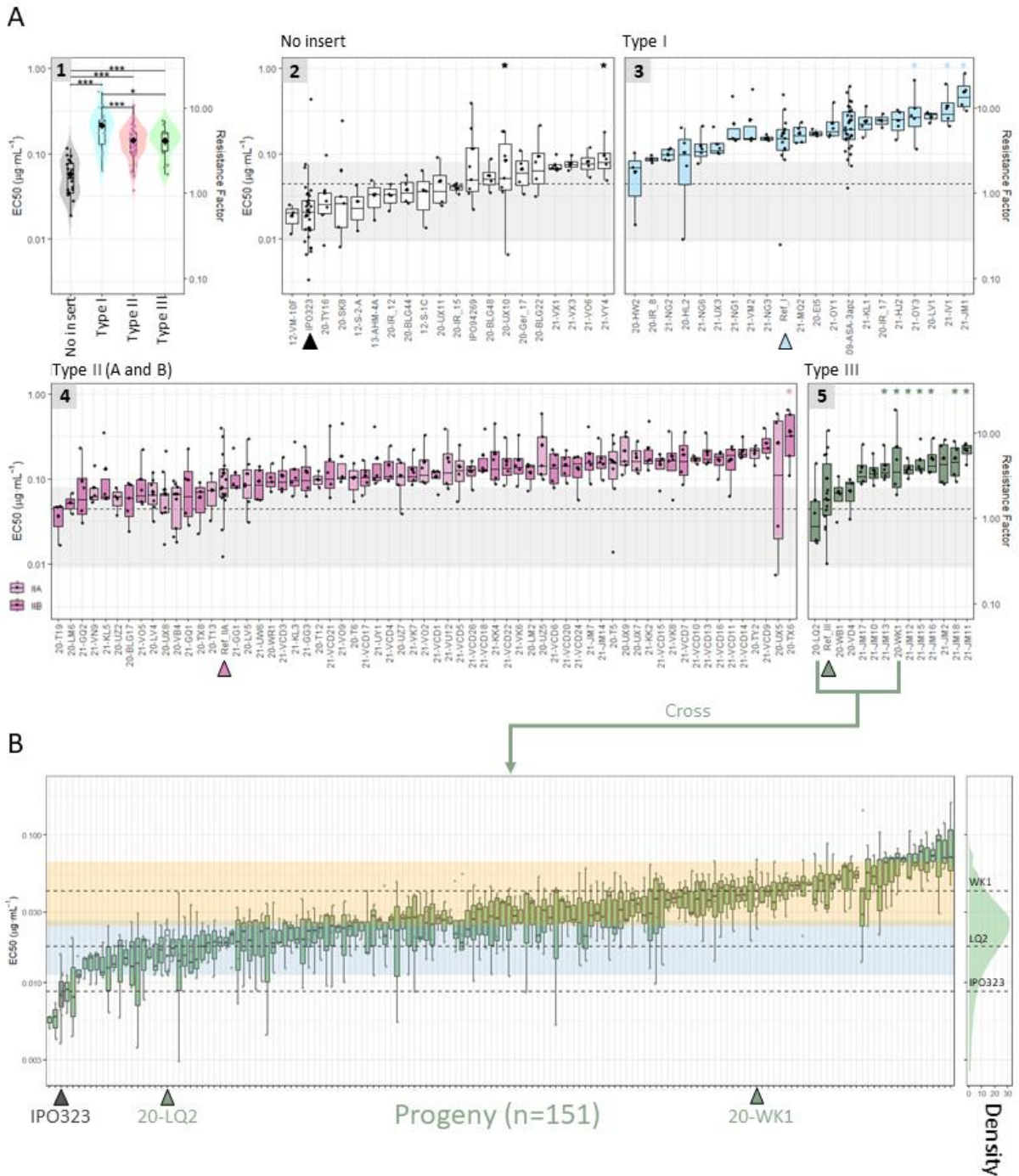


Fig.3. Distribution of terbinafine resistance demonstrate the quantitative nature of MDR. A. Variation of terbinafine EC_{50} among MDR field strains. (1) Violin plot showing the effect of P_{MFS1} genotype on terbinafine EC_{50} (EC_{50} distributions per genotype). Each violin represents the distribution of EC_{50} values for strains grouped by P_{MFS1} genotype. Each point corresponds to the mean EC_{50} of a strain (biological replicates ($n \geq 2$)), and the larger points represents the mean of the P_{MFS1} group. Genotype effects were assessed using a linear mixed-effects model on log-transformed EC_{50} , including biological variation and strain as random effects. Estimated marginal means were compared with contrast post-hoc tests and Bonferroni correction (* $p < 0.05$; * $p < 0.001$). (2-5) Boxplots of terbinafine EC_{50} per strain grouped by genotypes, showing intra-genotype variability independent of P_{MFS1} effect, including experimental variation. The experimental variability of biological replicates ($n \geq 2$) is showed by individual points and error bars (standard deviation of biological replicates). Diamond-shaped points indicate the mean EC_{50} per strain. Each groups display a reference standard, highlighted by colored arrows, carrying the corresponding P_{MFS1} genotype, i.e., Ref_I, Ref_II and Ref_III are IPO323 transformants with *in locus* type I, IIA, or III insertions. Statistical differences with the corresponding reference strain were assessed using a linear mixed-**

effects model with post-hoc contrasts and Bonferroni correction (* $p < 0.05$). Resistance factor (right y-axis) represents the ratio of a strain's mean EC_{50} relative to sensitive control IPO323. **B. terbinafine EC_{50} in progeny from 20-LQ2 \times 20-WK1 type III cross also shows a quantitative distribution.** EC_{50} values of individual progeny derived from sexual crosses between type III P_{MFS1} field strains 20-LQ2 and 20-WK1 follow a normal distribution. Individual points represent biological replicates ($n \geq 2$). Boxplots of parental strains are pointed out with green arrows and IPO323 susceptible control with grey one. Dotted lines indicate mean EC_{50} of IPO323, 20-LQ2, and 20-WK1. Orange and blue boxes indicate the IC95 boundaries of EC_{50} for 20-WK1 and 20-LQ2, respectively. Density curve of mean EC_{50} values is shown on the right, summarizing the distribution of mean EC_{50} values across all progeny. For both A and B EC_{50} determination, growth in liquid YPD medium supplemented with increasing terbinafine concentrations was monitored at OD_{405nm} after 7 days (18°C, dark), and expressed as percentage of growth relative to control. EC_{50} was computed from dose-response curves fitted to each technical replicate. All EC_{50} values are represented log₁₀-transformed for clarity.

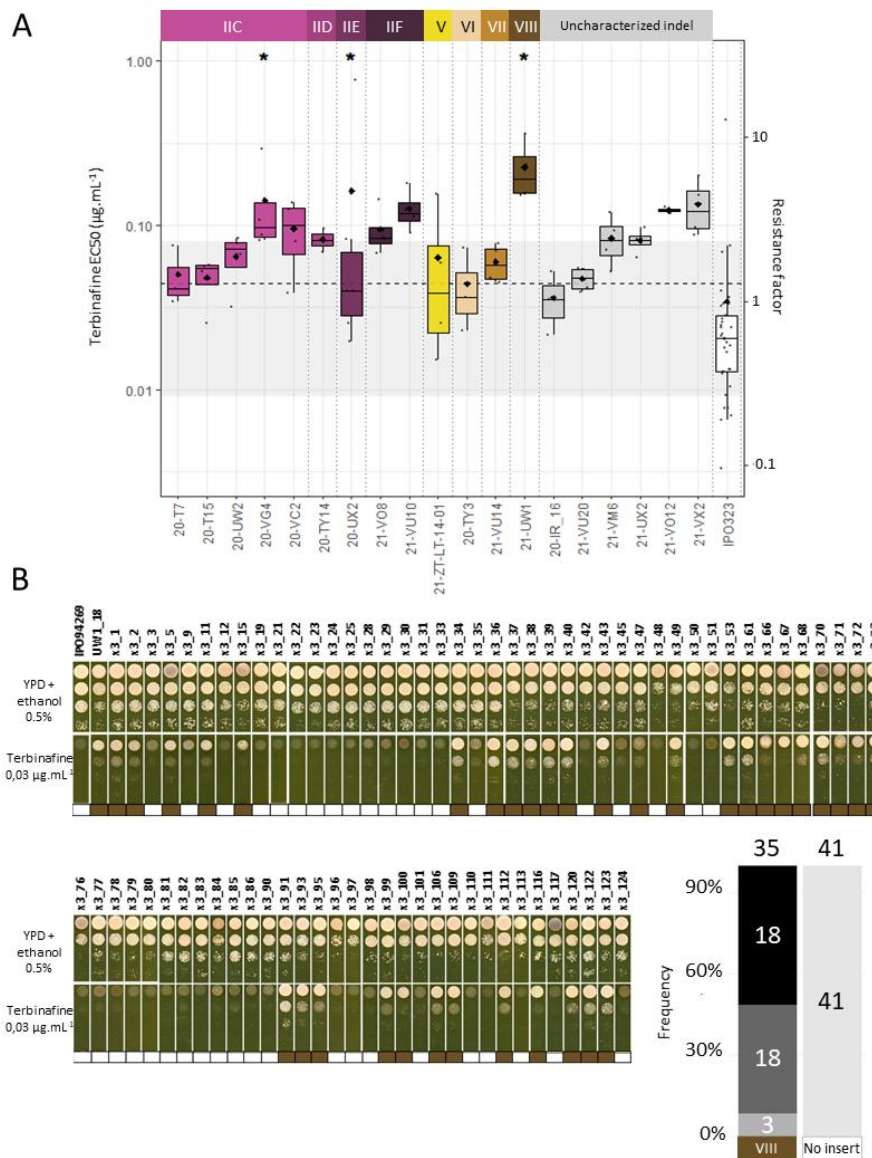
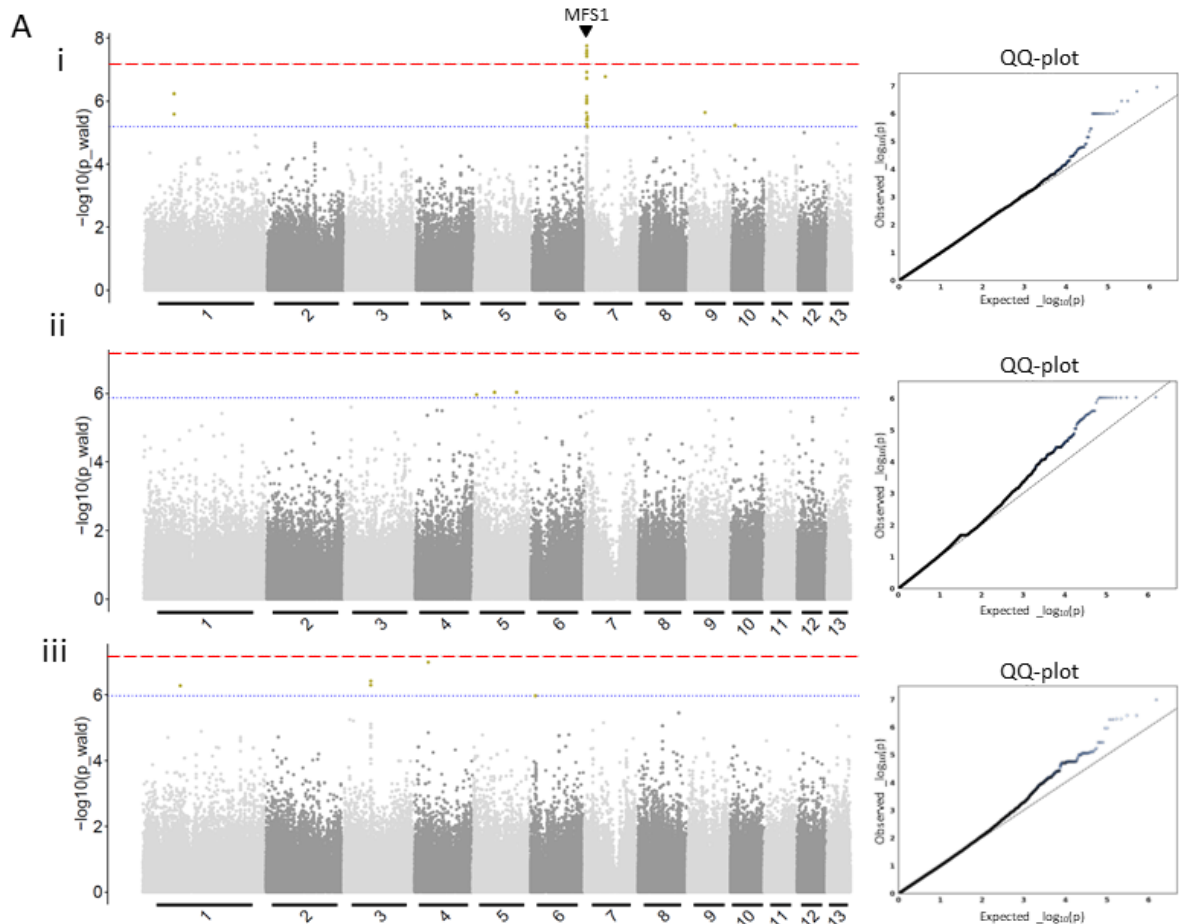


Fig.4: New $PMFS1$ insertions differentially impact MDR phenotypes in *Z. tritici*. **A. Terbinafine EC_{50} of strains carrying new P_{MFS1} insertion.** For EC_{50} determination, growth in liquid YPD medium supplemented with increasing terbinafine concentrations was monitored at OD_{405nm} after 7 days (18°C, dark), and expressed as percentage of growth relative to control. EC_{50} was computed from dose-response curves fitted to each technical replicate. Each strain was tested in at least 2 biological replicates (individual points in boxplots), each with three technical replicates. Statistical differences relative to the sensitive control strain IPO323 were tested on log-transformed values using a linear mixed-effects model including biological replicate variability as random effect, with estimated marginal means compared *via* contrast post-hoc tests and Bonferroni correction (* $p < 0.05$). Strains with uncharacterized indels (NA

in panel A) or no-insert but with upstream insertions detected by whole-genome sequencing (20-IR_16, 21-VM6, 21-UX2) are included. All EC_{50} values are represented log₁₀-transformed for clarity. **B. Validation of *PMFS1*^{typeVIII} contribution to MDR by forward genetics.** Growth of parental strains (IPO942696, *PMFS1*^{no insert}; 21-UW1, *PMFS1*^{typeVIII}) and their individual progeny was tested on solvent and terbinafine-supplemented YPD medium. Colored cells indicate *PMFS1* genotype of each progeny determined by PCR amplicon size: white = no insert, brown = VIII indel. Frequencies of HR (black), MR (grey), LR (light grey), and S (lighter grey) phenotypes associated with each genotype are shown in the bottom-right graph



B

	Position	MISS	REF	ALT	AF	Beta	SE	p_wald	Effect	Gene	Protein annotation
i	chr_1:1471109	5	T	G	0.057	0.4576	0.0888	5.85E-07	upstream gene variant	ZtIPO323_005000	Related to dehydrogenase/reductase SDR family member 7 precursor
	chr_1:1471141	5	G	C	0.057	0.4576	0.0888	5.85E-07	upstream gene variant	ZtIPO323_005000	
	chr_1:1471157	5	A	G	0.085	0.4224	0.0857	2.60E-06	upstream gene variant	ZtIPO323_005000	
	chr_7:63918	0	C	T	0.219	0.3863	0.0893	3.12E-06	intron variant	ZtIPO323_079190	Aspartyl protease
	chr_7:63992	0	G	A	0.273	0.3864	0.0758	3.88E-06	3 prime UTR variant	ZtIPO323_079190	
	chr_7:63994	0	C	T	0.273	0.3864	0.0758	3.88E-06	3 prime UTR variant	ZtIPO323_079190	HET-domain-containing protein
	chr_9:821139	2	G	C	0.159	0.4698	0.0948	2.32E-06	upstream gene variant	ZtIPO323_099700	
	chr_10:181759	0	T	G	0.078	0.4594	0.0971	5.98E-06	3 prime UTR variant	ZtIPO323_104670	Unnamed protein product
	chr_10:181761	0	A	G	0.078	0.4594	0.0971	5.98E-06	3 prime UTR variant	ZtIPO323_104670	
	chr_10:181767	0	T	C	0.078	0.4594	0.0971	5.98E-06	3 prime UTR variant	ZtIPO323_104670	
chr_10:181777	0	T	C	0.078	0.4594	0.0971	5.98E-06	3 prime UTR variant	ZtIPO323_104670	UPF0656 protein	
chr_5:1085256	0	T	C	0.118	0.4228	0.0778	9.40E-07	missense variant	ZtIPO323_064220		
chr_5:1085261	0	G	A	0.118	0.4228	0.0778	9.40E-07	missense variant	ZtIPO323_064220		
chr_5:1085263	0	T	C	0.118	0.4228	0.0778	9.40E-07	synonymous variant	ZtIPO323_064220		
chr_5:1085294	0	T	C	0.118	0.4228	0.0778	9.40E-07	missense variant	ZtIPO323_064220		
chr_5:1085297	0	G	A	0.118	0.4228	0.0778	9.40E-07	missense variant	ZtIPO323_064220		
chr_5:1085305	0	T	A	0.118	0.4228	0.0778	9.40E-07	synonymous variant	ZtIPO323_064220		
chr_5:1085308	0	C	T	0.118	0.4228	0.0778	9.40E-07	missense variant	ZtIPO323_064220		
chr_5:1085308	0	C	A	0.118	0.4228	0.0778	9.40E-07	synonymous variant	ZtIPO323_064220		
chr_5:1085312	0	A	G	0.118	0.4228	0.0778	9.40E-07	missense variant	ZtIPO323_064220		
ii	chr_5:2158920	0	A	G	0.074	0.5380	0.0988	9.34E-07	missense variant	ZtIPO323_088540	Unnamed protein product
	chr_5:2158944	0	T	A	0.074	0.5380	0.0988	9.34E-07	missense variant	ZtIPO323_088540	
	chr_1:1851292	0	G	A	0.25	0.4303	0.0758	5.28E-07	synonymous variant	ZtIPO323_006830	5'-nucleotidase like protein
chr_1:1851293	0	T	C	0.25	0.4303	0.0758	5.28E-07	synonymous variant	ZtIPO323_006830		
iii	chr_3:1336886	0	T	G	0.467	-0.4417	0.0776	5.05E-07	missense variant	ZtIPO323_041970	Unnamed protein product
	chr_3:1336887	0	T	G	0.467	-0.4417	0.0776	5.05E-07	missense variant	ZtIPO323_041970	
	chr_3:1339667	0	T	C	0.4	-0.4147	0.0719	3.81E-07	synonymous variant	ZtIPO323_041990	Dol-P-Man:Man(5)GlcNAc(2)-PP-Dol alpha-1,3-mannosyltransferase
chr_3:1339691	0	C	T	0.4	-0.4147	0.0719	3.81E-07	synonymous variant	ZtIPO323_041990		

Fig.4. Genome-wide association confirms that *MFS1* remains the major locus associated with multidrug resistance (MDR), but additional loci may contribute to resistance variance. Genome-wide association tests for terbinafine resistance phenotypes. Manhattan plots (a, c, e) and Q-Q plots (b, d, f) are shown for each GWAS testing the association between SNPs and terbinafine resistance. Estimated marginal means of log-transformed EC_{50} ($\text{Log}_{EC_{50}}$) were extracted from a mixed linear model ($\text{Imer}(\text{Log}_{EC_{50}} \sim \text{Souche} + (1|\text{Date}))$) and used as quantitative phenotypes for 128 strains (a, b) and corresponding subsets (c, e). Subsets were designed to mask the effect of the

PMFS1 locus through nested GWAS, performed separately on two groups of strains defined by the genotype of the top SNP from the complete GWAS: the reference allele group (c, 68 strains) and the alternative allele group (e, 60 strains). Complete, reference allele–nested, and alternative allele–nested GWAS analyses were performed using **GEMMA** on 758,800; 774,740; and 756,655 SNPs, respectively, fitting a linear mixed model (LMM). The first three principal components of the SNP-based PCA were included as covariates to account for population structure. On Manhattan plots, red and blue dashed lines represent Bonferroni and 10% FDR significance thresholds, respectively. SNPs surpassing these thresholds are highlighted in green. **B. Candidate SNPs for additional mechanisms contributing to MDR.** Candidate SNPs surpassing the 10% FDR threshold were extracted from each GWAS (a: complete dataset, b: reference-allele nested GWAS, c: alternative-allele nested GWAS). Only SNPs located outside the *MFS1* region and within 5 kb of annotated genes are shown. Columns indicate: **Missing**: number of strains with missing genotypes; **REF / ALT**: reference and alternative alleles; **Beta**: estimated effect size of the SNP on phenotype; **p_wald**: Wald test p-value for SNP–trait association; **Effect**: predicted impact of the SNP on gene expression or protein structure (predicted with SNPEff); **Gene**: nearest affected gene; **Protein annotation**: predicted protein function according to *Lapalu et al.*, 2025.

Supplementary figures

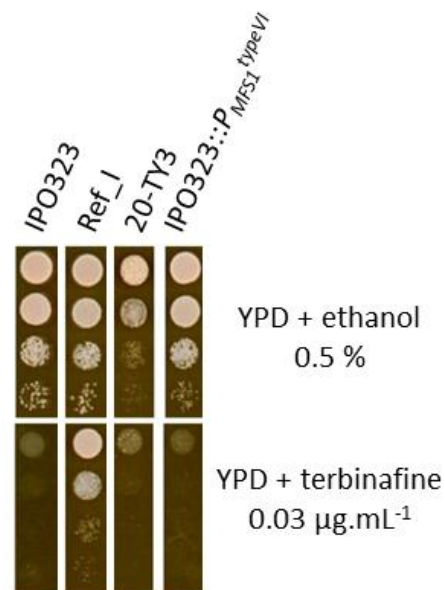


Fig.S1: P_{MFS1}^{typeVI} transposon insertion is not sufficient to induce terbinafine resistance in the IPO323 genetic background. For these droplet tests, IPO323: P_{MFS1}^{typeVI} mutant, natural P_{MFS1}^{typeVI} strain 20-TY3, susceptible control IPO323 and positive MDR control Ref_I were precultured for 7 days and approximately 5 µL of serial spore dilutions (10^7 , 10^6 , 10^5 and 10^4 spores.mL⁻¹) were spotted onto solvent of terbinafine-supplemented YPD medium. Growth was assessed after 7-day culture. IPO323: P_{MFS1}^{typeVI} appears as susceptible as IPO323 control strain.

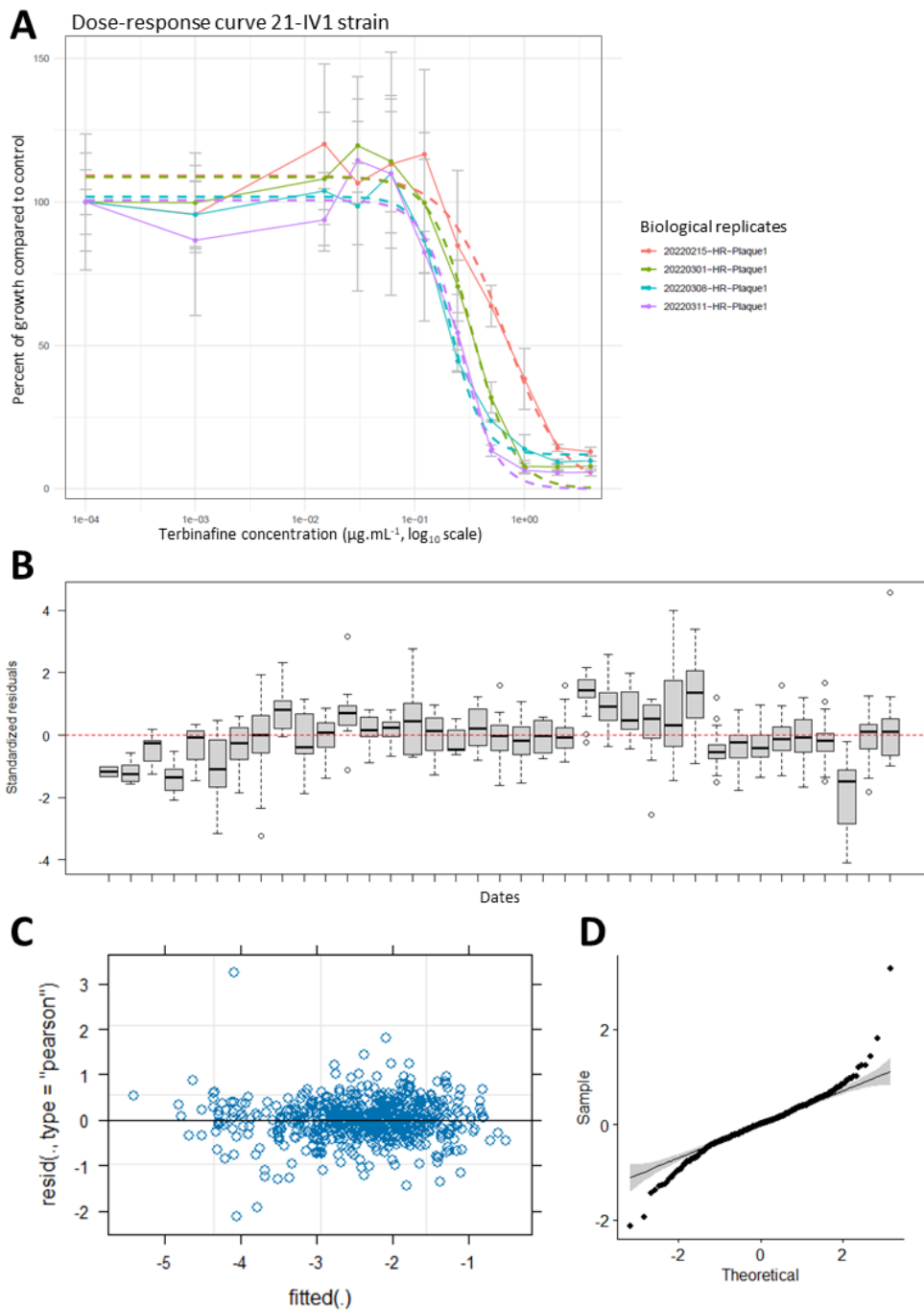


Fig.S2: Assessment of experimental variability and statistical modeling of terbinafine EC_{50} data.

A: Example of terbinafine dose–response curves obtained for the 21-IV1 strain. Each curve was fitted for one biological replicate based on three technical replicates per terbinafine concentration (error bars represent the standard deviation of technical replicates). B: Standardized residuals from the linear model $\text{Log}EC_{50} \sim \text{Strain}$ plotted for each biological replicate (experimental date), highlighting substantial experimental variability. This observation justified the inclusion of Date as a random effect in the mixed linear model used for statistical analyses. C and D: Diagnostic plots of the mixed linear model used for statistical comparisons of terbinafine EC_{50} in field strains, including experimental date as a random effect ($\text{Log}EC_{50} \sim \text{Strain} + (1|\text{Date})$). The distribution of Pearson standardized residuals versus fitted values (C) and the QQ plot (D) indicate acceptable homoscedasticity and approximate normality of residuals.

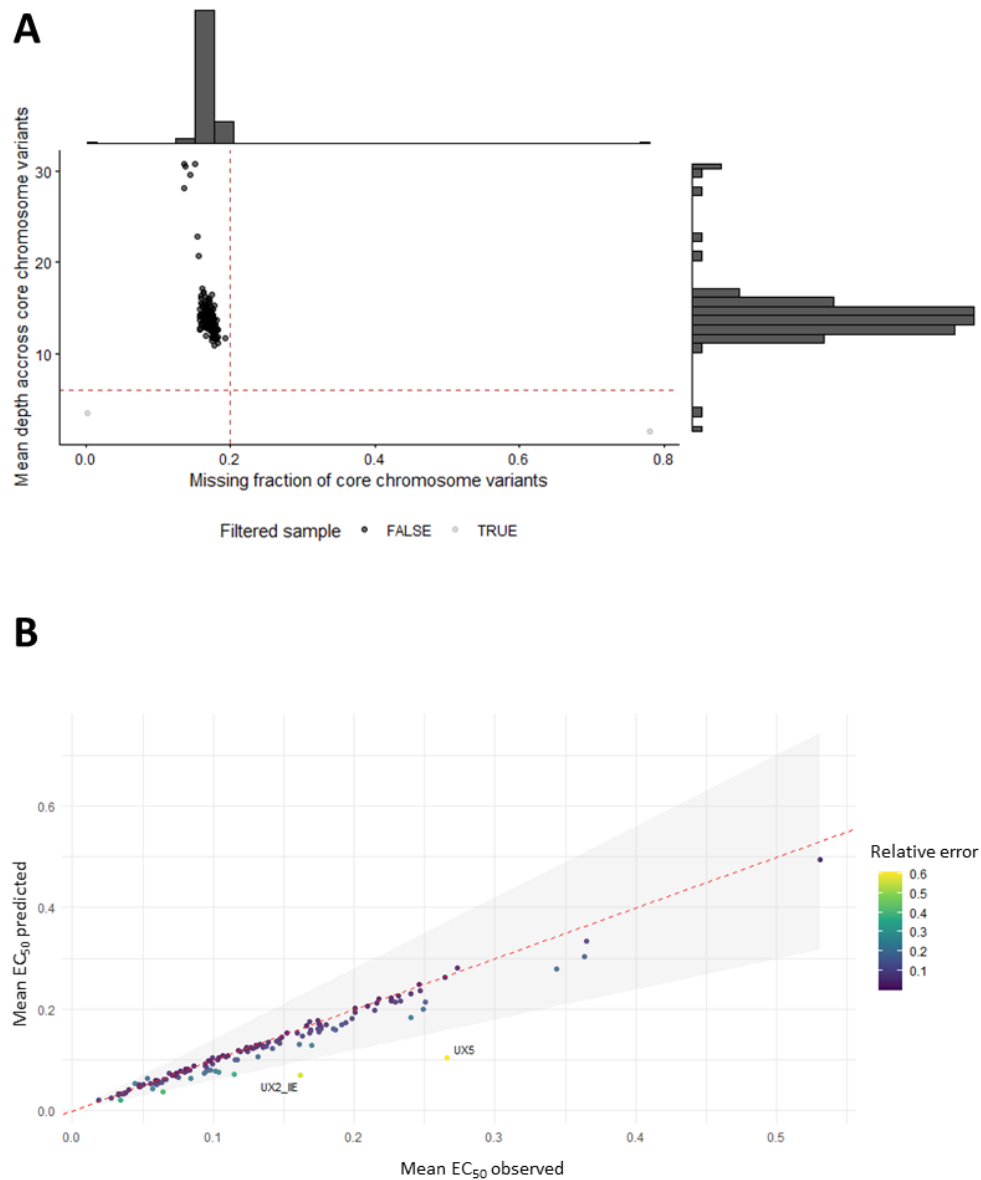


Fig.S3: Sample quality control for GWAS. A: Quality control based on missing data and sequencing depth. Each point represents one strain, plotted according to the fraction of missing genotypes (F_{MISS}) and the mean sequencing depth across core chromosome variants. Dashed red lines indicate the filtering thresholds used for GWAS analyses ($F_{MISS} \geq 0.2$ and mean depth ≤ 6). Strains failing at least one of these criteria are shown in grey, whereas retained strains are shown in black. Marginal histograms display the distribution of missing data and sequencing depth across strains. This filtering step was applied to exclude low-quality samples prior to downstream GWAS analyses. **B: Quality control based on quality of mean EC₅₀ prediction.** Observed versus predicted mean EC₅₀ values colored according to relative prediction error. The shaded grey area represents a $\pm 40\%$ deviation from the identity line. Strains exceeding this threshold (UX2-IIE and UX5) were considered poorly predicted by the model and excluded from downstream GWAS analyses.

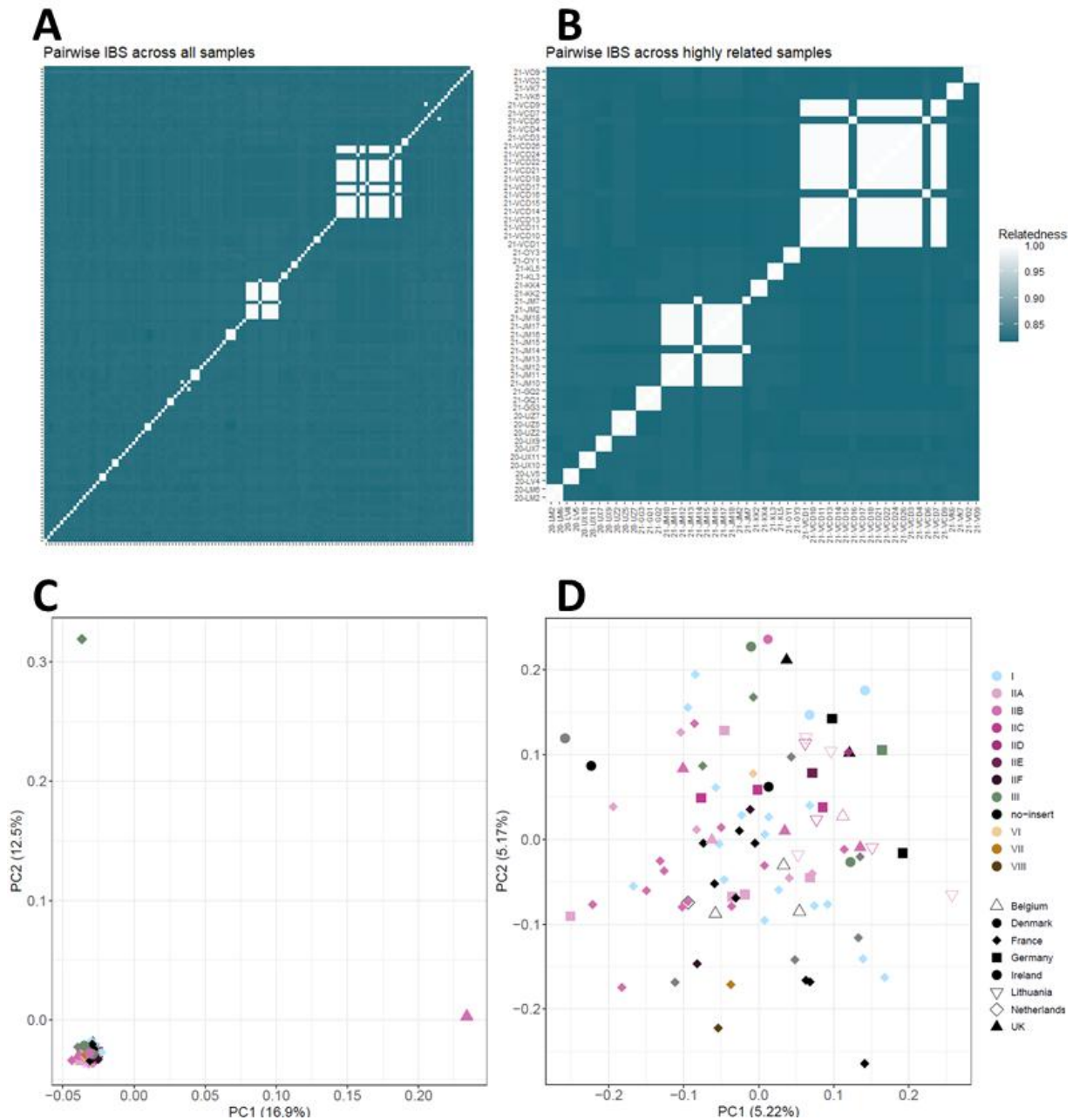


Fig.S4: Kinship and population structure analyses. (A, B) Pairwise genetic relatedness among MDR field strains. (A) Heatmap of pairwise identity-by-state (IBS) values computed from genome-wide SNP data for all strains retained after quality filtering. lighter colors indicate higher genetic relatedness. (B) Heatmap restricted to strains involved in highly related pairs ($IBS \geq 0.99$). This representation highlights clusters of near-clonal or highly related strains. **(C, D) Principal component analysis (PCA) of population structure** performed on LD-pruned, biallelic SNPs ($MAF \geq 5\%$, no missing data, thinned to one SNP per 1 kb). Each point represents a strain, colored according to *PMFS1* genotype and shaped by country of origin. (C) PCA including clonal strains. The presence of tight clusters reflects the contribution of clonal or near-clonal strains to population structure, motivating the inclusion of the first three principal components as covariates in GWAS analyses. (D) PCA after random selection of a single representative per clonal cluster reveals that population structure is not driven by *PMFS1* insertion type nor by geographic origin.

Limits of individual approaches

Our work highlights several mechanisms in populations of *Zymoseptoria tritici* that stimulate resistance acquisition, including pre-adaptation via aneuploidy and hypermutator phenotypes. In this context, it is important to recognize the limitations of using experimental evolution with the IPO323 reference strain. Although this strain lacks a functional Dim2 enzyme and is therefore prone to transposition, it originates from 1989 and may not fully represent the more recent evolutionary dynamics occurring in the field (Kema and van Silfhout, 1997).

Another limitation of experimental evolution is its tendency to over-select non-synonymous adaptive mutations compared to natural evolution (Remigi et al., 2019). This is largely because experiments are performed in simplified environments. While mutant competition for nutritional resources is possible, other fitness constraints, such as pathogenicity, are not included in the experimental setup used by Ballu and collaborators. As a result, findings from experimental evolution may not be fully transferable to field populations or reflect natural mutation acquisition dynamics. Consistently, the ZtAtrR mutation we identified *in vitro* was not detected in our MDR field strain collection. Even though *in vitro* growth assays did not reveal fitness costs for this MDR mutation, it may impact other fitness components, such as pathogenicity. Future pathogenicity tests of ZtAtrR mutants will address this possibility. Importantly, even if a fitness cost exists, the mutation could still appear in field populations and be maintained through compensatory mutations, as observed in other cases (Hawkins and Fraaije, 2018).

Finally, another limitation of our work is the difficulty of identifying candidate genes in field strains from GWAS analysis. In this study, we estimated terbinafine EC₅₀ for more than a hundred strains through growth assays and dose-response modeling. This approach is time-consuming, which limited the number of phenotyped strains. Consequently, the subsequent statistical associations with genotypes suffered from limited power. Given the polygenic nature of MDR we described in this work, supported by the multiple candidate genes identified from experimental evolution, it is likely that many minor contributors participate in the overall phenotype. Identifying and functionally validating these contributors will therefore be challenging, potentially requiring higher throughput phenotyping methods.

ADDITIONAL RESULTS: CLUES FOR HYPERMUTATION AND ANEUPLOIDY INVOLVED IN MDR

Context and experimental approach

In the first chapter of this thesis, we reported *de novo* mutations selected through experimental evolutions from two separated studies, Ballu et al., 2023 and Ballu et al., 2024. We primarily focused our analysis on IPO323-evolved isolates, carrying few mutations. However, it is important to note that the experimental design of the second experimental evolution differed from the first one (Ballu 2021). Indeed, in order to assess the durability of anti-resistances strategies during the selection phase (i.e., once resistance has emerged), the authors initiated experimental evolution, not only with IPO323 as in 2023, but with synthetic populations as a mix of strains combined in different proportions (Table VII). The isolates used in the mix were qualified as “semi-isogenic” (Ballu 2021) because derived from the progeny of a cross between IPO323 and a field resistant strain (G. Gazeau, unpublished; codes and phenotypes in Table VII) and displayed either resistance to SDHIs (R_SDHI, with H152R substitution in SDHC) or DMIs (R_DMI, carrying different SNPs in *CYP51*). The “S” strain, constituting 95 % of the initial PopR population, corresponds to the IPO323_Ancessor_2024 described in Chapter I. Fungicide resistant isolates made up 5% of the PopR population, approximately 2.5% each.

Table VII: Isolates and derived ancestral populations used in the experimental evolution. Simplified from Ballu 2021. Genotypes of *SDHC* and *CYP51* (respectively encoding the targets of SDHIs and DMIs) loci are indicated for each resistance phenotype.

Isolates	Genotypes	SDHI	DMI	PopS	PopR
S	<i>sdhc</i> ^{wt} ; <i>cyp51</i> ^{wt}	S	S	100%	95%
R_SDHI	<i>sdhc</i> ^{H152R} ; <i>cyp51</i> ^{wt}	R	S	-	2.50%
R_DMI	<i>sdhc</i> ^{wt} ; <i>cyp51</i> ^{I381V}	S	R	-	2.50%

In order to identify MDR mutations and underlying mechanisms, we assessed the resistance spectra of 51 isolates from this particular experiment as described in Chapter I. Among them, 24 were retained for whole genome sequencing because of their wide resistance spectrum. With the genomic sequences, processed as described in Chapter I, we were able to

discriminate *a posteriori* IPO323-evolved isolates from those derived from other ancestors. We identified 11 isolates evolved from the R_SDHI ancestral strain. In this complementary chapter, we report the results of resistance and efflux phenotyping as well as genomic sequence information gathered from these isolates, that provide additional relevant insights in MDR genetic basis and mechanisms. The fungicide selection regimes under which these isolates were selected are illustrated in Table VIII (Ballu *et al.* 2024). For every experimental information detail regarding the following results section, see Chapter I (experimental procedures).

Table VIII: Name of fungicide selection regimes of experimental evolution carried out by Ballu and collaborators (2024) and corresponding number of cycles, referring to isolates studied in the present work. Alternation of molecules is represented by fully colored cells alternated and mixing by colored gradients inside the same cell (i.e., the same cycle).

Cycle number (week)	1	2	3	4	5	6	7	8
Alt_MF	Green	Blue	Green	Blue	Green	Blue	Green	Blue
Alt_CPB	Green/Blue	Pink	Green/Blue	Pink	Green/Blue	Pink	Green/Blue	Pink
Mixt_CPB	Pink/Blue/Green gradient							
Mixt_MF	Blue/Green gradient							
Color legend: Fungicide used and molecular targets								
Benzovindiflupyr (B), Fluxapyroxad (F): Complex II succinate-dehydrogenase inhibitors (Succinate DeHydrogenase Inhibitors)								
Prothioconazole-Destio (P), Mefentrifluconazole (M): C14-demethylase in sterol biosynthesis inhibitors (DeMethylation Inhibitors, SBI : Class I)								
M05, chloronitriles (unspecified mechanism) : Chlorothalonil (C)								

Results

The 11 isolates derived from the R_SDHI ancestral strain displayed a broad resistance spectrum against fungicides of various modes of action (as illustrated for two of them in Fig. 43A), including terbinafine, while their ancestor, R_SDHI, is susceptible to all MoAs tested (except SDHI, data not shown). Some of them also demonstrated a significant reduction in Nile Red accumulation compared with the ancestral strain, indicating enhanced transport activity, which was further supported by the synergy pattern between terbinafine and chlorpromazine on fungal growth in DDST (Figs 43B and 43C). These results pointed to contrasting MDR mechanisms selected, including transport modulation as well as other mechanisms. Thus, the genome sequences of these 11 isolates were further analyzed for MDR mutation identification.

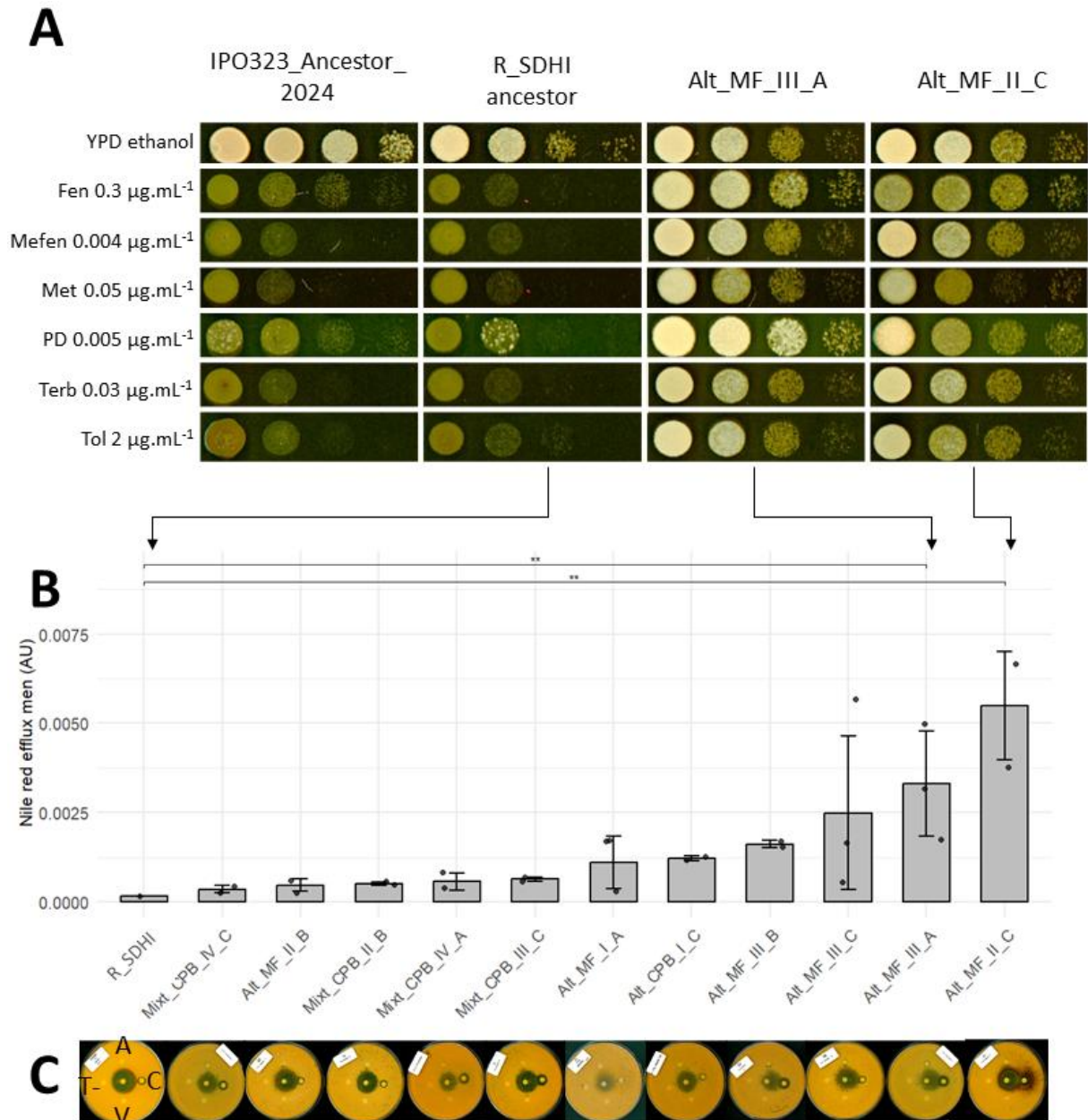


Fig. 43: Resistance spectra and efflux activity of *R*_SDHI-evolved multidrug resistant isolates. A: Fungal growth assessed by drop spore dilution assay (10^7 , 10^6 , 10^5 , 10^4 spores.mL⁻¹ in 5 μ L drops) on solid YPD medium supplemented with ethanol -0.5% v/v as control or the discriminatory doses of fungicides indicated in the Fig. legend. Fen: fencicoxamid, Mefen: mefentrifluconazole, Met: metyltetraprole, PC: prothioconazole-desthio, Terb: terbinafine, Tol: tolnaftate. B: Efflux capacity of the isolates assessed by a Nile Red efflux assay. The bar represents the mean efflux per strain, \pm standard deviation ($n \geq 2$ per strain), expressed as the inverse of Fluorescence_{600nm}/OD_{405nm}. *: Bonferroni corrected p-values < 0.05 of pairwise contrasts against the ancestral *R*_SDHI strain, extracted from a linear mixed model. C: Double disk synergy test (DDST) results for corresponding isolates, assessing synergy between terbinafine (T, central disk) and transport modulators (lateral disks, A: Amytriptiline, V: Verapamil, C: Chlorpromazine) on fungal growth. T-: ethanol 80%. Interactions between terbinafine and modulators were inferred from the shape of the inhibition halo (Laishram et al., 2018).

The main observation of the genomic analysis was that the number of mutations identified in R_SDHI-evolved isolates was far higher than expected. Indeed, the number of mutations was more than 100 times higher in R_SDHI derived isolates than in IPO323-derived isolates. For example, the mean number of mutations in non-intergenic regions (defined as gene coordinates plus 5 kb upstream and downstream) across all these strains was about 350, compared with 2.8 for the 42 mutants derived from IPO323 ancestral strains described in Chapter 1 (Fig. 44).

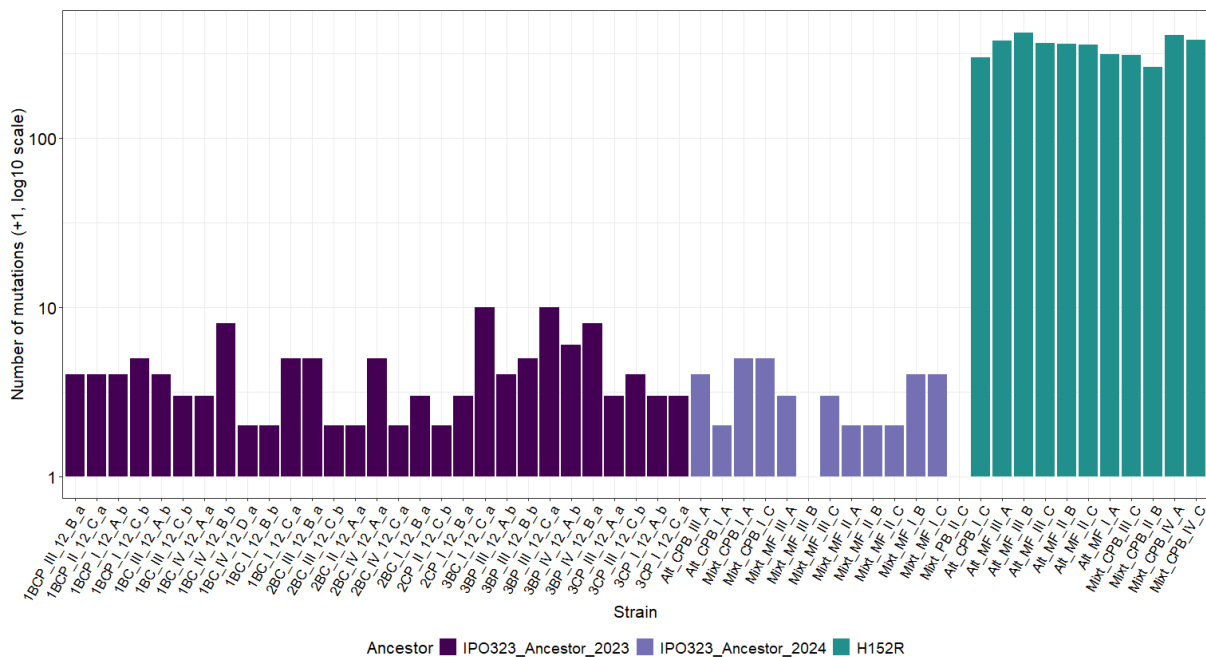


Fig. 44: Number of variants (SNP and small indels) detected in gene neighboring regions in isolates evolved from IPO323 (purple) or from R_SDHI (turquoise). Variants, called with GATK, were filtered for keeping only non-variant positions in ancestral strains and positions in a 5 kb window overlapping gene encoding regions.

This high number of mutations cannot be explained by the number of generations. Indeed, the isolates went through the same number of growing cycles as those evolved from IPO323 or even less (supplementary Fig.S1 of Chapter 1), and the growth rate of R_SDHI *in vitro* was even estimated lower than the IPO323 growth rate (Ballu et al., 2024). It is also unlikely to have been caused by a mutagenic effect specifically exerted by the fungicide treatment, since IPO323-derived isolates evolved under the same conditions. The most likely hypothesis to explain this elevated number of mutations is an elevated mutation rate in the R-SDHI isolates, in comparison with IPO323.

This characteristic may have been acquired under the fungicide selection pressure, by selection of mutations conferring higher mutation rate, or it may be inherent to the R_SDHI ancestor. A look at the mutations in the latter gives some clues to answer this question. Among the 356,581 variants identified in R_SDHI, 180 are associated with moderate and high effect on protein function (i.e., moderate: missense variants, high: frame-shift variants, stop-gain), in genes annotated as encoding DNA repair proteins (Table IX). As an example, R_SDHI carries 37 mutations in the *msh3* gene including 8 frameshift mutations, but also in *msh2*. DNA repair protein loss-of-function is often reported in mutator phenotypes, particularly in the mismatch repair pathway (*msh*) (Bottery et al., 2024; Boyce et al., 2017; Hunter et al., 2006).

How these mutations influence the mutation rate of the strain remains to be investigated and experiments are currently carried out by our team. Preliminary results of a fluctuation assay tend to confirm the elevated mutation rate of strain R_SDHI vs IPO323 (data not shown). Given that this strain is the result of successive crosses between IPO323 and field strains, it implies that it has acquired this mutator phenotype from a natural parent.

Table IX : Number of variants (SNP and small indels) of moderate and high effect identified in coding sequence of DNA repair protein in the R_SDHI genetic background.

Chromosome	Gene	Start	End	Sequence annotation	Number of mutations
chr_1	ZtIPO323_011450.1	2961810	2963596	DNA repair family protein	2
chr_1	ZtIPO323_019020.1	4816278	4818407	DNA repair protein Rad7	3
chr_1	ZtIPO323_020290.1	5152455	5156913	DNA repair protein Pso2/Snm1	3
chr_2	ZtIPO323_025820.1	658835	659991	DNA repair protein Nse1	3
chr_2	ZtIPO323_030290.1	1836833	1841293	DNA repair protein Rhp26/Rad26/DNA repair and recombination protein RAD26	2
chr_2	ZtIPO323_037120.1	3679566	3682902	DNA repair and recombination protein RAD54B-like	1
chr_4	ZtIPO323_051400.1	398580	401269	DNA damage response protein RcaA	1
chr_4	ZtIPO323_057610.1	2090077	2094780	DNA excision repair protein	1
chr_5	ZtIPO323_062610.1	625344	628373	DNA repair protein	8
chr_6	ZtIPO323_071830.1	392641	395662	DNA mismatch repair protein msh-2	8
chr_6	ZtIPO323_075020.1	1416935	1419629	DNA repair protein rhp54	1
chr_6	ZtIPO323_077330.1	2098064	2099924	DNA damage-binding protein cmr1	5
chr_6	ZtIPO323_078590.1	2523800	2530809	DNA repair protein rad8	12
chr_6	ZtIPO323_078670.1	2581012	2583758	DNA repair helicase	2
chr_7	ZtIPO323_079350.1	87036	90612	DNA mismatch repair protein msh3	37
chr_7	ZtIPO323_080660.1	481009	484681	DNA mismatch repair protein msh4	2
chr_8	ZtIPO323_092800.1	1199547	1202254	DNA repair helicase rad25 like protein	1
chr_8	ZtIPO323_095050.1	1726960	1730705	DNA repair protein rad16	27
chr_9	ZtIPO323_101130.1	1208302	1211723	DNA mismatch repair protein Mlh1	10
chr_9	ZtIPO323_101640.1	1335462	1338149	DNA repair exonuclease	6
chr_10	ZtIPO323_108710.1	1118025	1119855	DNA repair protein rhp57	37
chr_11	ZtIPO323_112390.1	578452	582331	DNA repair protein rad18	1
chr_12	ZtIPO323_117190.1	278768	280895	DNA recombination/repair protein Rad52	1
chr_12	ZtIPO323_118650.1	659400	663169	DNA mismatch repair protein MLH3	5
chr_16	ZtIPO323_128690.1	149114	152149	DNA damage response protein RcaA	1

We intended to investigate further the mutations identified in the genomes of the evolved isolates and their putative links to MDR. The table X gives a summary of genes identified as mutated in at least one R_SDHI-evolved isolate potentially associated with MDR. Several transporter genes show mutations, either in upstream, downstream, or CDS regions across the different isolates, such as ZtIPO323_012290 encoding a putative MFS transporter, or the ABC transporter ZtIPO323_088500, potentially affected by mutations found in 7 different isolates. Beyond transporters, other MDR associated genes were unveiled, including enzymes that may be involved in fungicide metabolism, such as Glutathione S-transferases, cytochrome p450 oxidase and short chain dehydrogenases.

Table X: Subset of genes likely to be impacted by mutations found in R_SDHI-evolved isolates.

	Chromosome	Gene	Start	End	Sequence annotation	Mutation impact (higher)	Number of mutants
Transport	chr_1	ZtIPO323_004580	1359868	1365150	ABC transporter	MODIFIER	1
	chr_1	ZtIPO323_012290	3160060	3163512	MFS general substrate transporter	MODIFIER	3
	chr_1	ZtIPO323_015880	4069788	4072715	Putative transporter	MODIFIER	2
	chr_1	ZtIPO323_018720	4751098	4752330	MFS transporter	MODIFIER	1
	chr_1	ZtIPO323_020340	5162955	5164817	General substrate transporter	LOW	1
	chr_2	ZtIPO323_026660	861615	863830	MFS general substrate transporter	MODIFIER	1
	chr_2	ZtIPO323_030930	2059468	2060972	Putative Mg ²⁺ transporter-like Zinc transport protein	HIGH	9
	chr_2	ZtIPO323_036640	3521377	3523488	General substrate transporter	MODIFIER	2
	chr_4	ZtIPO323_050540	41788	43948	MFS1 putative major facilitator superfamily transporter	MODIFIER	2
	chr_4	ZtIPO323_051050	316990	318409	MFS monocarboxylate transporter like protein	MODIFIER	1
	chr_6	ZtIPO323_071080	167618	170525	Putative magnesium transporter NIPA1	MODERATE	3
	chr_6	ZtIPO323_075050	1423723	1425385	C4-dicarboxylate transporter/malic acid transport protein	MODIFIER	3
	chr_7	ZtIPO323_079180	56483	61058	Sugar transporter STL1	MODIFIER	5
	chr_7	ZtIPO323_088500	2597454	2602185	ABC transporter	MODIFIER	7
	chr_8	ZtIPO323_094570	1603205	1605709	MFS sugar transporter like protein	MODIFIER	1
	chr_9	ZtIPO323_098800	612491	615168	Putative major facilitator superfamily transporter	MODIFIER	7
chr_10	ZtIPO323_108770	1151871	1154144	MFS general substrate transporter	MODIFIER	2	
chr_10	ZtIPO323_109440	1373888	1376120	Putative transporter	MODIFIER	2	
chr_14	ZtIPO323_126810	398137	399243	MFS general substrate transporter	MODIFIER	5	
Metabolization	chr_7	ZtIPO323_088370	2552425	2553317	Glutathione S-transferase family protein	LOW	1
	chr_10	ZtIPO323_110050	1546018	1547419	Glutathione S-transferase	MODIFIER	5
	chr_4	ZtIPO323_059140	2454703	2456123	Short-chain dehydrogenase like protein	MODIFIER	1
	chr_4	ZtIPO323_059170	2463050	2464104	Putative oxidoreductase	LOW	5
	chr_8	ZtIPO323_096910	2372320	2374340	Beta-lactamase-like protein ARB_00930	MODIFIER	2
	chr_11	ZtIPO323_114390	1109170	1110466	Short chain dehydrogenase like protein	MODIFIER	2
	chr_13	ZtIPO323_124470	708073	710002	Cytochrome P450 monooxygenase like protein	MODIFIER	1

Number of mutants: number of R_SDHI derived isolates displaying at least one mutation in the corresponding position. Mutation Impact (higher): higher effect on protein function or gene expression among all mutations identified across isolates (scored with SNPEFF); MODIFIER: upstream or downstream gene variant, LOW: splice region, intron or synonymous variant, MODERATE: missense or in-frame indel variant, and HIGH: start loss, stop gained or frameshift variant. Gene IDs and annotations were retrieved from Lapalu et al. (2025).

All these genes constitute highly relevant candidates for explaining the MDR phenotype of R_SDHI evolved isolates, but the effect of the identified mutations remains to be dissected. Moreover, association between phenotype and genotype can hardly be achieved due to the number of mutations unveiled in comparison with IPO323-evolved isolates. In that sense we chose not to pursue functional validation, as it was considered too risky and time-consuming.

Nevertheless, these strains were still a source of particularly relevant findings, as the sequencing depth analysis (Fig. 45) revealed aneuploidy in these isolates, and more precisely a partial or complete duplication of chromosome 7 (in addition to chromosome 1, and some accessory chromosomes) as we reported before for IPO323-evolved isolates (chapter 1). These aneuploid strains were selected under mefentrifluconazole (DMI) and fluxapyroxad (SDHI) alternation treatment.

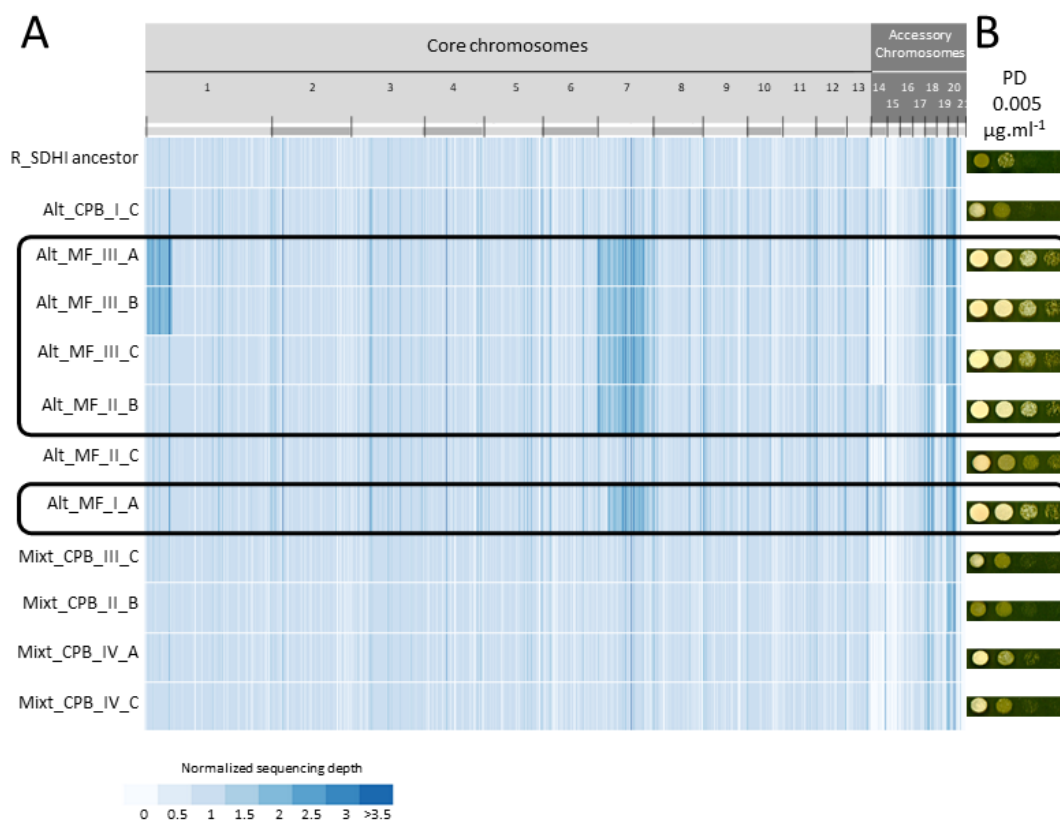


Fig. 45: Chromosomal number variation related to resistance to prothioconazole-desthio in *Zymoseptoria tritici* MDR isolates evolved from R_SDHI ancestral strain. A: Normalized read depth across 21 chromosomes of Illumina sequenced reads were mapped against IPO323 reference genome and mean depth was calculated in consecutive 5 kb windows across all 21 chromosomes and normalized by the median depth of the core chromosomes (excluding chromosome 7) for each isolate. A normalized depth close to 0 across an entire chromosome indicates chromosome loss, whereas increased read depth suggests chromosome duplication. Intermediate values suggest heterogeneity in chromosome content within the sequenced cell population of the same isolate. B: Fungal growth assessed by drop spore dilution assay (10^7 , 10^6 , 10^5 , 10^4 spores.mL⁻¹ in 5 µL drops) on YPD solid medium supplemented prothioconazole-desthio (PD)

We found that this duplication was also closely associated with resistance to the DMI prothioconazole-desthio (Fig. 44), as we reported in IPO323-evolved isolates displaying chr_7 duplication. On this chromosome, different genes are related to the ergosterol biosynthesis pathway, including *CYP51*, the molecular target of prothioconazole, but also *MFS1*, encoding the transporter shown to be involved in field MDR (chapter 2). The increased dosage of these genes may drive their overexpression, explaining resistance.

These findings highlight that large structural rearrangements and copy number variations can arise in different genetic backgrounds in *Z. tritici*, under different fungicide selection pressure *in vitro*, and may be associated with its rapid adaptation toward resistance as reported in other pathogenic fungi (Khateb et al., 2023; Sionov et al., 2010; Tsai and Nelliati, 2019). For the phytopathogenic fungi however, it constitutes, to our knowledge, an unprecedented result.

No copy number variation of the chromosome 7 has been identified among our MDR field strains (chapter 2), nor reported elsewhere, although read depth analysis revealed duplication of chromosome 5 in two strains (20-IR_12 and 20-BLG17, data not shown). The stability of aneuploidy, its fitness cost and importance in fungicide adaptation in the field remain to be elucidated.

Overall, our results, including these additional ones, raise questions about how genomic plasticity, through CNV, structural variants, TIP, and mutations (including increased mutation rate) unveiled in this study shape the landscape of MDR in *Z. tritici*, and the molecular pathways involved.

GENERAL DISCUSSION AND CONCLUDING REMARKS

In this study, we highlighted multiple genetic bases and molecular mechanisms underlying multidrug resistance (MDR) in *Zymoseptoria tritici*, using two complementary approaches: analyses of experimentally evolved strains and of natural field populations. The results presented throughout this thesis contribute to a broader understanding of the processes driving the acquisition of a generalist resistance phenotype, providing insights into the factors involved in its emergence under diverse fungicide selection pressures.

We identified features at the genome-scale that may act as drivers of *Z. tritici*'s adaptive potential. These include chromosome copy number variation, likely to contribute to fungicide resistance, and an increased mutation rate which we hypothesize may fuel the pace of adaptation of *Z. tritici* under fungicide pressure. Beyond these global mechanisms, our complementary approaches allowed us to identify stable and heritable genetic polymorphisms associated with MDR, including single-nucleotide polymorphisms (SNPs) and transposon insertion polymorphisms (TIPs), present in both field-derived MDR strains and experimentally evolved isolates.

Our analyses further demonstrate that MDR in field populations is a polygenic trait, supported by the diversity of candidate genes identified through GWAS, whose functions converge with those of candidates identified in experimentally evolved strains. In the following sections, we discuss how these factors may interact to generate a complex and multifactorial adaptive landscape, ultimately giving rise to a generalist resistance phenotype through distinct genetic and molecular trajectories.

Genomic plasticity and mutation rate in MDR acquisition

Copy number variations in MDR acquisition

We observed duplication of chromosomes in all sets of strains studied in this work. Notably we observed chromosome 5 duplication in two field strains. But more interestingly, we noted a duplication of chromosome 7 in two sets of experimentally evolved mutants from different genetic backgrounds. In the last case, this duplication was associated with prothioconazole resistance. The role of aneuploidy and copy number variations (CNVs) in drug adaptation has been documented in various model organisms. Experimental evolution under

fungicide or drug selection pressure has repeatedly triggered CNVs in clinical fungi, such as *A. fumigatus*, *C. albicans*, and *Cryptococcus neoformans*, as well as in other unicellular organisms including *Leishmania donovani* (Dumetz et al., 2018; Gerstein et al., 2015; Muñoz et al., 2018; Selmecki et al., 2008; Sionov et al., 2010; Todd and Selmecki, 2020). For example, recurrent amplification of the left arm of chromosome 5 in *C. albicans* is sufficient to confer azole resistance through increased copy number of *TAC1*, a transcriptional activator of the MDR transporter genes *CDR1* and *CDR2*, as well as *ERG11*, the azole target (Ford et al., 2015; Selmecki et al., 2008). This parallels our observations in *Z. tritici*, since the duplication of chromosome 7 implies increased copy number of the DMI target gene *CYP51* and the MDR transporter gene *MFS1*, potentially associated to their overexpression.

CNVs are rapidly acquired structural variations capable of conferring resistance to a wide range of compounds in a single step. By increasing chromosome copy number and overall genome size, they also elevate the probability of *de novo* mutations occurring in the extra copies relative to the rest of the genome. In this way, CNVs reshape the mutational and adaptive landscape of many organisms and constitute a preadaptation step in resistance selection (Bayer et al., 2018; Otto, 2007; Sun et al., 2023; Yona et al., 2012; Zhou et al., 2011).

To our knowledge, such mechanism has not previously been reported in a phytopathogenic fungi, highlighting the novelty of our results, suggesting that aneuploidy may serve as an early step in MDR acquisition in *Z. tritici*, preceding the fixation of point mutations.

A hypermutator unveiled

Hypermutation is a convergent feature of several human and plant fungal pathogens, often associated with rapid acquisition of antifungal resistance. It can arise through multiple molecular pathways, including reactivation of transposable elements (TEs) and defects in DNA mismatch repair (MMR) (Baril and Croll, 2025). In this study, we identified evidence of both types of hypermutator phenotypes in *Zymoseptoria tritici*.

First, the extensive transposon insertion polymorphism (TIP) we observed in *P_{MFS1}* from European MDR natural populations, whether linked to MDR or not, fits within the context of recent deregulation of TE activity in populations spread outside the species' center of origin. Specifically, the recent loss of Dim2 activity, and consequently the inactivation of the Repeat-

Induced Point mutation (RIP) defense against TE activity, has been associated with TE-mediated adaptation (S. Fouché et al., 2022; Lorrain et al., 2021; Oggenfuss et al., 2021; Oggenfuss and Croll, 2023). This appears to be a very recent phenomenon, with the highest wave of TE activity occurring in the last 25 years (Baril et al., 2025). The TIP in *P_{MFS1}* may therefore be a direct consequence of stress-driven TE transposition in Dim2-defective strains, contributing to the overall adaptive potential of the fungus.

Second, we observed evidence of an elevated mutation rate in one strain used in Ballu's experimental evolution study (Ballu, 2021) (R_SDHI). In *Aspergillus fumigatus*, multi-azole-resistant hypermutator isolates carry a SNP in the mismatch repair protein encoding gene *msh6* (Bottery et al., 2024). Similar patterns are seen in obligate plant pathogens such as *Erysiphe necator*, *E. pisi*, *E. pulchra*, and *Blumeria graminis*, which each lack 5-21 MisMatch Repair (MMR) genes and this loss is associated with hypermutator phenotypes (Phillips et al., 2021). In these fungal species, the hypermutator phenotype could be associated with rapid adaptation to environmental stress (e.g., azoles in the case of *A. fumigatus*). Mechanistically, MMR deficiency prevents correction of replication-induced base mispairing and thus leads to increased mutation rate. In our study, two missense mutations were identified in *msh2* in strain R_SDHI, alongside with multiple missense and nonsense mutations in other DNA repair genes, consistent with a loss of DNA repair capacity in this strain. Isolates experimentally evolved from these ancestral strains under fungicide selection accumulated approximately 100 times more mutations than IPO323-evolved isolates over the same number of growth cycles. According to the origin of this strain, obtained through crosses between IPO323 and a SDHI-resistant field strain, this hypermutator phenotype was likely inherited from a field ancestor. To our knowledge, this is the first report of such a phenotype in *Z. tritici*.

Together, our findings support a role for hypermutation, both via TE deregulation and elevated mutation rate, in the rapid acquisition of MDR in *Z. tritici*.

Short and long-term impacts of genomic plasticity

Whether considering aneuploidy or hypermutator phenotypes, both carry costs ranging from short- to long-term. Copy number variations, and particularly whole-chromosome duplications, are frequently associated with fitness penalties. The increased protein production and resulting imbalance are often invoked to explain the loss of aneuploid chromosomes under

non-selective conditions (Torres et al., 2007; Tsai and Nelli, 2019; Yang et al., 2021). In this sense, aneuploidy may represent a transient state, acting as an initial step toward the fixation of more stable, perennial resistance through point mutations. CNVs may be common in *fungi*, as evidenced by the high rate of large duplications in *S. cerevisiae* relative to the base mutation rate (Zhu et al., 2014) but they may be transient and therefore underreported. This may explain why we did not observe chromosome 7 aneuploidy in field strains of *Z. tritici*, while it may have played, play or will play a role in fungicide resistance acquisition. Bennet and collaborators (Bennett et al., 2014), in their model proposed an explanation how aneuploidies selected under fungicide pressure increase the potential for mutation accumulation without associated fitness penalty. Accumulation of point mutations in each copy of resistance driving genes allows the additional copies to be lost, as euploid cells carrying only one copy with the beneficial mutation(s) are fitter than their aneuploid counterparts. Transient aneuploidies have been reported in clinical isolates from *C. albicans* (Ford et al., 2015). These observations and our results underscore an important advantage of experimental evolution, potentially enabling detection of such early adaptive and transient mechanisms, difficult to be caught in nature.

Also, hypermutator phenotypes have, in theory, a high cost for genome integrity. Some studies argue that it constitutes a dead end, or a “devil’s bargain” conferring short-term benefits at the cost of long-term perils for genome’s sake, as reported by Fouché and collaborators (S. Fouché et al., 2022). However, Baril and Croll described situations in which hypermutators do not necessarily meet an evolutionary dead end: functional recovery may be possible if hypermutator lineages are selected for re-acquiring machinery to control mutation rates, through compensatory mutations or sexual recombination helping to restore these MMR and TE regulation machineries (Baril and Croll, 2025). In that sense, the hypermutator phenotype observed in this study, leading to such an increase in the adaptation pace is not necessarily going to be a short-term issue, even less under conditions where it confers a selective advantage for stress adaptation, such as exposure to fungicides.

Active role of fungicide stress

Fungicide exposure may drive acquisition of these aneuploid and hypermutator phenotypes, by directly promoting TE mobilization or mutagenic processes. It has been shown

that stress conditions, including exposure to antifungal compounds, trigger transposon mobilization, as observed in *Monilinia fructicola* (Chen et al., 2015). Some fungicides have also been demonstrated to induce aneuploidy by disrupting normal cell division. For instance, exposure to fluconazole often leads to improper bud separation in *C. albicans* and related species, resulting in aneuploid progeny (Harrison et al., 2014). This was also reported for *C. neoformans* (Altamirano et al., 2017). Moreover, carbendazim, a fungicide inhibiting microtubule polymerization used for a long time in agriculture for STB control is also known to promote aneuploidy in *Z. tritici* (Habig et al., 2017). All the IPO323-derived strains of this study displaying duplication of chromosome 7 were selected under a selection regime including this last fungicide, strongly supporting an active role of carbendazim in generating aneuploidy. Together, these observations suggest that fungicide stress may not only select for pre-existing adaptive variants but also actively contribute to the generation of genetic diversity through transposon mobilization, hypermutation, and aneuploidy, thereby accelerating the emergence of multidrug resistance.

The recent gains in genome plasticity of *Z. tritici* witnessed in this work has the potential to dramatically increase the load of resistance mutations in fungicide exposed field populations which is critical for subsequent resistance selection, both fueling adaptation and resulting in rapid MDR acquisition. In the next section, we will address the MDR mutations we identified in this thesis.

Perennial acquisition of resistance: Mutations and molecular pathways unveiled

In this study, we have demonstrated that multidrug resistance (MDR) in field populations is a quantitative trait. This quantitative variation arises not only from polymorphisms in the *MFS1* promoter, which are associated with distinct levels of MDR, but also from additional heritable determinants. The polygenic architecture of MDR revealed by our GWAS approach reflects perennial adaptation to fungicide selection pressure and is consistent with the diversity of mutations identified in experimentally evolved strains. Notably, we validated the involvement of two newly identified mutations in MDR: a gain-of-function mutation in a transcription factor selected *in vitro*, and a novel indel in the *MFS1* promoter ($P_{MFS1}^{typeVIII}$) identified in a field strain. Altogether, these results shed light on components of the regulatory

pathways underlying MDR in *Zymoseptoria tritici* as an adaptive response to fungicide exposure.

Mutations in transcriptional regulator encoding genes and stress response

In independent isolates selected under fungicide alternation regimes, we identified ZtAtrR-C740Y, a missense substitution in a protein homologous to the *A. fumigatus* transcription factor AtrR. In *A. fumigatus*, AtrR is a key regulator of oxidative stress responses and azole resistance, acting through the transcriptional control of genes involved in ergosterol biosynthesis as well as the ABC transporter Cdr1B (Hagiwara et al., 2017; Paul et al., 2023, 2019), in cooperation with several coactivators (Paul et al., 2023, 2022). Notably, the C-terminal region of AtrR has been shown to be critical for *Cdr1B* expression regulation (Ror et al., 2024).

Although we did not observe a comparable role for ZtAtrR in oxidative stress response, the C740Y mutation, located in the C-terminal region of the protein, conferred resistance to multiple fungicide classes, including DMIs (prothioconazole, mefentrifluconazole), SBI class IV compounds, and inhibitors of mitochondrial respiration. These results suggest that ZtAtrR may fulfill a conserved function in fungicide resistance, potentially through interactions with coactivators and the regulation of functionally similar target genes. Consistent with this hypothesis, we identified orthologs of known AtrR coactivators and target genes in *Z. tritici*. Ongoing analyses of their expression patterns in the wild type and the *ZtAtrR-C740Y* background should provide further insight into the extent of functional conservation between these two fungi.

Beyond future prospects for dissecting the regulatory pathways associated with ZtAtrR-C740Y, one particularly intriguing finding of this thesis helps to propose potential links between ZtAtrR, fungicide resistance, and global stress response pathways. Specifically, we identified a strong antagonistic interaction between the efflux pump inhibitor chlorpromazine and terbinafine on fungal growth, observed exclusively in ZtAtrR-C740Y strains. To our knowledge, such an interaction between these two compounds has not been previously reported. In contrast, most studies have described either synergistic or neutral effects on fungal development, often highlighting the potential of chlorpromazine to re-sensitize MDR pathogens to antifungal drugs (Hayashi et al., 2003; Marchetti et al., 2000; Roohparvar et al., 2007b).

However, beyond its role as a transporter inhibitor, chlorpromazine exerts a wide range of cellular effects, notably through alterations of membrane structure and the induction of oxidative and endoplasmic reticulum (ER) stress responses (Maruoka et al., 2007; Matteoni et al., 2021; Smith, 1977). Importantly, chlorpromazine has also been reported to inhibit calcineurin, thereby stimulating Ca^{2+} influx and activating MAP kinase signaling in budding yeast (Bonilla and Cunningham, 2003; Kamada et al., 1995). In another study, transient cytoplasmic Ca^{2+} accumulation resulting from mitochondrial dysfunction were shown to promote persistent nuclear localization of the zinc-finger transcription factor CrzA in *C. albicans*, leading to global overexpression of multidrug transporter genes and, ultimately, azole resistance (Song et al., 2020). Although the precise mechanisms underlying ZtAtrR-C740Y mediated resistance remain to be elucidated, these observations allow us to hypothesize the involvement of a similar regulatory pathway. In the model we propose, chlorpromazine-induced Ca^{2+} influx acts as a signaling trigger, and the downstream response, requiring ZtAtrR-C740Y but not the wild-type ZtAtrR, leads to overexpression of transporter genes (Li et al., 2023; Song et al., 2020). This response could account for the reduced toxicity of terbinafine observed under chlorpromazine exposure. Supporting this hypothesis, ZtAtrR-C740Y strains display altered morphology compared to the wild type under fungicide treatment, notably a white, filamentous growth suggestive of modified melanin production. Melanin biosynthesis is known to be induced in response to multiple stresses and is regulated by mitogen-activated protein kinase (MAPK) pathways, including Ca^{2+} /calcineurin signaling, as reported in *Alternaria alternata* (Chhoker et al., 2025; Y. Yang et al., 2022). Taken together, the antagonistic interaction between terbinafine and chlorpromazine may therefore indicate a specific role for ZtAtrR-C740Y in coordinating responses to global cellular stresses linked to Ca^{2+} signaling and MAPK-dependent regulatory networks.

Beyond ZtAtrR, we identified a diverse set of mutated transcription factors in both the IPO323 and R_SDHI experimentally evolved strains. Investigating the roles of these factors will contribute to a deeper understanding of the molecular mechanisms underlying MDR, including the regulatory networks controlling transporter genes, other transcription factors, and genes encoding metabolic enzymes.

Mutations in transporter and enzyme encoding genes

We also identified mutations in detoxification genes and in their promoter regions, constituting additional candidate determinants of MDR shared between field populations and experimentally evolved strains.

In field strains, we reported an extended transposon insertion polymorphism in P_{MFS1} that is responsible for quantitative variation in terbinafine resistance, together with candidate SNPs identified by GWAS located near transporter genes that may contribute to the quantitative shifts in MDR levels observed in natural populations. Among these transporters, the Flu1-like transporter is of particular interest, as Flu1 has been shown to confer resistance not only to fluconazole but also to terbinafine in *C. albicans* (Calabrese et al., 2000; Sanglard et al., 1997). Beyond transporters, we identified several candidate enzymes potentially involved in fungicide detoxification. Although metabolic resistance mechanisms have not frequently been reported in fungal human and plant pathogens, they are well documented contributors to MDR in insects and plants (Amezian et al., 2021; Cummins et al., 2013; De Rouck et al., 2023). Notably, genes encoding short-chain dehydrogenase/reductases (SDRs) emerged as candidates in both the GWAS and the analysis of experimentally evolved strains, alongside genes encoding glutathione S-transferases (GSTs) and cytochrome P450 monooxygenases (CYP450s) in the latter. While GSTs and CYP450s are widely recognized for their roles in drug resistance across diverse organisms (De Rouck et al., 2023; Karunker et al., 2008; Pavlidi et al., 2017; Sang et al., 2018), the contribution of SDRs to drug and pesticide resistance has been largely underexplored. However, their overexpression in resistant spider mites suggests that they may represent strong and underexplored candidates for MDR in fungal pathogens as well (De Rouck et al., 2023; Xue et al., 2020).

Our findings reveal a multigenic determinism of MDR in natural populations, mechanistically supported by the diversity of candidate genes identified through our complementary approaches. In addition, the wide range of efflux phenotypes observed in experimentally evolved strains points to the coexistence of MDR mechanisms that are either dependent on, or independent from, enhanced drug transport. This observation supports a potential contribution of metabolic detoxification enzymes to MDR in both *in vitro*-evolved isolates and field strains.

Altogether, our results resonate with the landmark study by Sang et al. (2018), which uncovered the role of a master transcriptional regulator of MDR in *Claviceps homoeocarpa* (ShXDR1). This regulator was shown to control the expression of multiple genes involved in a global detoxification pathway, encompassing both transporters and detoxification enzymes. Prior to that work, such an organized detoxification system, structured into phases I, II, and III as described in plants and insects, had not been reported in *fungi*. Building on this conceptual framework, identifying a comparable master regulator in *Z. tritici* may prove instrumental in elucidating the regulatory links among the candidate genes involved in MDR. This includes trans-acting regulatory factors controlling the expression of the major MDR determinant *MFS1*, as well as the contribution of the newly described transcription factor ZtAtrR.

Implications for resistance risk management

In this work, we documented that duplication of chromosome 7, associated with prothioconazole resistance, was obtained *in vitro* under both benzovindiflupyr (SDHI) / carbendazim (MBC) and mefentrifluconazole (DMI) / fluxapyroxad (SDHI) alternation regimes. In contrast, the ZtAtrR-C740Y mutation arose only under benzovindiflupyr (SDHI) / prothioconazole (DMI) alternation regimes. Notably, combinations of DMI and SDHI are commonly recommended for second wheat fungicide treatments in France (ARVALIS, 2016). Although NTSR can also emerge under single-fungicide selection, as reported by (Gutiérrez-Alonso et al., 2017), through overexpression of ABC transporters and GSTs under SDHI pressure, heterogeneous environments are known to enhance genetic diversity (Bell and Reboud, 1996; Kassen, 2002). While heterogeneous fungicide selection pressure strategies can slow down the evolution of specialist resistance, alternation and mixtures favor the selection of generalist phenotypes by shaping novel mutational landscapes (Ballu et al., 2024, 2023, 2021; Comont et al., 2020; Kim et al., 2014; Lagator et al., 2013).

Although often overlooked in field studies, NTSR substantially increase resistance factors (RF) when combined with TSR. In French populations, MDR reaches an annual frequency of approximately 25% (until 40 %) (INRAE, ANSES, ARVALIS, 2025), potentially posing a greater threat to future fungicide efficacy than TSR alone. Similar patterns are observed in weeds and insects, where NTSR combined with TSR is associated with high RFs and unexpected resistance profiles (Bass et al., 2015; De Rouck et al., 2023; Gaines et al., 2020; Mitchell et al., 2014).

These findings underscore the need for more in-depth investigations of MDR mechanisms in *Z. tritici* to better understand field selection dynamics and accurately evaluate the risk posed to current and future active ingredients (AIs), including biocontrol molecules. Indeed, our lab has shown that overexpression of *MFS1* can confer resistance to a natural compound currently under development for biocontrol (data not shown, personal communication). Consequently, even if synthetic fungicides were to be phased out in the future, MDR could still compromise alternative disease control strategies.

Altogether, these results highlight the importance of integrating MDR risk into resistance management strategies. The high genomic plasticity witnessed in this study and reported in many others, allows the fungus to explore a broad adaptive landscape, potentially accelerated by fungicide exposure. Additional studies are needed to deepen our understanding of this phenotype and to identify reliable markers for field monitoring, as conventional genotyping based solely on P_{MFS1} length polymorphism insufficient to capture the dynamics of MDR evolution in natural populations.

General conclusion and prospects

In this work, we highlighted key genetic drivers and molecular mechanisms underlying MDR in both experimentally evolved strains and contemporaneous European field populations of *Zymoseptoria tritici*. By combining resistance phenotyping, efflux capacity assessment, and genomic analyses, we highlighted the diversity of non-target-site resistance (NTSR) mechanisms contributing to MDR, and identified factors potentially accelerating their acquisition, including copy number variations and hypermutation.

From a practical standpoint, we further validated the critical role of P_{MFS1} plasticity in field MDR, uncovering new transposon insertion polymorphisms (TIPs) in its promoter. We also concluded that quantitative variations, likely driven by polygenic determinism, underlie shifts in MDR levels among contemporary European field strains. These observations highlight the need for careful monitoring to accurately assess the risks associated with this generalist resistance phenotype. Moreover, as suggested by Ballu and collaborators (2023, 2024), we demonstrated that balanced fungicide selection pressures preferentially select NTSR, as no target-site resistance (TSR) was identified in IPO323 evolved isolates. In these isolates, resistance phenotypes are solely driven by NTSR, whose broad spectrum underscores their potential impact on the sustainability of chemical control strategies.

Our results call for better integration of MDR considerations into resistance management strategies. This requires fine-scale monitoring of the phenotype in the field, development of new molecular markers, and quantitative phenotyping to track shifts in MDR in natural populations. Future work should dissect the role of ZtAtrR as a gateway to characterize MDR regulatory pathways, enabling marker development and elucidation of interactions among the molecular actors identified in this study. Additionally, it will be crucial to validate the role of aneuploidy in MDR acquisition, its stability under selective and non-selective conditions, and its contribution to adaptation *in natura*, as well as to assess the prevalence and impact of hypermutator phenotypes in field populations.

Finally, our findings emphasize that the mechanisms selected in *Z. tritici* are shared with other fungal pathogens and even across organisms exposed to drugs or pesticides. This comparative perspective facilitates functional validation, as exemplified by the homology with an *A. fumigatus* protein, that guided the successful characterization of ZtAtrR-C740Y in MDR. Broadly, these results situate this work within a global One-Health framework: MDR represents a worldwide health and food security concern, highlighting the importance of cross-disciplinary cooperation, where advances in clinical research can inform phytopathology and *vice versa*.

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