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Assessing the risk of resistance selection towards QiI fungicides in *Zymoseptoria tritici*

Guillaume Fouché

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Estimation du risque de sélection de la
résistance aux fongicides de type Qil chez
Zymoseptoria tritici.
*Assessing the risk of resistance selection towards
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Thèse de doctorat de l'université Paris-Saclay

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Spécialité de doctorat : Biologie
Unité de recherche : Université Paris-Saclay, INRAE, AgroParisTech, UMR BIOGER,
78850, Thiverval-Grignon, France.
Réfèrent : Faculté des sciences d'Orsay

**Thèse présentée et soutenue à Paris-Saclay,
le 24/06/2021, par**

Guillaume Fouché

Composition du Jury

Marie Dufresne Professeure (Université Paris-Saclay)	Présidente
Bart Fraaije Program leader, HDR (NIAB, UK)	Rapporteur & examinateur
Francesca Zito Directrice de recherche (Université Paris Diderot)	Rapportrice & examinatrice
Jérôme Clain Maître de conférence, HDR (Université Paris Descartes)	Examineur
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David Young Principal scientist (Corteva™ Agriscience, USA)	Co-encadrant (tuteur en entreprise)

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*In loving memory of
Catherine and Gilbert Fouché.*

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LIST OF ABBREVIATIONS

- ABC:** ATP-binding cassette
- ADP:** adenosine diphosphate
- AOX:** alternative oxidase
- ATP:** adenosine triphosphate
- AUC:** area under the curve
- AZ:** azoxystrobin
- BEN:** benzovindiflupyr
- bp:** base pairs
- BSA:** bovine serum albumin
- CCCP:** m-chlorophenylhydrazine
- CoA:** coenzyme A
- Cyt *c*:** cytochrome *c*
- ddPCR:** digital droplet PCR
- DMI:** demethylation inhibitor
- DMSO:** dimethyl sulfoxide
- DNA:** deoxyribonucleic acid
- EDTA:** ethylenediaminetetraacetic acid
- ETC:** electron transport chain
- FAD(H₂):** (dihydrogenated) flavin adenine dinucleotide
- FHB:** *Fusarium* head blight
- FPX:** fenpicoxamid
- FRAC:** fungicide resistance action committee
- GWAS:** genome-wide association study
- Ha:** hectare
- HIV:** human immunodeficiency virus
- I₅₀:** 50% inhibitory concentration of enzyme activity
- IC₅₀:** 50% inhibitory concentration
- IMM:** inner mitochondrial membrane

IMS: inter-membrane space

kDa: kilo Dalton

Mb: megabases

MDR: multidrug resistance

MFS: major facilitator superfamily

Mia: billion

MIC: minimal inhibitory concentration

MoA: mode of action

NAD(H): (hydrogenated) nicotinamide adenine dinucleotide

OD: optical density

OMM: outer mitochondrial membrane

OXPHOS: oxydative phosphorylation

PAMP: pathogen-associated molecular pattern

PCR: polymerase chain reaction

pH: potential hydrogen

PRO: prothioconazole desthio

q: quintal (equal to a thousand tonnes)

Q_i site: ubiquinone inner binding site

Q_iI: Q_i site inhibitor

Q_{io}I: inhibitor of the Q_i and Q_o sites

Q_o site: ubiquinone outer binding site

Q_oI: Q_o site inhibitor

QTL: quantitative trait locus

rDNA: ribosomal DNA

RF: resistance factor

ROS: reactive oxygen species

SC: supercomplexes

SDH: succinate dehydrogenase

SDHI: succinate dehydrogenase inhibitor

SHAM: salicylhydroxamic acid

SNB: *Stagonospora nodorum* blotch
SNP: single nucleotide polymorphism
STB: *Septoria tritici* blotch
StrR: strobilurin resistance
TCA: tricarboxylic acid
TE: transposable element
UQ: ubiquinone
UQH₂: ubiquinol
UV: ultraviolet
WT: wild-type

SYNTHESE

La résistance aux fongicides représente un défi majeur pour la protection des plantes, et soulève la question théorique de la prédiction et de la réduction de l'adaptation chez les champignons. Cette thèse illustre cette problématique en étudiant l'évolution du pathogène fongique *Zymoseptoria tritici*, responsable au champ de la septoriose du blé, et du fenpicoxamide, représentant un nouveau mode d'action anti-septoriose en cours de développement, et inhibant la respiration mitochondriale en se liant au cytochrome *b*, au niveau du site de fixation interne de l'ubiquinone (site Q_i). Nous avons abordé trois questions principales : 1/ Quel(s) est/sont le(s) mécanisme(s) de résistance le(s) plus susceptibles(s) d'émerger ? ; 2/ Quel est le risque pratique de résistance au fenpicoxamide en conditions contrôlées ? ; 3/ Quelles seraient les stratégies optimales pour réduire ce risque de résistance au fenpicoxamide ?

En utilisant l'évolution dirigée, nous avons pu identifier la résistance de cible, et en particulier une substitution au niveau du site Q_i du cytochrome *b* : G37V, comme le mécanisme de résistance le plus probable. Ce résultat est cohérent avec le fait que la résistance de cible est le mécanisme de résistance le plus fréquemment décrit chez les champignons phytopathogènes, le fait que G37V est compatible avec la substitution du site Q_o G143A qui domine dans les populations européennes, le fait que G37V est la seule substitution que nous ayons sélectionnée, indépendamment du fond génétique, et le fait que G37V est sujet à l'évolution parallèle observée chez d'autres taxons résistants aux QiIs. Les isolats G37V présentaient des facteurs de résistance élevés contre le fenpicoxamide, à la fois au niveau cellulaire et enzymatique. En effet, les tests de croissance *in vitro* ont montré que G37V entraînait de forts niveaux de résistance au fenpicoxamid, mais accroissait également la sensibilité aux QoIs. Les doubles mutants G37V+G143A étaient également résistants aux QiIs mais avec des facteurs de résistance plus faibles comparés aux simples mutants. Ces observations tendent à montrer que les substitutions des deux sites influent sur le transfert des électrons d'un site du cytochrome *b* à l'autre. Au niveau enzymatique, les observations faites sur la croissance ont été confirmées. En l'absence de fongicides, la substitution G37V entraînait également une réduction de l'activité enzymatique de 50%, sans toutefois avoir d'impact visible sur la croissance *in vitro*. Les premiers résultats ont également révélé l'absence d'effet de cette réduction d'activité enzymatique sur la pathogénicité, chez des mutants portant les altérations du cytochrome *b* G37V et G143A. Les tests *in planta* indiquaient par ailleurs un fort impact de la substitution G37V sur l'efficacité du fenpicoxamide. L'ensemble de ces résultats suggère un risque élevé de résistance en pratique

pour le fenpicoxamide, et souligne l'importance primordiale d'implémenter des stratégies efficaces à retarder l'évolution de la résistance au champ. Par évolution expérimentale, nous avons donc évalué divers scénarios de déploiement de fongicides, incluant le fenpicoxamide, mais différant par le nombre de différentes substances actives, leurs risques inhérents de sélectionner la résistance, et la stratégie d'application (par exemple, en alternance ou en mélange). Les premiers résultats indiquaient que le développement de la résistance peut être significativement retardé par des mélanges incluant un partenaire avec un mode d'action différent et présentant une forte synergie avec le fenpicoxamide. En effet, la synergie augmente la dose relative de fongicide appliquée, et permet un contrôle suffisant des génotypes résistants. Nous discutons cependant des limites de cette stratégie.

Les résultats de cette thèse ont démontré que l'évolution expérimentale est un outil précieux chez *Z. tritici* pour établir des hypothèses quant à l'évolution des champignons pathogènes, pour ce qui concerne la résistance aux fongicides. D'un point de vue opérationnel, elle a ouvert de nouvelles perspectives pour mettre en œuvre des recommandations responsables préalablement à la mise sur le marché de nouveaux fongicides, en accord avec la demande d'une utilisation plus prudente des produits phytosanitaires d'origine synthétique.

INTRODUCTION

1. WHEAT IN WORLD CEREAL PRODUCTION.

1.1. Economic importance of wheat.

Cereals are annual monocotyledonous grasses producing grains from the *Graminaceae* (syn. *Poaceae*) family, and commonly referred to collectively as grasses. 700 million hectares (Mha) of cereals are cultivated all over the world, representing almost half of arable land. The biggest cereal producers are China, the United States (US) and the European Union (EU). The US is also the world's largest cereal exporter, while Japan is the world's largest importer. In terms of crop, corn is the most cultivated cereal in the world, followed by wheat and rice (Fig. 1). In the EU, the trend is slightly different as wheat stands as the most commonly cultivated cereal. Together, wheat, corn and barley represent almost 90% of the European cereal production. Three-quarters of this production are concentrated in just eight countries, of which France (26% of total European production), Germany and Romania are the most important producers (Fig. 1) (Passion céréales, 2019).

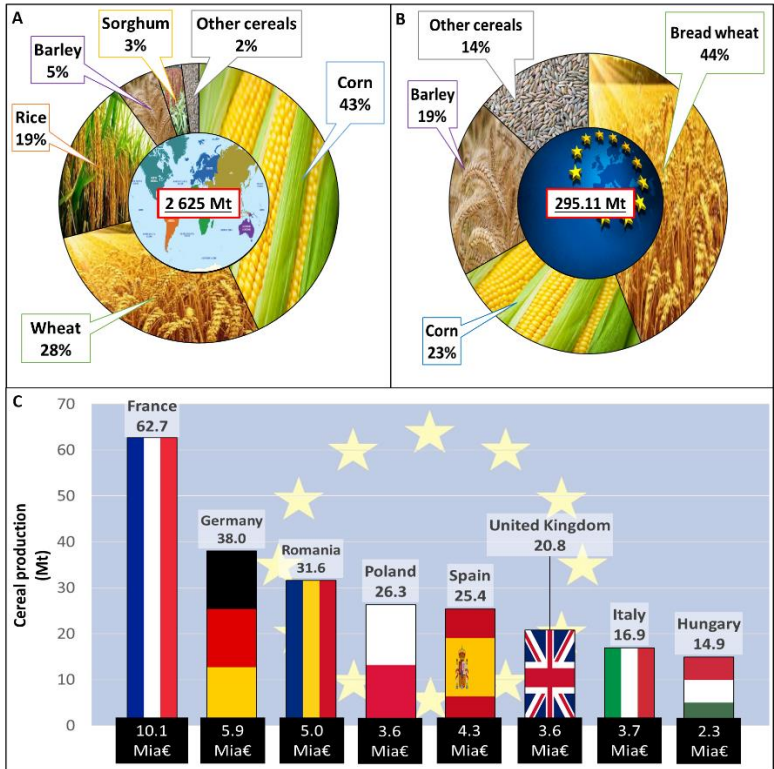


Figure 1. Distribution of cereal production in the world and Europe (adapted from Passion céréales, 2019). **A.** Worldwide cereal production in 2018 (USDA, campaign 2018/2019). **B.** EU (28 countries) cereal production in 2018 (Eurostat). **C.** Largest cereal producing countries in the EU in 2018 (Eurostat).

As the world’s second most important cereal crop, wheat represents 28% of the world’s cereal production with an annual production of about 700 Mt worldwide. The biggest exporters of wheat are Russia, Canada and the US (International Trade Center), while the EU is the world’s

biggest wheat producer. In the EU, wheat represents 44% of cereal production (Fig. 1) and is therefore the most important crop economically (Passion céréales, 2019).

In Europe, France is the largest producer of bread wheat, followed by Germany and the United Kingdom (UK) (Fig. 2). France is also the biggest European exporter of wheat, and the fourth largest exporter in the world. France ranks second among European flour producers and tenth in the world (ANMF) (Passion céréales, 2019).

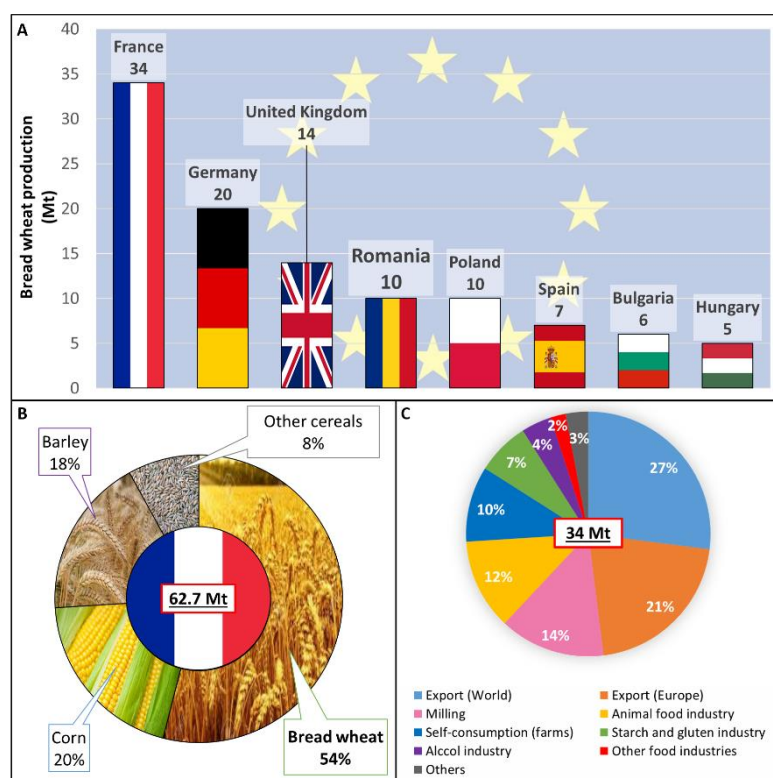


Figure 2. Bread wheat production in Europe and France (adapted from Passion céréales, 2019). **A.** Bread wheat production in Europe in 2018 (Eurostat). **B.** French cereal production in 2018 (SSP récolte 2018). **C.** Destination of the French bread wheat production in 2018 (ANMF).

1.2. Wheat and agriculture.

“Wheat” is a general term used to refer to several cereal species belonging to the *Triticum* genus. The most widely cultivated species is the common wheat or bread wheat, *Triticum aestivum*. This hexaploid hybrid species represents more than 90% of the world wheat production, followed by durum wheat, *Triticum durum*, a tetraploid hybrid species. Bread wheat is mainly cropped to produce flour, for the manufacture of various types of breads and other baking products, while durum wheat is mainly used to produce semolina for pasta and couscous production. Approximately 60% of the world production is intended for human food, and 30% for animal food. The remaining 10% of the production goes to the industrial production of starch, gluten and biofuels (Peña, 2002).

Winter wheat and spring wheat can be differentiated according to the time of year when they are cultivated. Both bread and durum wheat can be winter or spring wheats. Winter wheat is sown during fall and harvested in late spring or early summer of the following year (Fig. 3). It is grown in countries with moderate winters like Western Europe and most of the American production areas. Winter wheat needs a period of cold dormancy (generally 30 to 60 days with a temperature ranging from 0 to 5°C) before it can tiller and head. Spring wheat is sown during spring and harvested in late summer or early fall of the same year. It does not need cold dormancy and is preferentially cultivated in countries with very cold winters like Canada or Russia.

The BBCH stage 39 (Fig. 3), called the flag leaf stage, is a particularly critical stage of wheat development and corresponds to the full unrolling of the flag leaf. The flag leaf, also designated as the F1, is on its own responsible for about 50% of the grain filling, which justifies that wheat protection is focused on the protection of this leaf (Wazziki *et al.*, 2015). Combined with weather data, identifying the development stages helps in determining the adapted timing to apply treatments onto cereal fields, especially against fungal diseases.

2. MAIN WHEAT DISEASES.

The main diseases affecting wheat are detailed in Table 1. This list does not pretend to be exhaustive but focuses on the major pathogens and associated diseases, describing their most important traits.

2.1. Bacteria.

Bacterial diseases do not have a big impact on cereal production. With the exception of certain regions in the world with specific climates, bacterial diseases are not common and do not cause severe damage in cereal fields. The deployment of resistant crop varieties has been an efficient way to control these pathogens, even though most of them are ubiquitous bacteria.

Two main bacterial diseases can be observed on wheat. *Pseudomonas syringae* pv. *atrofaciens*, causing basal glume rot (Table 1) produces several antimicrobial and phytotoxic substances (syringomycin E and G, syringopeptin 25A and 25B), likely involved in the infection process (Vassilev *et al.*, 1996). The disease caused by *Xanthomonas campestris* pv. *translucens* is called bacterial leaf streak when happening on leaves, and black chaff when occurring on spikes (Table 1). Its impact on yield remains unnoticeable except in regions with high humidity like South America where it remains a serious threat (Sapkota *et al.*, 2020).

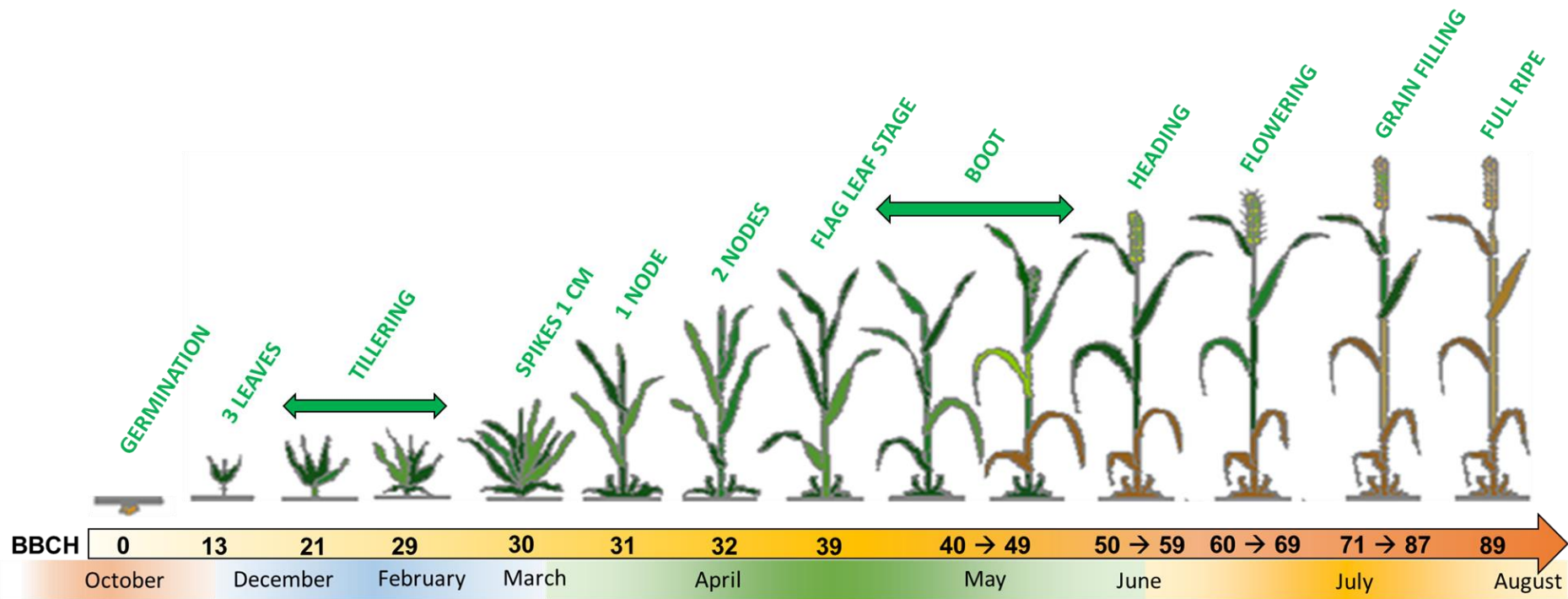


Figure 3. Developmental stages of winter wheat (Credits: Arvalis). The BBCH scale is used to score the developmental stages of various crops. The scale is divided into 10 main stages, numbered from 0 to 9, and 10 secondary stages for each main stage, also numbered from 0 to 9. The result is a two-digit code (from 00 to 99) that designates each stage of development (Lancashire *et al.*, 1991; Witzemberger *et al.*, 1989).




2.2. Viruses.


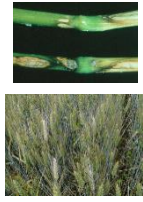





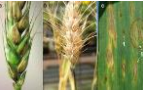
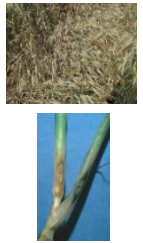
Contrary to bacteria, a large number of viral species are able to infect wheat. Virus transmission can occur in various ways but most of the time, fungi, nematodes, plasmodiophorids or insects are vectors (Lapierre and Signoret, 2004). The most common viral diseases are mosaic type diseases and yellowing diseases.




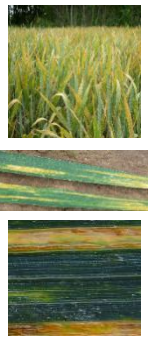



The wheat mosaic-causing viruses are transmitted by a soil plasmodiophorid called *Polymyxa graminis*, an obligate parasite of cereal roots (Table 1). The only way to control these viral diseases is the deployment of virus-resistant cultivars, as *P. graminis* resistance is not available in cereals. Managing *P. graminis* or its spores by chemical treatment of the soils is not possible due to obvious environmental concerns. Moreover, resting spores can survive in the soil for decades, so crop rotation is of no use either (Kanyuka *et al.*, 2003).

The *Barley yellow dwarf virus (BYDV)* can be transmitted by at least twenty-five aphid species, the most efficient vector being *Rhopalosiphum padi* (Table 1). The best management strategy in the case of *BYDV* is again the deployment of resistant cultivars, coupled with the control of aphid populations (Miller *et al.*, 1997).

Table 1. Main bacterial, viral and fungal diseases affecting wheat. (Picture credits Arvalis, INRAE and APS).

Type	Disease	Causing agent	Hosts	Symptoms	Geographic distribution	Impact	Disease management	Literature
Bacteria	Basal glume rot	<i>Pseudomonas syringae</i> pv. <i>atrovaciens</i>	Wheat, barley, rye, triticale, oats and other grasses		Central and Eastern Europe, regions with humid climates	Yield losses between 5 to 60%	Cultivar resistance	(Vassilev <i>et al.</i> , 1996)
	Bacterial leaf streak (leaves)	<i>Xanthomonas campestris</i> pv. <i>translucens</i>	Wheat, rice, triticale and barley		Ubiquitous	Low except in regions with highly humid climates	Seed treatment	(Xin <i>et al.</i> , 2018) (Sapkota <i>et al.</i> , 2020)
	Black chaff (spikes)							
Viruses	Mosaic	<i>Wheat yellow mosaic virus</i> <i>Soil-borne wheat mosaic virus</i>	Wheat		Vector: <i>Polymyxa graminis</i> (soil plasmodiophorid) North America, Western Europe	Yield losses can reach 100% depending on cultivar susceptibility	Cultivar resistance	(Kanyuka <i>et al.</i> , 2003)

Type	Disease	Causing agent	Hosts	Symptoms	Geographic distribution	Impact	Disease management	Literature
Viruses	"Yellowing"	<i>Barley yellow dwarf virus</i>	Wheat, barley, corn, oats, rice and other grasses		Vector: <i>Rhopalosiphum padi</i> and other aphid species Ubiquitous	Yield losses range between 5 to 90% depending on cultivar susceptibility	Cultivar resistance, control of aphid populations	(Pike, 1990) (Miller <i>et al.</i> , 1997)
Fungi	Sharp eyespot	<i>Rhizoctonia cerealis</i>	Cereals, turf grasses, sugar beet, cotton, potato, several legumes		Ubiquitous	Yield losses range from 5 to 25%	Seed fungicide treatment	(Hamada <i>et al.</i> , 2011) (Lemańczyk and Kwaśna, 2013) (Li <i>et al.</i> , 2017)
	Take-all	<i>Gaeumannomyces graminis</i>	Wheat, triticale, barley, rye and oat		Ubiquitous	Yield losses up to 50%	Crop rotation, tillage, seed treatment	(Freeman and Ward, 2004)
	<i>Stagonospora nodorum</i> blotch	<i>Parastagonospora nodorum</i>	Wheat, occasionally barley or rye		Main wheat producing regions of the world	Yield losses range from 5 to 30%	Seed and field fungicide treatments Agricultural practices	(Bearchell <i>et al.</i> , 2005) (Solomon <i>et al.</i> , 2006)
	<i>Fusarium</i> head blight	<i>Fusarium spp.</i> <i>Microdochium spp.</i>	Wheat and barley		Ubiquitous	Most of the time low but can reach 30 to 70% Grain contamination by mycotoxins	Seed treatment Cultivar resistance Fungicide treatments	(Aoki <i>et al.</i> , 2012) (Ji <i>et al.</i> , 2019)
	Common bunt of wheat	<i>Tilletia caries</i>	Wheat		Ubiquitous	Yield losses comparable to rusts (30-70%) if seeds are not treated	Seed treatment	(Schlaich <i>et al.</i> , 2006) (Mourad <i>et al.</i> , 2018)
	Loose smut of cereals	<i>Ustilago tritici</i>	Wheat, barley and rye		Ubiquitous	Yield losses can reach 40% if seeds are not treated	Seed treatment Cultivar resistance Late sowing	(Quijano <i>et al.</i> , 2016)
	Wheat blast	<i>Pyricularia graminis-tritici</i>	Wheat and several other grasses		Mainly Brazil and South America but recently detected in Bangladesh	High yield losses	Fungicides (seeds and field) Cultivar resistance	(Ceresini <i>et al.</i> , 2018)
	Eyespot or strawbreaker	<i>Oculimacula yallundae</i> <i>Oculimacula aciformis</i>	Wheat, barley, rye and triticale		Mainly North Western Europe and US, New Zealand	Yield losses range from 10 to 30% but can double in case of lodging	Resistant cultivars Culture rotation Prophylactic practices Fungicides	(Crous <i>et al.</i> , 2003) (Burt <i>et al.</i> , 2010) (Lewien <i>et al.</i> , 2018)

Type	Disease	Causing agent	Hosts	Symptoms	Geographic distribution	Impact	Disease management	Literature
Fungi	Black rust or stem rust	<i>Puccinia graminis f. sp. tritici</i>	Wheat		Mostly Africa, the Middle East and Southern and Western Europe	Yield losses range between 5 to 50%	Breeding for resistant cultivars Fungicide treatments	(Bhattacharya, 2017) (Soko <i>et al.</i> , 2018)
	Cereal powdery mildew	<i>Blumeria graminis f. sp. tritici</i>	Wheat		Main wheat producing regions of the world	Yield losses about 10%, 20% when spikes are contaminated, up to 40% for spring wheat	Resistant cultivars Prophylactic practices Fungicide treatments	(Leath, 1989) (Draz <i>et al.</i> , 2019)
	Tan spot or helminthosporiosis	<i>Pyrenophora tritici-repentis</i>	Wheat, occasionally barley or rye		North America, Europe	Yield losses range from 10 to 50%	Breeding for resistant cultivars Culture rotation (with soybean) Residue burial Fungicide treatments	(Rees and Platz, 1983)
	Yellow rust or stripe rust	<i>Puccinia striiformis f. sp. tritici</i>	Wheat		Asia, Europe, North America, South America, the Middle East and Africa	Yield losses range from 2% to 60%	Breeding for resistant cultivars Avoiding early and dense sowing Destruction of sprouting cereals Fungicide treatments	(Wellings, 2011) (Vergara-Diaz <i>et al.</i> , 2015) (Ye <i>et al.</i> , 2019)
	Brown rust or leaf rust	<i>Puccinia triticina</i>	Wheat, triticale and some grasses		Ubiquitous	Yield losses around 15% but can reach 40%	Breeding for resistant cultivars Avoiding early and dense sowing Destruction of sprouting cereals Fungicides	(Bolton <i>et al.</i> , 2008)
	Septoria leaf blotch	<i>Zymoseptoria tritici</i>	Wheat		Every wheat-growing region	Yield losses around 10% but can reach 50%	Breeding for resistant cultivars Prophylactic practices Fungicide treatments	(Goodwin <i>et al.</i> , 2011) (Fones and Gurr, 2015) (Steinberg, 2015)
	Ergot	<i>Claviceps spp.</i>	Rye mostly, triticale, wheat, barley, oats and forage or wild grasses		Ubiquitous	Low yield losses but harvest contamination by alkaloid-containing sclerotia	Residue burying Culture rotation	(Miedaner and Geiger, 2015) (Pažoutová <i>et al.</i> , 2015)

2.3. The main threat: fungal diseases.

By far, fungi and oomycetes represent the biggest threat for crop production worldwide. With the number of phytopathogenic species estimated at 8.000 (as compared with only 300 human pathogenic species), fungi and oomycetes are responsible for 70% of diseases affecting crops. Annual losses due to these species (pre- and post-harvest) represent a third of all the global food production, representing enough food to feed 600 million people (Savary *et al.*, 2019). Cereals, and especially wheat, are not spared by fungal pathogens.

2.3.1. Seed-borne diseases.

2.3.1.1. Diseases mainly affecting the roots and/or the stem.

Wheat sharp eyespot, triggered by the polyphagous basidiomycete *Rhizoctonia cerealis*, causes symptoms mainly on stems (Table 1; Fig. 4). Within 30 years, *R. cerealis* has become ubiquitous and can now be found on every continent, probably because of climate change, the use of susceptible wheat cultivars and its ability to infect many different hosts (Hamada *et al.*, 2011). Earlier, not considered as a serious threat in temperate regions, its importance in Europe is increasing annually. The fungus destroys the transport tissues of the plant, which leads to lodging and important yield losses. As no resistance to *R. cerealis* has been found in wheat cultivars yet, disease control relies on seed or early seedling fungicide treatments (Hamada *et al.*, 2011).

The “take-all” disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* is one of the most important root diseases of cereals and grasses. This soil-borne fungus causes root necrosis resulting in low growth, bad tillering and whitening of the whole plant. No cultivar resistance is available and foliar fungicide treatments are ineffective. The disease can only be controlled by agricultural practices and preventive seed treatment, especially by silthiofam (see 4.2.6.) (Freeman and Ward, 2004).

2.3.1.2. Diseases mainly affecting leaves.

Parastagonospora nodorum (former name *Stagonospora nodorum*) is an ascomycete causing the *Stagonospora nodorum* blotch (SNB) of wheat (Table 1; Fig. 4). Although it can be seed-borne, contaminations can also occur through both sexual and asexual spores produced on crop residues. SNB is mainly a foliar disease, but it can affect also grains, sheaths, leaves, spikes and

nodes. First symptoms appear on leaves, as small necrotic lesions containing pycnidia. Pycnidiospores are spread through splashing to upper foliar stages and to the nearest plants (Solomon *et al.*, 2006). Interestingly, SNB was the most important foliar blotch disease of wheat during the major part of the twentieth century, until 1980, in the UK (Bearchell *et al.*, 2005). In 1980, there was a switch between *Septoria tritici* blotch (STB) and SNB, with STB becoming largely predominant in fields. This switch may be correlated to changes in atmospheric pollution, due to human industrial activity and resulting SO₂ emissions. However, the underlying explanation for this correlation remains unclear, and a re-emergence of SNB cannot be excluded (Bearchell *et al.*, 2005). Today, SNB can still be found in French fields, with higher prevalence in southern and eastern regions, but with moderate impact on yields.

2.3.1.3. Diseases mainly affecting spikes.

The ascomycete species complex formed by *Fusarium spp.* and *Microdochium spp.* are responsible for *Fusarium* head blight (FHB) on spikes, but sometimes symptoms can be visible also on leaves (Table 1; Fig. 4). Infected seeds can germinate but seedlings often die. Surviving plants still carry the disease. *Fusarium* species secrete many important mycotoxins, highly toxic for animals (trichothecenes, deoxynivalenol, nivalenol and zearalenone) that can lead to the production of grains unfit for consumption (Ji *et al.*, 2019). These toxins are the biggest concern for FHB, because, despite its high prevalence, associated yield losses are of lesser importance.

Two basidiomycete species, *Tilletia caries* and *Ustilago tritici*, are also responsible for spike diseases (Table 1; Fig. 4). Grains infected by *T. caries* (common bunt) become swollen and are easily crushed during harvest. In this process, spores infect healthy grains, therefore transmitting the disease to the next generation. Common bunt used to be a devastating disease at the beginning of the twentieth century, but is now well controlled and only occasionally observed (Mourad *et al.*, 2018). Spikes infected by *U. tritici* (loose smut) are covered in a black powdery mass of chlamydospores, readily apparent. However, sometimes grain may appear healthy but is infected, allowing disease transmission to the seedling (Quijano *et al.*, 2016).

Thanks to seed treatments, grain contamination is now controlled most of the time in developed countries where large-scale farming is common. Nevertheless, seed contamination can remain a real problem in developing countries where small-scale farmers use their own seeds for sowing, or in organic farms where seeds are not treated (Mourad *et al.*, 2018).

Wheat blast, caused by *Pyricularia graminis-tritici* is a major threat in Brazil and South American countries. However, the detection of an epidemic breakthrough in Southeast Asia after the import of contaminated Brazilian wheat grains led to the implementation of systematic quarantine measures for Brazilian wheat intended for export (Ceresini *et al.*, 2018).

2.3.2. Air-borne and rain-borne diseases.

2.3.2.1. Diseases mainly affecting the stem.

Oculimacula yallundae and *Oculimacula aciformis* are ascomycete species responsible for cereal eyespot (Fig. 4), also referred to as strawbreaker (Table 1). Lesions are very similar to those of sharp eyespot and lead to the lodging of infected plants, increasing yield losses (Lewien *et al.*, 2018).

Puccinia graminis f. sp. tritici is a basidiomycete causing black rust or stem rust on wheat (Table 1; Fig. 4). *Puccinia spp.* have complex life cycles involving secondary hosts and several different spore types. Stem rust is rare in France but the recent outbreak of a virulent race of stem rust in Sicily in 2016 suggests that climate change, coupled with fungal adaptation to resistant wheat varieties, could potentially lead to the re-emergence of stem rust in Europe (Bhattacharya, 2017; Soko *et al.*, 2018).

2.3.2.2. Diseases mainly affecting leaves.

Blumeria graminis (former name *Erysiphe graminis*) is an ascomycete responsible for cereal powdery mildew (Table 1; Fig. 4). It can infect all cereal crops but under different *formae speciales* with strict host specificity. Symptoms usually start at the bottom of the plant where the humidity is higher. Mainly visible on leaves, symptoms can also develop on stems and spikes (Leath, 1989). However, this disease has become rare in France over the last few years due to the deployment of resistant cultivars (Walker A. S., personal communication).

Pyrenophora tritici-repentis is a necrotrophic ascomycete causing the tan spot disease also called helminthosporiosis (Table 1; Fig. 4). This species is characterized by its large ascospores that can be spread by the wind, but not far due to their size. After spore germination, the mycelium penetrates the leaf surface through apleria formation and symptoms are therefore mainly visible on leaves (Fig. 4). Tan spot is an important disease in North America and Europe with a low prevalence but capable of causing severe crop damage (Rees and Platz, 1983).

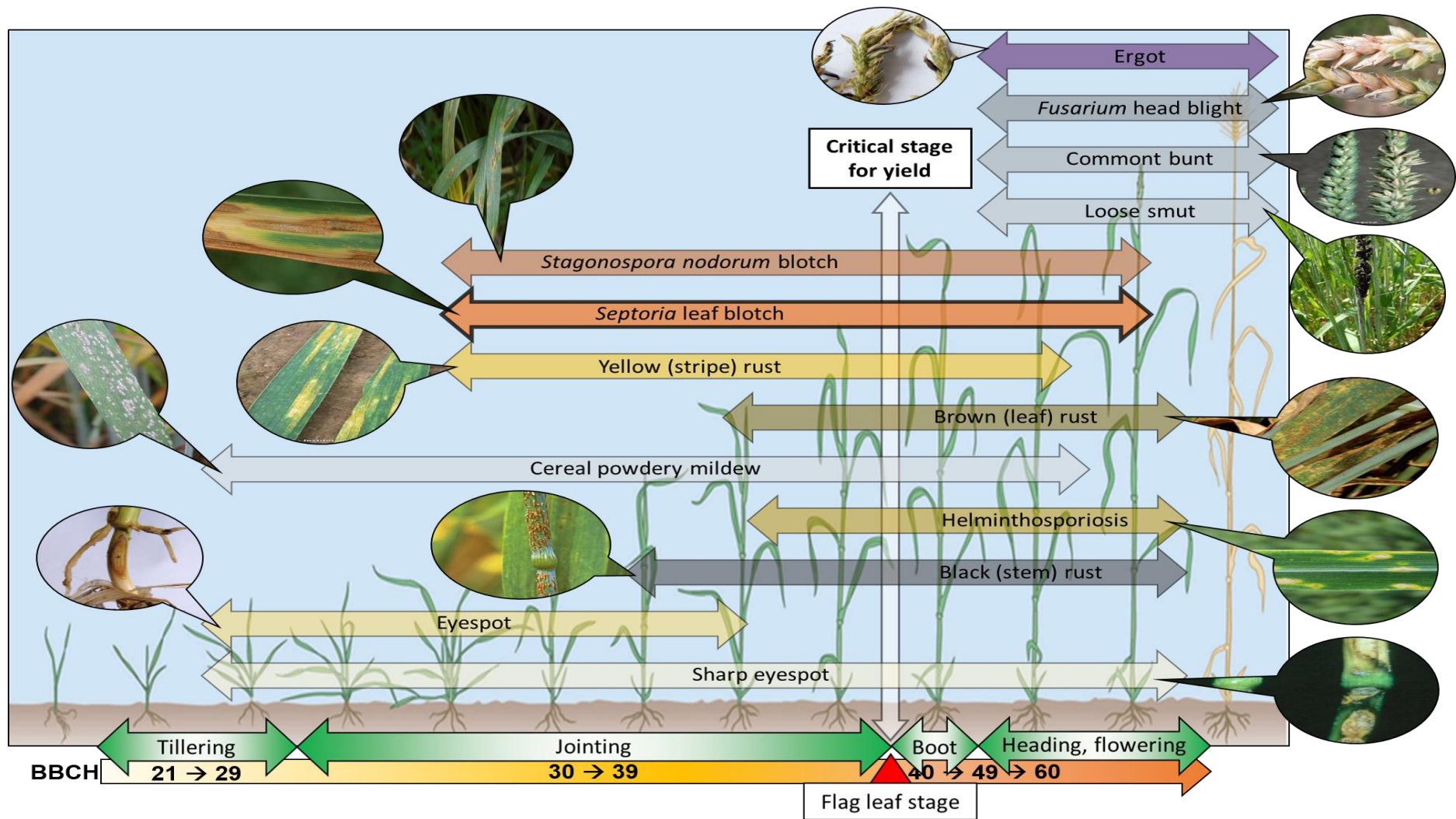


Figure 4. Main fungal diseases of wheat and corresponding disease periods. Arrows correspond to the periods during which the main disease symptoms can be visible, according to the wheat development stages. From bottom to top, diseases affecting stems, leaves and spikes.

The two species, *Puccinia striiformis* f. sp. *tritici* and *Puccinia triticina* are basidiomycetes responsible for wheat yellow rust and brown rust respectively (Table 1; Fig. 4). Symptoms are very similar and mainly visible on leaves for both species. Yellow rust symptoms are hard to detect at first, but suddenly spread very fast in the field, partly due to the high sporulation capacity of *P. striiformis*. Yellow rust frequency remains lower than that of other foliar diseases but is very damaging to the crop. Moreover, its importance has sharply increased in terms of frequency, geographic distribution and epidemic intensity over the past decade, partly due to climate change, and to the emergence of the “Warrior” races of *P. striiformis*, which are more aggressive, and more tolerant to UV and higher temperatures. In addition, a rapid loss of cultivar resistance poses major problems for rust control in the field (Vergara-Diaz *et al.*, 2015; Wellings, 2011).

Brown rust is a more common disease of wheat than yellow and black rusts. In cases of severe infection, brown rust pustules can appear on both stem and glumes (Fig. 4). Yield losses are mainly due to a decrease in the photosynthetic foliar surface, and sometimes to a decrease in grain quality when spikes are infected. Just like yellow rust, the status of brown rust is currently changing. For a long time, brown rust was not considered a major disease even though it was often present in fields. However, with climate change triggering milder winters and warmer springs, and the selection of *P. triticina* strains able to bypass cultivar resistance, brown rust has recently become a much more consistent threat (Bolton *et al.*, 2008).

Zymoseptoria tritici, causal agent of STB (Table 1; Fig. 4), is the most important wheat pathogen regarding disease prevalence, yield losses and cost of disease control in Western Europe. Section 3 is the focus of this species and associated disease.

2.3.2.3. Diseases mainly affecting spikes.

The major air-borne spike disease is the ergot caused by *Claviceps spp.* (Table 1; Fig. 4). Long attributed to *Claviceps purpurea* alone in temperate regions, recent studies showed that the disease is triggered by at least four cryptic species (Pažoutová *et al.*, 2015). *Claviceps spp.* are ovarian parasites and contaminations can occur through air-borne ascospores, air-borne infected pollen grains, insects, rain splashing or head to head contacts (Miedaner and Geiger, 2015). The biggest problem with ergot is not yield losses, usually ranging between 5 to 10%, but the production of toxin-containing sclerotia on spikes that contaminate the harvest. Sclerotia contain toxic alkaloids, causing a disease called “ergotism” in humans and animals consuming

contaminated grain or flour. Therefore, ergot content after harvest is legally limited for both human and animal food (Haque *et al.*, 2020; Miedaner and Geiger, 2015). In the field, disease management relies mostly on prophylactic measures, but the recent discovery of QTLs associated with cultivar resistance in durum wheat may lead to development of new tools for disease control (Gordon *et al.*, 2020). Well controlled for many years, ergot has recently reappeared in France, mostly due to a loosening of soil preparation practices, and to a reduction in fungicide use on rye (Miedaner and Geiger, 2015).

3. ZYMOSEPTORIA TRITICI, CAUSAL AGENT OF SEPTORIA TRITICI BLOTCH.

3.1. Economic impact of *Septoria tritici* leaf blotch in Europe.

The economic importance of wheat outlined in the previous chapter makes any potential yield loss a serious concern for Europe. Northern France, Germany and the UK are part of a geographic zone where sea greatly influences weather. The resulting humid climate creates favorable conditions for *Z. tritici* development (see section 3.5.). Coupled with large-scale wheat-growing areas in these regions, these climatic conditions have contributed to make *Z. tritici* the number one threat to European wheat production. In spite of all control methods deployed in European fields, *Z. tritici* remains a persistent pathogen that causes variable epidemics every year. In fields where STB-tolerant cultivars are grown and fungicide treatments are applied, annual yield losses due to STB range between 5 to 10% (120-700 M€). Losses may reach 20% with cultivars more susceptible to STB, and even 50% during high disease pressure years (Fones and Gurr, 2015; Savary *et al.*, 2019).

In addition to potential yield losses, disease control incurs heavy costs, mainly because of chemical control. The cereal fungicide market was estimated at 1.8 Mia€ in 2014 in Europe, with more than half (1 Mia€) dedicated to STB control alone (Torriani *et al.*, 2015). The main difficulty is in balancing the cost of fungicide treatments with the potential damage from STB each year. In France, for example, due to a rather dry spring in 2019, STB appeared later, triggering low yield losses. The average loss was 10.2 q/ha in commercial fields, slightly lower than the 11.5 q/ha assessed in trials (Fig. 5) (Arvalis, 2019). Average severity is generally estimated in trials while comparing yields of treated plots and untreated plots in the same conditions (cultivar, weather...). Yield gain in the treated plots is therefore a proxy for disease severity, including STB but also other diseases. This explains why STB severity might be overestimated in trials. Arvalis has conducted more than 400 such trials in 20 French regions

between 2004 and 2019. During this period, annual yield gains due to fungicide treatments were very variable (Fig. 5), mostly because STB epidemics strongly depend on weather conditions (see section 3.5.). This underlines the difficulty in predicting the ratio between the cost of fungicide treatments and the gains to be made each year (Arvalis, 2019).

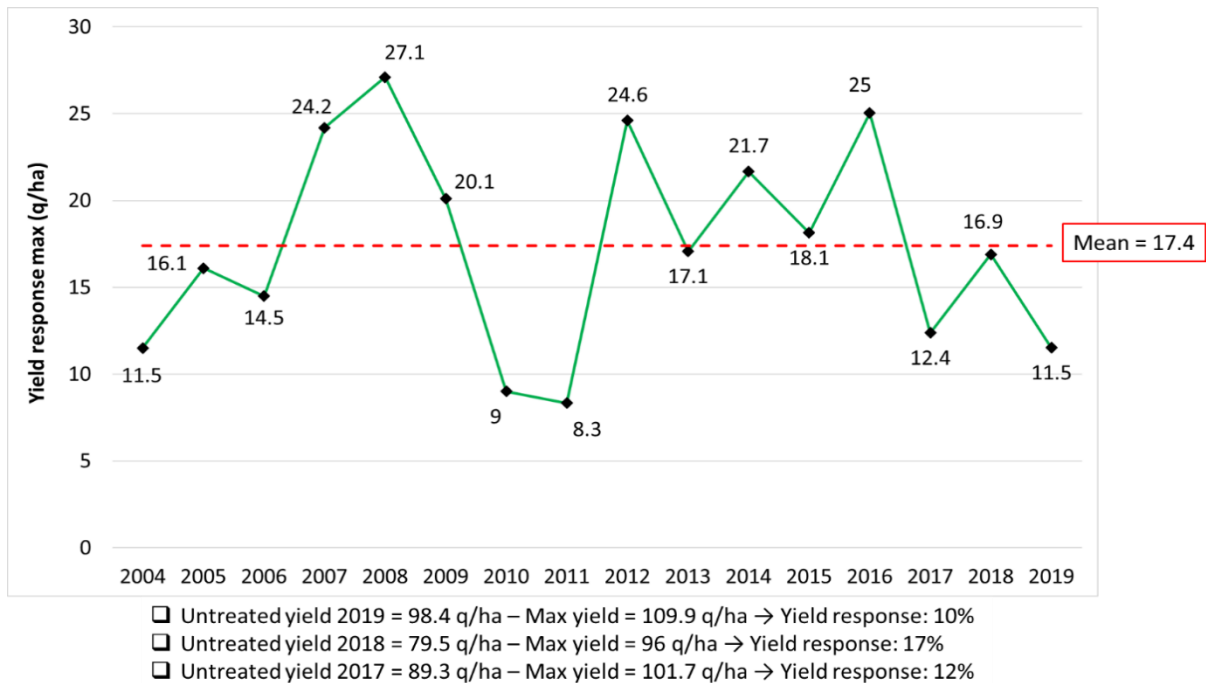


Figure 5. Yield gain associated with optimal fungicide treatments in France over the past fifteen years (Arvalis, 2019). Yield response to treatments is used as an estimate of yearly disease severity, in particular for STB.

3.2. Systematics.

Zymoseptoria tritici is the name commonly used today for the pathogenic species responsible for STB on wheat, since the nomenclature of fungi was unified (Wingfield *et al.*, 2012). Formerly called *Mycosphaerella graminicola* (teleomorph) or *Septoria tritici* (anamorph), it is an ascomycete species belonging to the *Dothideomycete* class and the *Mycosphaerellaceae* family. *Dothideomycetes* encompass several thousand phytopathogenic species, along with opportunistic human and animal pathogens. *Dothideomycetes* are generally specialized plant pathogens, with narrow host ranges, but they can infect very different plant species and are therefore distributed all over the world. Most of them are hemibiotrophs causing leaf spot symptoms without killing their host (Goodwin *et al.*, 2004). The genus *Mycosphaerella* first regrouped many species, which are now divided into different genera like *Cercospora*, *Septoria*

or *Ramularia*. Based on 28S rDNA, species with *Graminaceae* hosts now cluster outside the genus *Septoria*, and regroup inside the new genus *Zymoseptoria* (Fig. 6).

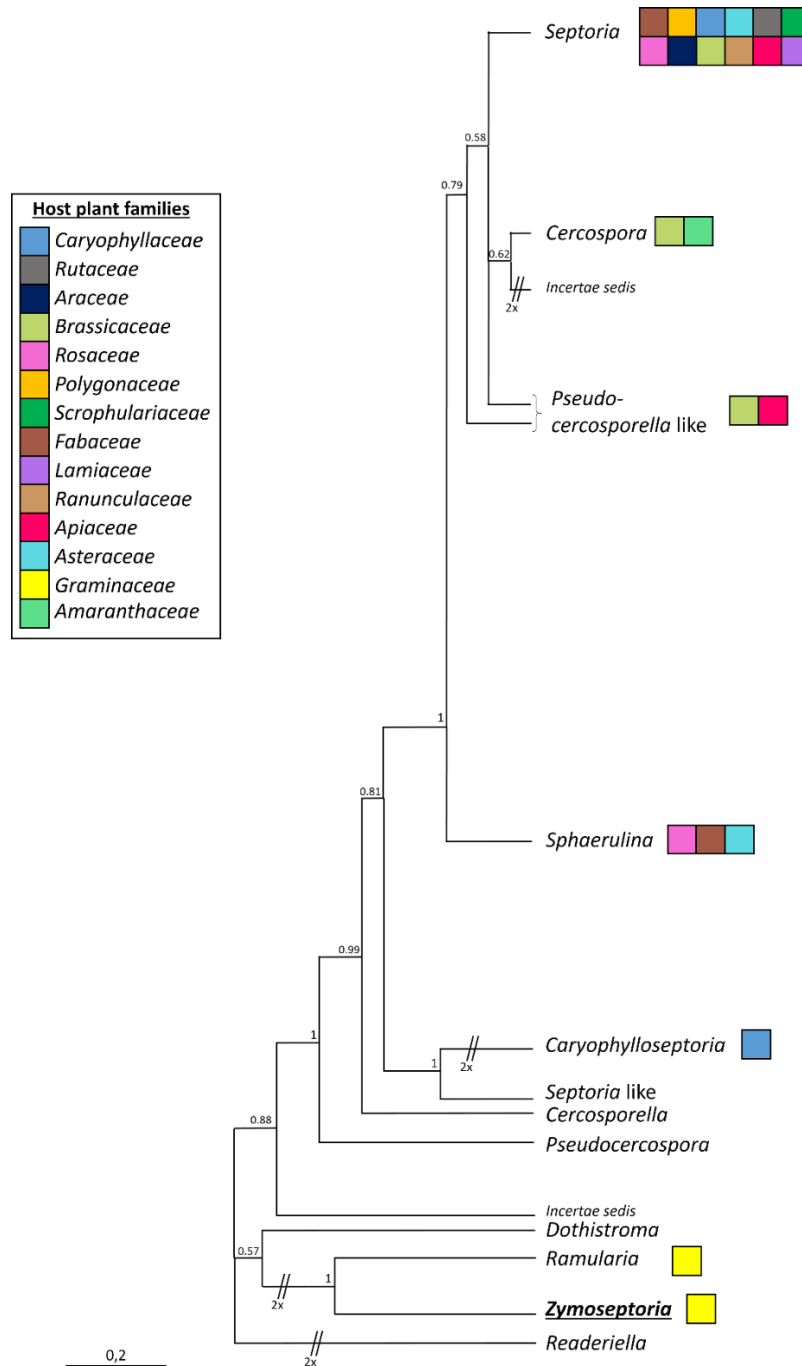


Figure 6. Simplified phylogenetic tree showing the distribution of *Zymoseptoria* and its closest genera (adapted from Verkley *et al.*, 2013). Tree was built using a Bayesian analysis of seven combined loci. Bayesian posterior probabilities values are indicated on branches. The scale bar indicates 0.2 expected changes per site. The tree was rooted to *Readeriella mirabilis* (*Teratosphaeriaceae*). Colored squares show the host plant families identified for species belonging to each genus.

Zymoseptoria species can be distinguished from *Septoria* ones by their yeast-like form under axenic culture conditions, and different spore types (Quaedvlieg *et al.*, 2011). Moreover, it emerged that *Zymoseptoria* is closer to the *Ramularia* genus than to the *Septoria* one as shown by the latest phylogenetic studies (Fig. 6) (Verkley *et al.*, 2013). Aside from *Z. tritici* and *Zymoseptoria passerinii*, the agent causing barley speckled leaf blotch, the *Zymoseptoria* genus also encompasses species like *Zymoseptoria brevis*, *Zymoseptoria halophila*, *Zymoseptoria ardabiliae* and *Zymoseptoria pseudotritici* (Quaedvlieg *et al.*, 2011). These last two species have been isolated from wild grasses in Iran, and are closely related to *Z. tritici*. They are believed to be two progenitor species of *Z. tritici*, confirming the Middle East origin of this pathogen (Stukenbrock *et al.*, 2012).

3.3. Genome description, origin and evolution.

Z. tritici is a haploid species, as are most ascomycetes during the asexual cycle. Its genome is approximately 40 Mb. It was the first phytopathogenic filamentous fungus to have a completely sequenced genome, from telomere to telomere. The reference strain is referred to as IPO323. Its genome is distributed over 21 chromosomes, eight of which are dispensable (Fig. 7). The *Z. tritici* genome displays extraordinary plasticity through numerous polymorphisms due to different chromosome numbers and size, genome size and sequence variations as well as gene content (Goodwin *et al.*, 2011).

Firstly, chromosome number can be variable as strains can easily gain or lose accessory chromosomes during meiosis (sexual reproduction), as well as during simple mitotic events (Möller *et al.*, 2018). Therefore, accessory chromosomes often show presence/absence polymorphisms, while core chromosomes are always present, sometimes in multiple copies acquired during meiosis. Secondly, strains can display genome size polymorphisms in both types of chromosome. This is mainly due to frequent recombination events (Goodwin *et al.*, 2011), or to insertion or deletion of entire clusters of transposable elements (TEs) (Oggenfuss *et al.*, 2020; Plissonneau *et al.*, 2018). Indeed, the presence of TEs greatly influences genome size. TE content in the *Z. tritici* genome is at least 17%, and is significantly higher in accessory chromosomes than in core chromosomes (Dhillon *et al.*, 2014; Lorrain *et al.*, 2020). This explains why size variation in core chromosomes never exceeds 5% but can reach 25% in accessory chromosomes. This leads to variable genome sizes among *Z. tritici* isolates, even in the same population. For example, a study based on five isolates including IPO323 gave genome sizes ranging between 37.9 to 40.7 Mb (Plissonneau *et al.*, 2018). The number of genes may also be highly variable. Among the five studied isolates, the biggest difference in gene

content between two strains was as high as 400 genes. In another comparison of 123 isolates, the largest difference in the number of functional genes between two isolates was a thousand genes. The number of genes in the core genome is around 9 000 and is quite stable between isolates. In contrast, in the dispensable part of the genome, gene number is highly variable and evolves rapidly (Plissonneau *et al.*, 2018).

To understand *Z. tritici* evolution, its genome was compared to that of its closest known progenitor species isolated from wild grasses in Iran and referred to as S1 (Stukenbrock *et al.*, 2010). This comparison revealed that reproductive isolation between the two species occurred recently. The genomic divergence observed between the two species was 7%, which is consistent with an 11.000-year speciation time, and mainly concerned accessory chromosomes (Fig. 7). Based on the broader host range of S1 and its lower pathogenicity on wheat, it is likely that the speciation process was triggered by *Z. tritici*'s host specialization on wheat. This co-evolution between a crop species and a pathogen during crop domestication is called host-tracking. Evolution through host-tracking is a longer process than other evolutionary mechanisms but, in the case of *Z. tritici*, it is supported by the fact that crop and pathogen have the same center of origin (Stukenbrock and McDonald, 2008). Through host-tracking, *Z. tritici* populations reached ever larger sizes as wheat-growing areas spread, while S1 populations decreased as wild lands were replaced by agricultural fields. The larger the populations, the greater the genetic diversity and adaptability, leading to sympatric divergence of the two fungal populations, characterized by a strong genetic differentiation. In the end, this evolutionary process led to the reproductive isolation of *Z. tritici* through ever-increasing host adaptation (Stukenbrock *et al.*, 2006).

Accessory chromosomes were also present in the S1 genome (except for chromosome 18) meaning that their acquisition preceded *Z. tritici* speciation (Stukenbrock *et al.*, 2010). Their origin remains unclear but it seems that they were acquired through horizontal transfer from a distantly related species. Indeed, dispensable chromosomes display differences in codon usage compared to core chromosomes. Moreover, when the *Z. tritici* genome was compared to the genomes of *P. nodorum* or *Aspergillus fumigatus*, the core genome and the dispensable genome tend not to cluster together, corroborating the hypothesis of a horizontal transfer. Nevertheless, the species from which these accessory chromosomes originate is not yet known (Goodwin *et al.*, 2011).

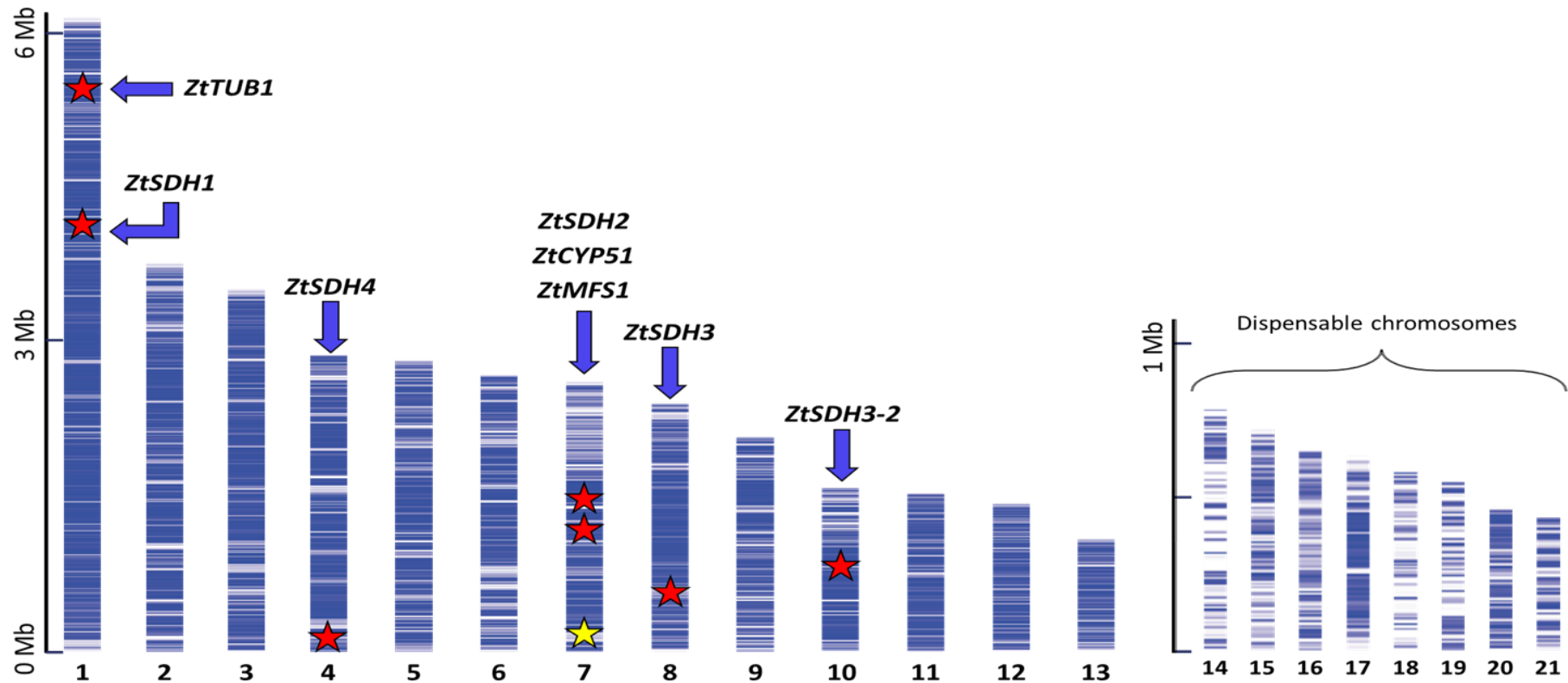


Figure 7. *Zymoseptoria tritici* core and accessory genomes (adapted from Stukenbrock *et al.*, 2010). The blue bars represent the parts of the genome shared by *Z. tritici* and S1 (closest known progenitor species isolated from wild grasses in Iran, the world area from which *Z. tritici* is supposed to originate). Most of the genetic divergence is visible on the accessory chromosomes. Red stars indicate the position of genes encoding important fungicide targeted proteins: *ZtCYP51* (sterol 14 α -demethylase), *ZtTUB1* (β -tubulin) and *ZtSDH1*, 2, 3 and 4 (A to D subunits of the mitochondrial succinate dehydrogenase (SDH)). *ZtSDH3-2* is a paralog of *ZtSDH3* discovered recently in some field isolates, encoding an alternative C subunit of the SDH (see section 5.8.4.). *ZtMFS1* (yellow star) encodes a membrane transporter from the major facilitator superfamily involved in multi-drug resistance (MDR) (see section 5.8.3.). The cytochrome *b* encoding gene *ZtCYTb* is not shown as it belongs to the mitochondrial genome.

3.4. Morphology.

For a long time, *Z. tritici* was considered a dimorphic fungus, because it is able to grow as a “yeast-like” form and a filamentous form. Actually, the “yeast-like” form is where the “zymo” part of the genus name comes from, as *zy*mo means *sourdough* or *ferment* (Francisco *et al.*, 2019). However, recent studies showed that *Z. tritici* actually displays several other morphotypes, and that it should instead be considered a pleomorphic fungus.

The “yeast-like” form, easily observed under laboratory conditions, is a combination of two cellular types: the asexual pycnidiospores, and the blastospores (Fig. 8) (Francisco *et al.*, 2019). Pycnidiospores are produced by pycnidia (Fig. 9), the asexual fruiting bodies. They were formerly called macropycnidiospores as opposed to the micropycnidiospores, which in fact designated the blastospores (Steinberg, 2015). Pycnidiospores are multicellular, as they are composed of 4 to 8 elongated cells. On the contrary, blastospores are unicellular and formed by lateral budding from hyphae or pycnidiospores (Steinberg, 2015). The third morphotype consists of bi-cellular ascospores, produced in pseudothecia (Fig. 9), the fruiting bodies resulting from the sexual reproduction.

Recently, chlamydospores have been described as a new cell type (Figs. 8 and 9). Chlamydospores are spherical cells with a thicker cell wall and a high lipid droplet content. They are formed intercalated between two fungal hyphae, or at their tip, and are known to be long-term survival structures able to resist environmental stresses like desiccation or low temperatures (Francisco *et al.*, 2019).

These four cellular structures are able to germinate and then grow as hyphae. *Z. tritici* can produce two types of hyphae: true fungal hyphae and pseudohyphae (Fig. 8 and 9). True fungal hyphae are preferentially formed under leaf surface conditions and their formation is a mandatory requirement for plant penetration (Steinberg, 2015). Pseudohyphae are thicker, display septal junctions, hence no cytoplasmic continuity, and are thought to ease the pathogen’s motility inside its host (Francisco *et al.*, 2019).

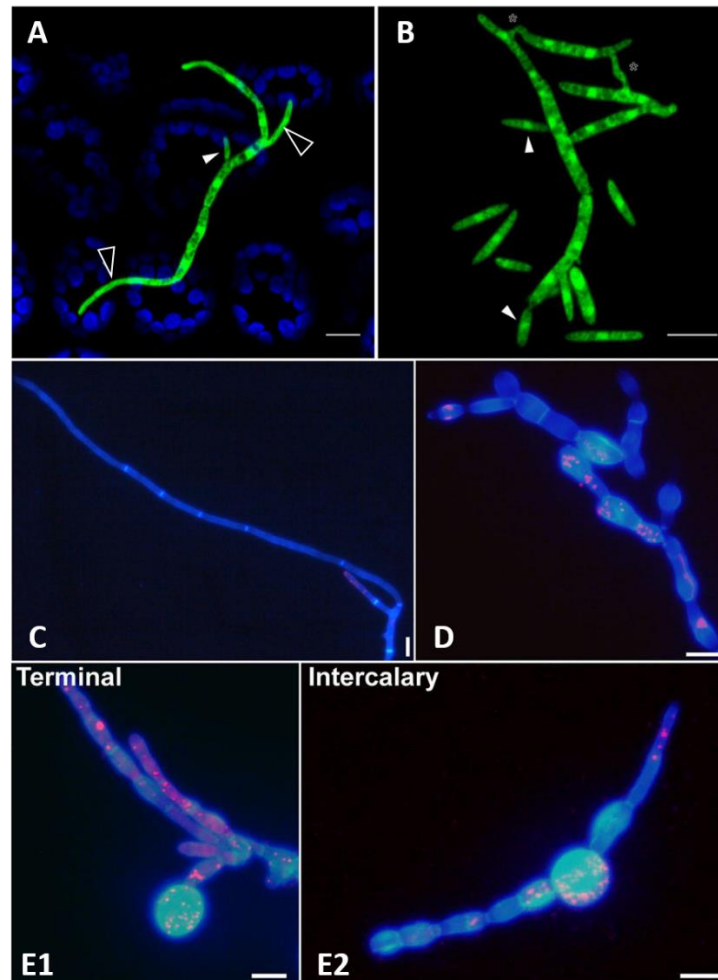


Figure 8. *Z. tritici* cellular types (Francisco *et al.*, 2019). **A.** Germinating pycnidiospore (GFP) producing hyphae (black triangles) and a blastospore (white triangle). **B.** Blastospores (white triangles) multiplying by blastosporulation. **C.** True fungal hyphae. **D.** Pseudohyphae. **E1-E2.** Chlamydospores. Bars represent 10 μ M.

3.5. Life cycle and epidemiology.

Sexual reproduction can only occur when two strains of opposite mating types infect the same plant (Suffert *et al.*, 2019). Interestingly, the frequency of both mating types remains almost equal whatever the spatial scale and maximizes the probability of successful sexual reproduction (Morais *et al.*, 2019). Ascospores, that can be spread by wind over hundreds of kilometers, usually constitute the main primary source of inoculum (Orton *et al.*, 2011; Suffert *et al.*, 2011). In Europe, *Z. tritici*'s life cycle follows the development of winter wheat (Fig. 4; Fig. 9). It overwinters mainly on crop residues, but also on autumn sown crops or on volunteer hosts. It can survive as mycelium, chlamydospores, pycnidia or pseudothecia.

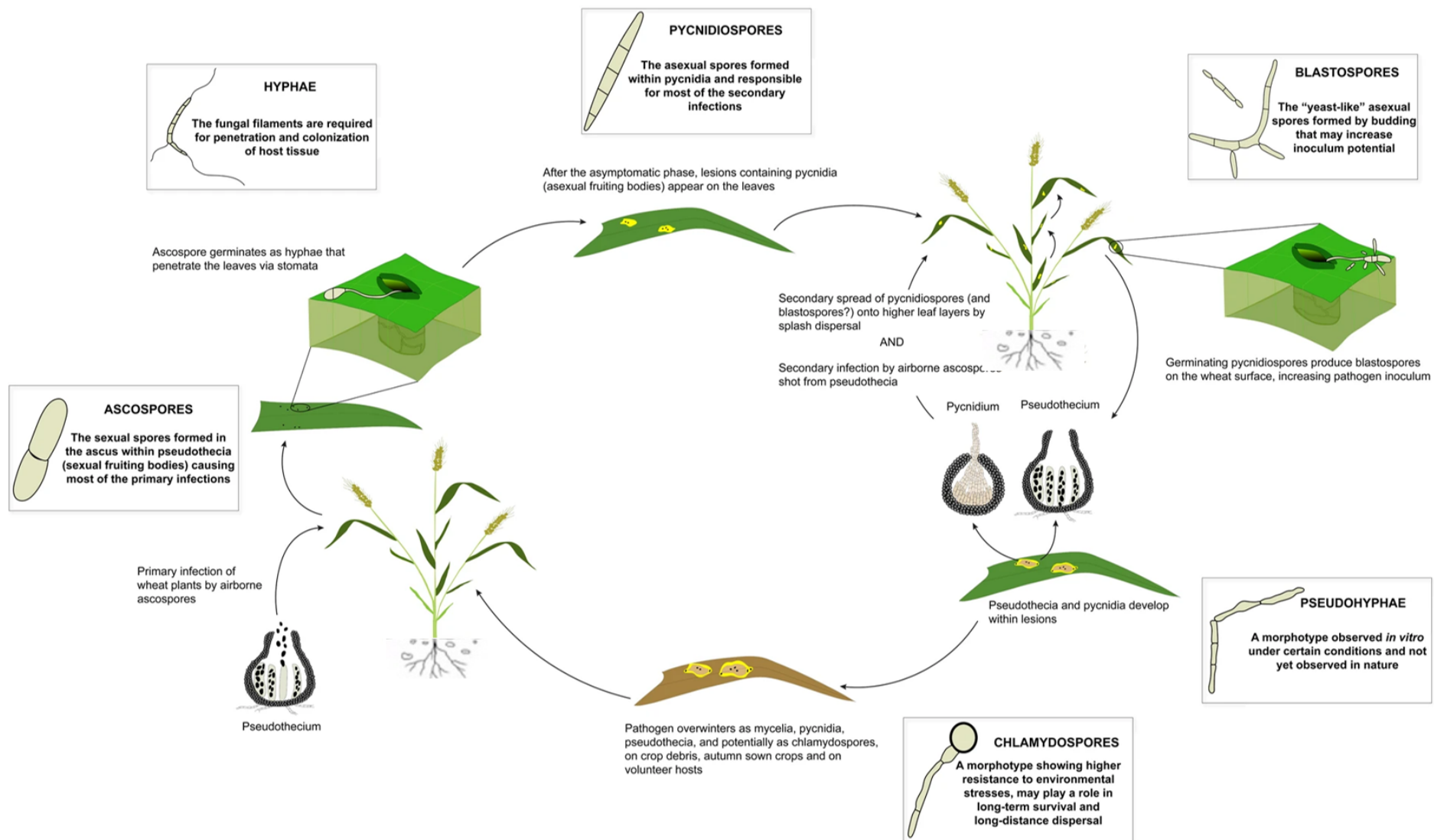


Figure 9. Disease cycle showing the role of each cellular type in STB epidemics (Francisco *et al.*, 2019).

Figure 9 shows that the *Z. tritici* life cycle consists of both asexual and sexual reproduction. As mentioned in section 3.1., *Z. tritici* is a persistent pathogen and first symptoms can be visible as early as spring, on the lowest leaves. On these first lesions, pycnidia appear and pycnidiospores are produced. Pycnidiospores are spread to upper foliar stages and to the neighboring plants through rain splash, making the epidemic development highly dependent on rainfall initially (Fig. 9). If the epidemic development is successful, throughout the wheat-growing season, pycnidiospores, blastospores, ascospores and sometimes chlamydospores will be responsible for new infections. After harvest, the fungus goes through another overwintering period, until the following season (Suffert *et al.*, 2011).

3.6. Pathogenicity.

When a competent spore lands on a wheat leaf, it germinates and produces true fungal hyphae (mycelium) (switch from yeast to filamentous growth; Fig. 9 and Fig. 10). Starvation and temperature increase on the leaves have been identified as stimuli inducing this morphological switch, a transition essential for infection (Steinberg, 2015). On the leaf surface, pycnidiospores also bud to form new blastospores that increase the inoculum size and multiply the infectious potential from rain splash (Francisco *et al.*, 2019). *Z. tritici* does not produce appressoria, therefore the fungal hyphae penetrate into the host tissue through substomatal openings (Steinberg, 2015). Remaining exclusively extracellular, the fungal hyphae colonize the neighboring stomatal cavities where they grow, branch off and perform hyphal fusions, creating pre-pycnidial structures (Fig. 10).

This infection phase can last from 9 to more than 30 days and remains completely asymptomatic, which led *Z. tritici* to be considered a hemibiotrophic fungus for a long time (Steinberg, 2015). However, recent data tend to show that *Z. tritici* is in fact a poor biotroph and should probably better be considered as a necrotrophic fungus with a long latency period (Hane *et al.*, 2020). This hypothesis is supported by the fact that it does not develop haustoria, arbuscules or any other feeding structure inside the host tissue, and colonizes only the apoplast more like an endophyte than a biotroph (Sánchez-Vallet *et al.*, 2015). Moreover, during the colonization phase, there is an upregulation of the genes involved in lipid and fatty acid metabolism, showing that the fungus rather uses its own lipids as a source of carbon (Rudd *et al.*, 2015). During this period, growth is therefore slow, a trait that may explain the extended latency period.

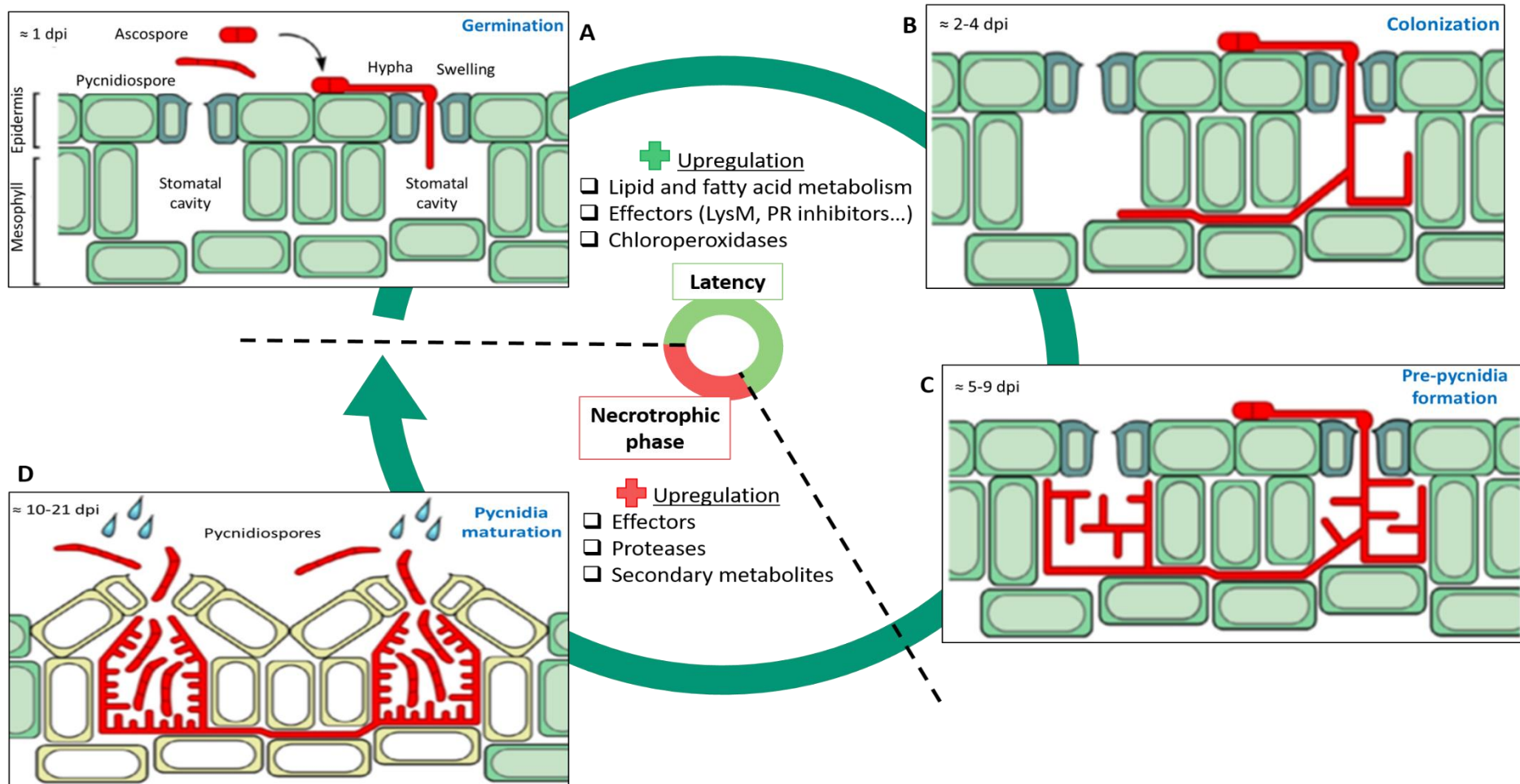


Figure 10. Wheat leaf tissue colonization by *Z. tritici* (adapted from Steinberg, 2015). **A:** Spores (asco- or pycnidiospores) germinate on the leaf surface and the resulting hypha penetrates the stomatal cavity. **B:** Remaining in the apoplast, the fungal hyphae colonize the neighboring substomatal cavities. **C:** Hyphae start invading the whole cavities to form pre-pycnidia. **D:** When pycnidia are mature, chlorotic lesions appear on leaves while pycnidiospores are ready to be spread through splashing. **A to C:** latency (“biotrophic” phase). **D:** Necrotrophic phase. Upregulated gene categories of both phases are shown.

Z. tritici is able to hijack the plant's immune system through the secretion of several effectors (Fig. 10) such as LysM effectors that suppress PAMP-triggered immunity through chitin recognition (Rudd *et al.*, 2015), or effectors inhibiting the expression of genes involved in plant defense, like the pathogenesis-related (PR) genes (Duba *et al.*, 2018). The fungus also secretes chloroperoxidases that protect against the oxidative stress resulting from reactive oxygen species (ROS) secreted by the plant (Rudd *et al.*, 2015). An interesting finding is that the decreased expression of genes involved in plant immunity not only happens locally, but is transmitted to other leaves, a phenomenon recently discovered and called “systemic induced susceptibility” (Seybold *et al.*, 2020). It is noteworthy that differences in virulence between strains have been correlated with differences in the expression level of genes encoding putative effectors or peroxidases (Palma-Guerrero *et al.*, 2017).

The symptomless latency period ends with the formation of mature pycnidia (Fig. 10). This process is governed by the branching of secondary hyphae able to fuse with other surrounding hyphae inside the stomatal cavities. Any defect in hyphal fusion results in the loss of pycnidia production and subsequently of asexual reproduction (Francisco *et al.*, 2020). During pycnidia maturation, there is a switch in *Z. tritici* lifestyle towards necrotrophy. At this point, the secretome of *Z. tritici* changes. There is a peak in effector and protease secretion, and an activation of the expression of secondary metabolism gene clusters. The large number of effectors and secondary metabolites probably protects the fungus, but also stimulates plant defense and activates programmed cell death, thereby favoring necrotrophic colonization (Rudd *et al.*, 2015). Interestingly, secondary metabolism profiles can be very variable among strains, in both the presence/absence of polymorphisms in gene clusters, or in the level of gene expression. These differences in the expression level of secondary metabolism genes have also been found to correlate with variations in virulence of strains (Palma-Guerrero *et al.*, 2017). Once the switch to necrotrophy is complete, there is a decrease in protease secretion in favor of cell wall-degrading enzymes, which is typical of necrotrophy. In addition, there is an increase in mannitol production, known to be related to asexual reproduction (Rudd *et al.*, 2015).

Differences observed in strains' pathogenicity are likely due to host adaptation. Indeed, host adaptation introduced sequence variations that have contributed to shaping the genome of *Z. tritici*. Genomic sequence analyses of 13 isolates showed that approximately half of the single nucleotide polymorphisms (SNPs) found in orthologous regions are located in protein-coding regions, and half of the non-synonymous substitutions are considered adaptive. The rate of non-synonymous SNPs is significantly higher in genes encoding putative effectors, which are small

cysteine-rich secreted proteins that promote infection (Grandaubert *et al.*, 2019). In some cases, effector encoding genes are avirulence genes (*Avr* genes), when secreted effectors trigger an immune response in resistant cultivars, resulting in a strain's absence of virulence. In the plant, the corresponding genes (encoding the proteins that recognize the effectors) are called resistance genes, generally named after the disease (*Stb* genes in wheat for *Septoria tritici* blotch). The evolution of these effector-encoding genes is more than twice as fast as of the rest of the genome, showing their importance in host adaptation. Genes contributing the most to adaptation are located in genomic regions with higher recombination rates, placing recombination and sexual reproduction as the most important drivers of *Z. tritici*'s rapid evolution and adaptation (Grandaubert *et al.*, 2019). One example is the *Avr3D1* (now referred to as *AvrStb7*) gene in *Z. tritici* that encodes a putative effector recognized by wheat cultivars bearing the *Stb7* resistance gene. So far, 30 different variants of the *AvrStb7* encoded protein have been identified, and several amino-acid substitutions make the protein undetectable by *Stb7*-carrying wheat cultivars. The *AvrStb7* gene is located in a highly dynamic region of chromosome 7, a region likely derived from a fusion between an accessory chromosome and a core chromosome (Meile *et al.*, 2018).

Contrary to other pathosystems, it seems that in *Z. tritici*, host-specific virulence is more of a quantitative trait due to the expression of many small effect *loci* (QTLs), whose expression is regulated by highly polymorphic regions (Hartmann *et al.*, 2017). Most of the time, polymorphism in these regions is due to the presence of TEs nearby. It has been shown that, under stress conditions, some TE families are de-repressed in the *Z. tritici* genome, leading to the appearance of polymorphisms like deletions or insertions that can be selected in case they are beneficial (Fouché *et al.*, 2020). For example, TE-mediated adaptation has been shown for the *Avr3D1* locus in host adaptation (Fouché *et al.*, 2020), or for the *MFS1* locus involved in fungicide resistance (Omrane *et al.*, 2017).

3.7. Population diversity and adaptation.

Due to this adaptive potential to local environment, *Z. tritici* populations display high genotypic diversity among individuals but clear genetic structuration at larger geographic scales. Indeed, different genotypes are frequently encountered within a single leaf lesion (Linde *et al.*, 2002). High degree of regional gene flow, frequent sexual recombination (25% of the in-season inoculum results from sexual reproduction) and “efficient” population size (> 3 000 individuals in a 50 m² wheat field) contribute to the high genetic diversity (Zhan *et al.*, 2003). In contrast, natural selection and genetic drift are the evolutionary forces that decrease the allele diversity

within populations (Zhan and McDonald, 2004). At the local scale, population size and diversity are sufficient to prevent genetic drift, while short distance migration maintains the genetic diversity (Morais *et al.*, 2019). At the regional scale and the continental scale, populations show clear genetic differentiation, probably triggered by local adaptation and not compensated by a sufficiently low gene flow (Hartmann *et al.*, 2020; Siah *et al.*, 2018).

TEs play a major role in local adaptation influencing intraspecific genome size variations. Indeed, since *Z. tritici* migrated from the Middle East, its genome has been shaped by TEs. As a result, TE content was found three times more important in isolates from current European populations than in isolates originated from Middle East populations, and even two-fold more important in isolates from a North American population (Oggenfuss *et al.*, 2020). These differences in TE content are likely due to TE-mediated adaptation. This phenomenon highlights the fact that some *loci*, linked to TEs, probably contribute to strains' rapid adaptation to local selection pressures, and are therefore selected. Nevertheless, this TE-mediated adaptation is continually in balance with a strong purifying selection, to avoid uncontrolled colonization of the genome by TEs (Oggenfuss *et al.*, 2020).

Local adaptation can result in the rapid selection of different traits, even within a single season, depending on which trait is the more beneficial at each time point. This includes the selection of greater sporulation ability during winter conditions, and shorter latency period in spring conditions, two traits selected for optimal epidemic efficiency (Suffert *et al.*, 2015). Another study highlighted the existence of a wide range of biological responses to temperature changes among *Z. tritici* field populations. Adaptation to temperature relies on many different loci, and most likely the wide range of response to temperature among strains is due to a combination of polymorphisms at these loci. This means that populations house the potential for easy and rapid adaptation to temperature variations, including global warming (Boixel *et al.*, 2019; Lendenmann *et al.*, 2016).

To conclude, sexual reproduction triggering frequent genetic recombination events, presence of TEs and their de-repression in stressful environments, or rapidly evolving accessory chromosomes provide many sources of genetic variation that contribute to the potential for rapid evolution and adaptation of field populations to biotic and abiotic stresses.

3.8. *Septoria tritici* blotch control methods.

Today, the control of STB in French wheat fields is based on integrated management, which is a combination of all available control methods while reducing chemical pesticide use to a

minimum (Stenberg, 2017). It relies on different components, mainly agricultural prophylactic measures, plant immunity and chemical control. Their use is optimized by technologies, like diagnostic methods and risk modeling for decision support tools. The aim of combining these complementary methods and tools is to reduce fungicide use to comply with environmental constraints while maintaining high yields for farmers (Arvalis, 2019). In France, the Ecophyto II + plan has given the guideline of a 50% reduction in the use of pesticides in fields by 2025. It also aims at achieving uniformity of agricultural practices in the European Union.

3.8.1. Agricultural practices.

Early sowing used to be a popular practice as it was supposed to trigger higher yields, in spite of increasing the risk of epidemics. However, in the UK, a study showed that disease severity was much higher with earlier sowing because humid and warmer autumn conditions favored primary contaminations, fungal development and spreading. The number of plots above the severity thresholds was at least five times greater in crops sown before mid-October than in crops sown after mid-November (Gladders *et al.*, 2001). Late sowing, on the other hand, has proven effective in limiting STB development, as it desynchronizes the development of wheat and the pathogen's infection cycle. This results in reduced inoculum at the end of winter and a lower risk of epidemics (Arvalis, 2019).

Soil preparation, *i.e.* grinding and burial of crop residues, has limited impact on STB epidemics, as does crop rotation. Indeed, for STB, local inoculum is never limiting as the ascospores remain the greatest source of primary inoculum, and migrate over long distances (see section 3.5.) (Arvalis, 2019; Suffert *et al.*, 2011).

Sowing density and nitrogen fertilization have also been identified as important parameters to reduce disease severity. In both cases, an optimum balance between yield and disease control is necessary (Baccar *et al.*, 2011; Précigout *et al.*, 2017).

3.8.2. Deploying resistant cultivars.

The choice of a STB-resistant cultivar remains the most efficient agronomic tool to control STB, as under optimal conditions it can be even more efficient than fungicide sprays. Resistant cultivars are obtained by breeding different wheat varieties, sometimes even with wild relative species, containing relevant resistance sources. Each newly developed resistant cultivar receives a grade reflecting its level of resistance to STB.

In wheat, defense against *Z. tritici* is a multigenic and quantitative trait, and complete resistance has never been described (Duba *et al.*, 2018). Twenty-one major STB-resistance genes have been identified in wheat so far, named from *Stb1* to *Stb21* (see section 3.6.), which deliver high levels of resistance to *Z. tritici* strains bearing the corresponding *AvrStb* genes (Saintenac *et al.*, 2018). *AvrStb* genes have been shown to be highly polymorphic (see section 3.6.) and cultivar resistance relying on known major resistance genes is usually rapidly overcome (Krattinger and Keller, 2016). *Stb6* is the only *Stb* gene characterized at the molecular level. It is therefore difficult to know which gene or combination of genes are present in the different cultivars. Consequently, their sustainable use and management optimization are difficult. However, major resistance genes remain at the heart of the more recent breeding programs (Krattinger and Keller, 2016).

STB resistance relies also on many quantitative trait loci (QTLs) which have little effect (Saintenac *et al.*, 2018). Interestingly, resistance based on minor QTLs is only partial but considered more sustainable because it does not involve a gene-for-gene interaction, so pathogen adaptation is supposedly slower. Breeders have exploited minor QTLs for decades, most of the time without even knowing it (Krattinger and Keller, 2016). Unfortunately, the phenotypic traits linked to QTLs have sometimes been associated with lower yields, and finding the optimum balance between yield and disease resistance is the real challenge for breeders (Duba *et al.*, 2018).

Combining genes of interest into a single plant is called “gene stacking”. An unfortunate consequence of such intensive breeding programs is an associated loss in genetic diversity (Duba *et al.*, 2018). For this reason, great interest has recently been paid to wild grass relatives of wheat, in order to perform introgression of ancient resistance gene in modern cultivars. The wheat varieties from such breeding programs are called “synthetic wheats” (Gorafi *et al.*, 2018).

In the field, mixtures of cultivars with quantitatively and qualitatively different resistance characteristics are commonly used. Indeed, because *Z. tritici* adapts rapidly to host resistance, using moderately resistant cultivars, mixtures or rotation of cultivars with different resistant genes, would be more efficient for long-lasting STB control in the field (McDonald and Linde, 2002). For example, studies have shown that mixtures of resistant cultivars deliver higher yields and reduce disease severity when compared to the average of the cultivars used separately (Kristoffersen *et al.*, 2020).

3.8.3. Chemical control.

Although breeding for resistant cultivars allows reduced fungicide use, chemical control is still the primary tool used to control STB in the field. In France, control relies on two fungicide applications per season, with even a tendency to reduce to one application due to lower disease pressures. In countries with more disease favorable conditions (like in the UK), three applications are authorized (Arvalis, 2019).

3.8.3.1. Multisite fungicides.

Multisite fungicides do not have a specific intracellular target (Fig. 11). They generally interact with various cellular components, especially with those containing thiol groups (Leroux, 2003). They mainly inhibit the germination of fungal spores. This inhibition is due to the impairment of oxygen consumption (mitochondrial respiration), lipid and sugar catabolism and sometimes plasma membrane integrity.

Multisite fungicides have been used in agriculture since the nineteenth century, and their earliest representatives contained elemental copper or sulfur. In the middle of the twentieth century, the development of synthetic chemistry allowed the discovery of new multisite fungicide groups including the dithiocarbamates (like mancozeb), the N-chloroalkylmercaptans (like folpet), and the phthalonitrile compound chlorothalonil, which is the most widely used multisite fungicide to control STB (Fig. 12).

Registered more than 40 years ago, chlorothalonil was used until 2019 in up to 50% of the wheat-growing areas in France, due to its superior anti-STB efficacy among multisite fungicides (Arvalis, 2019). However, multisite fungicides present some negative characteristics. Firstly, they display moderate antifungal activity and therefore have to be used at high concentrations in the field (up to 2 kg/ha for mancozeb for example). In addition, due to their physico-chemical properties, they hardly penetrate into the plant and are easily washed off, resulting in low persistence. This implies repeated treatments, causing ecotoxicology issues. Finally, the absence of specificity, due to their interaction with thiol-containing proteins, which are ubiquitous, triggers some toxicity to non-target organisms and has a negative impact on the environment (Leroux, 2003).

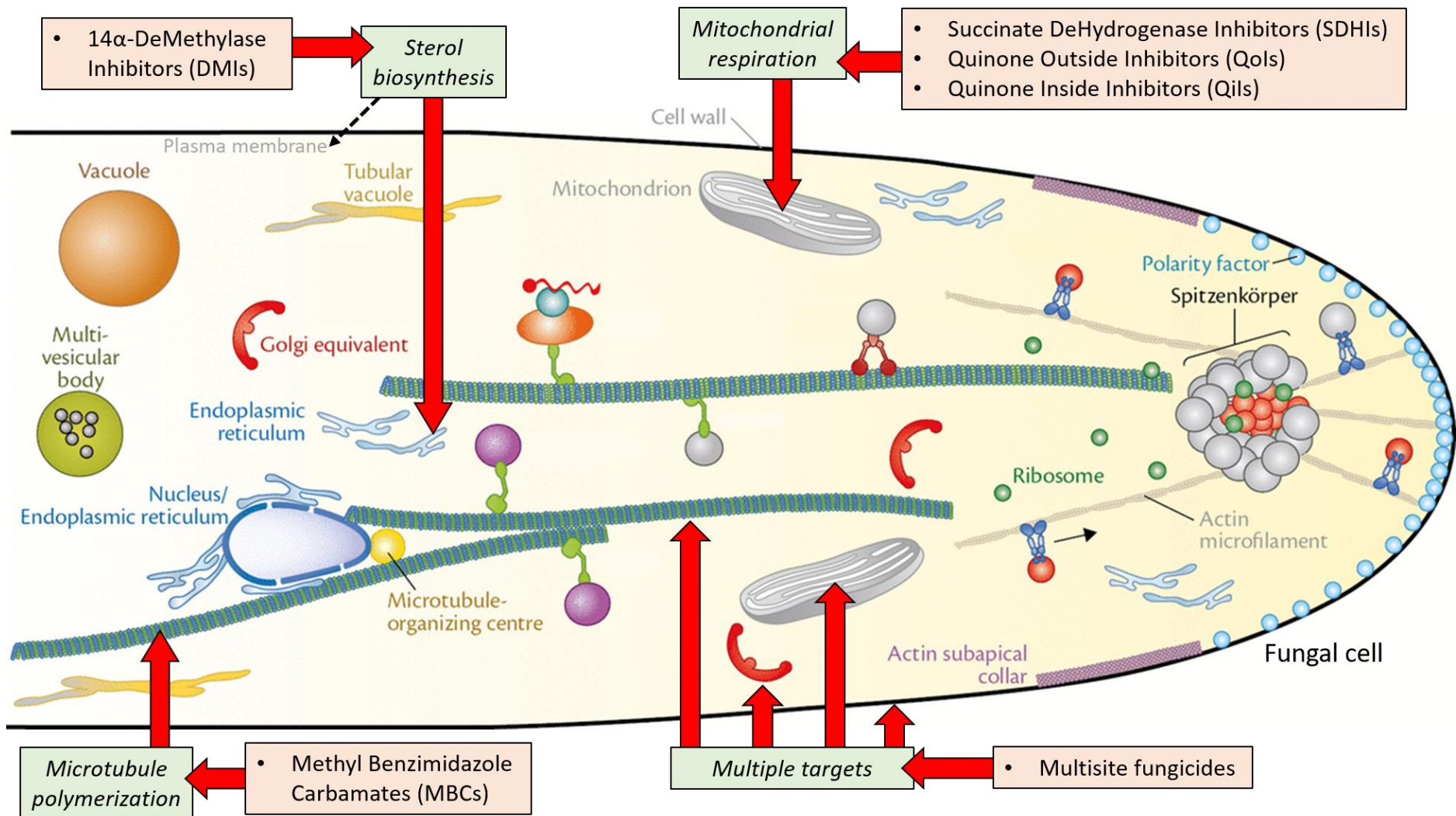


Figure 11. Organization of a fungal cell and principal targets of fungicides used against *Z. tritici* (adapted from Riquelme, 2013). Orange squares: main anti-STB fungicide classes. Green squares: targeted cellular processes.

Based on these characteristics, many multisite fungicides have been withdrawn from the European market. This includes chlorothalonil, which has been prohibited since May 2020 (Arvalis, 2019). Nevertheless, a few multisite fungicides are still authorized to control STB, like mancozeb, folpet and elemental sulfur. However, mancozeb has a recommendation from the commission for non-renewal and will be phased out completely in the EU in the near future. Indeed, doubts persist about its ecotoxicological characteristics and toxicity, while its status as a possible carcinogen is still debated (Arvalis, 2019; Runkle *et al.*, 2017). In France, elemental sulfur has become essential for STB control and is labelled as a biocontrol fungicide (Arvalis, 2019). Nevertheless, its antifungal activity remains lower than that of chlorothalonil, and its use at high doses leaves residual traces on wheat leaves that do not always align well with the image of organic farming (Walker A.-S., personal communication).

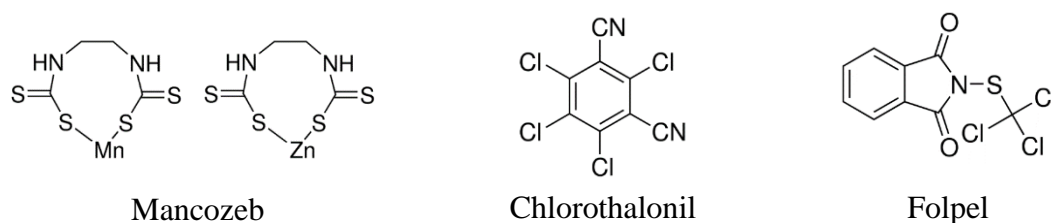


Figure 12. Chemical structures of the main multisite fungicides used against *Z. tritici*.

3.8.3.2. Methyl benzimidazoles carbamates (MBCs).

Developed in the late 1960s, MBCs, also referred to as benzimidazoles, represent the oldest class of systemic unisite fungicides. These include carbendazim, and its precursor, thiophanate-methyl, which were both authorized in France against *Z. tritici* until carbendazim was withdrawn from the market (Fig. 13; Table 2). Only thiophanate-methyl is still authorized to control *Fusarium* head blight, although little used on cereals.

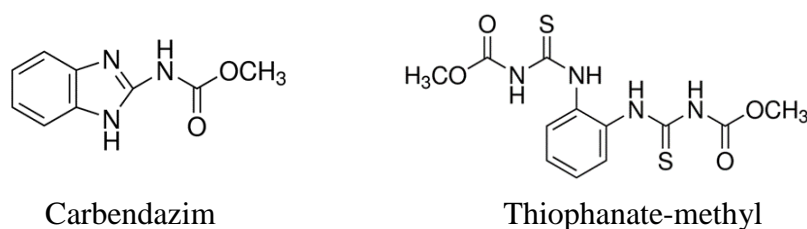


Figure 13. Chemical structures of the main benzimidazoles used against *Z. tritici*.

These active ingredients bind to β -tubulin, preventing microtubule polymerization, thus impairing intracellular vesicle trafficking, cellular division and hyphal growth. They display a broad antifungal spectrum, with the exception of oomycetes. Interestingly, their fungal specificity seemed to be linked to the phenylalanine residue at position 200 of the β -tubulin, present in fungi but not in plant or mammals (Leroux, 2003).

3.8.3.3. Sterol 14 α -demethylase inhibitors (DMIs).

Ergosterol is the most abundant sterol in most fungal species (Pichler and Riezman, 2004). In fungi, sterols are involved in the regulation of membrane permeability and fluidity, as well as other phenomena like endo/exocytosis or vacuolization (Pichler and Riezman, 2004). *CYP51* is the gene encoding sterol 14 α -demethylase, a key enzyme of ergosterol biosynthesis (Fig. 14), and the target of the demethylation inhibitors (DMIs).

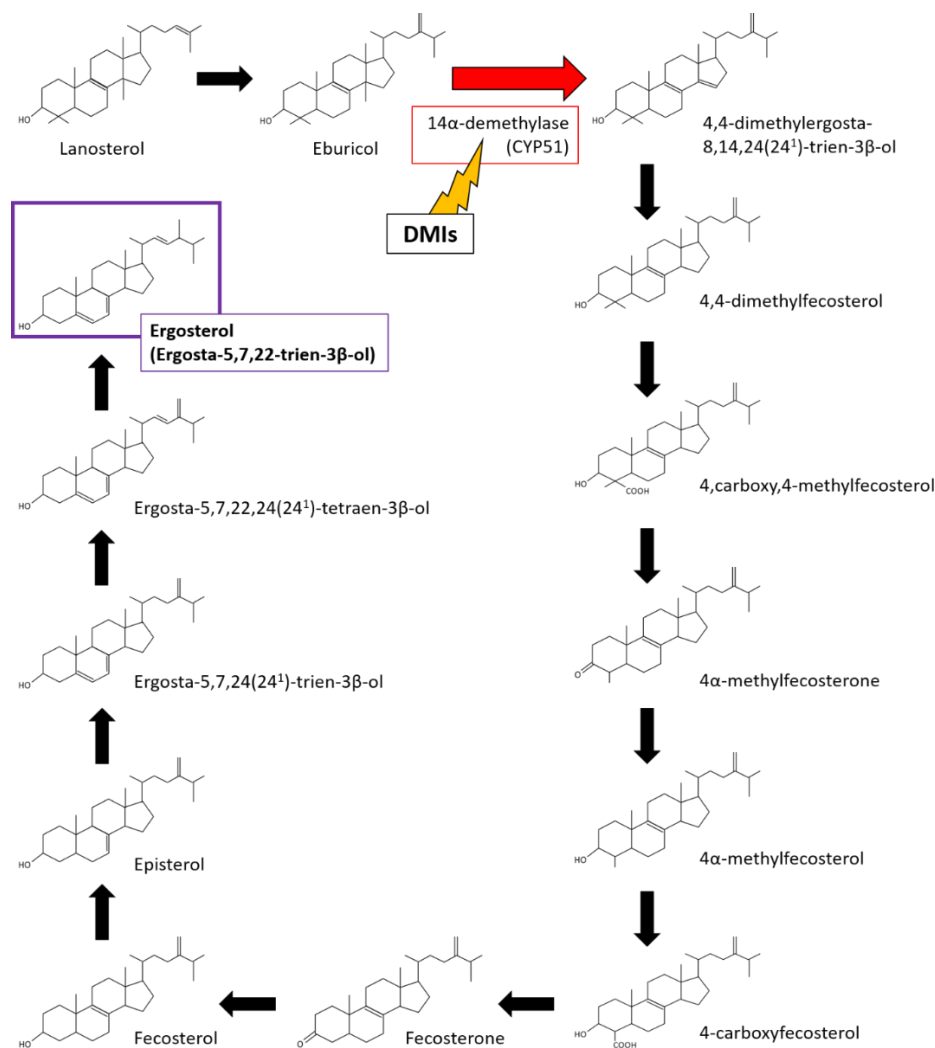


Figure 14. Ergosterol biosynthesis pathways in filamentous fungi (adapted from Billard, 2011).

DMIs do not prevent spore germination but impair germ tube elongation and hyphal growth. They decrease ergosterol content while C-14 methylated sterols accumulate, triggering some toxicity (Koeller, 2018). This fungicide class has been widely used in agriculture (and human medicine) since the 1970s, mostly because of its wide antifungal spectrum, good curative properties, and good plant penetration hence good persistence. DMIs now encompass numerous active ingredients, whose common feature is a nitrogen-containing heterocycle in their structure (Fig. 15). Consequently, this class is commonly referred to as the “azole” fungicides. Most anti-STB azoles are triazoles like tebuconazole, metconazole or epoxiconazole, but they also include the imidazole, prochloraz, and the triazolinethione, prothioconazole, which is a pro-fungicide (Fig. 15; Table 2). Triazoles constitute the majority of this antifungal class, but in agriculture, other chemical classes are also available like imidazoles, triazolinethiones, piperazines, pyridines and pyrimidines.

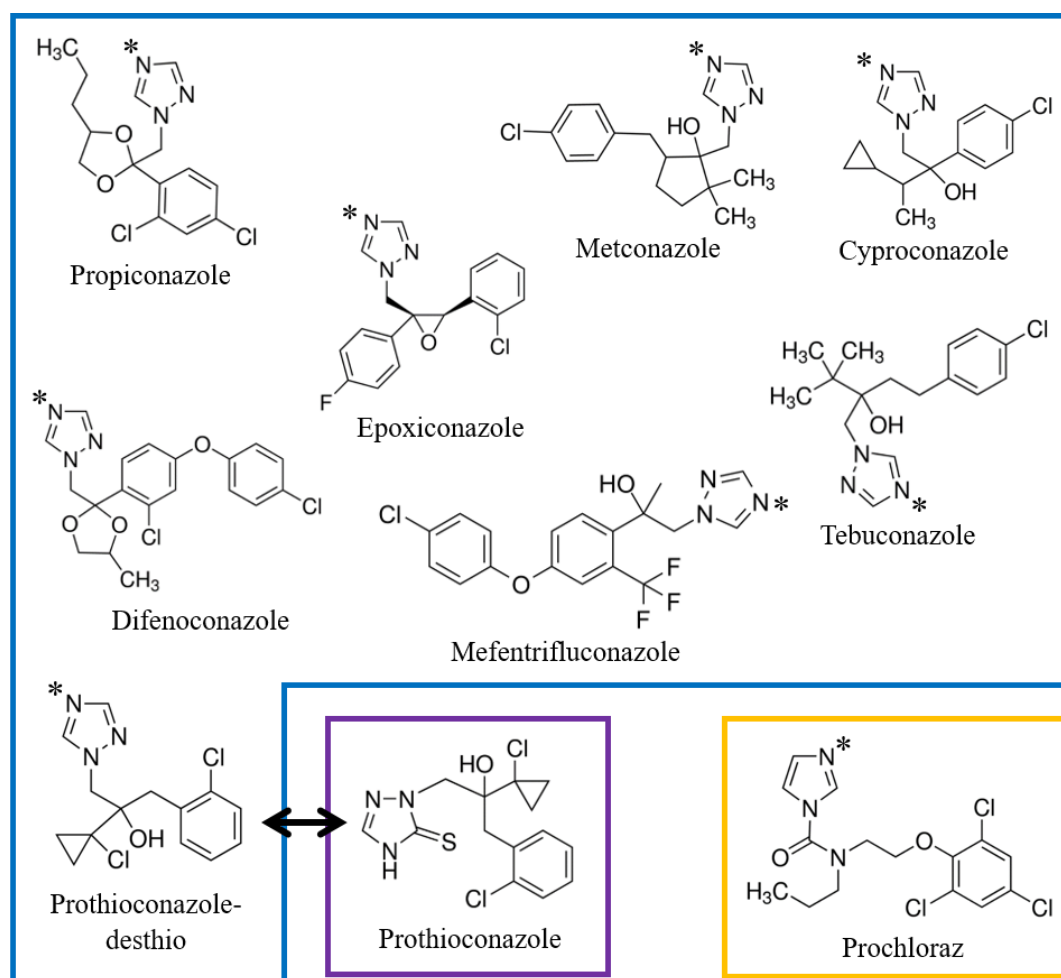


Figure 15. Chemical structures of the main DMIs used against *Z. tritici*. Blue frame: triazoles; Purple frame: triazolinethione; Orange frame: imidazole; *: heme iron-interacting nitrogen within the azole heterocycle.

Based on their ability to interact with the heme iron, some azoles may also interact with other cytochrome P450 enzymes producing effects in non-target organisms. Indeed, studies have shown the phytotoxicity of some azoles or even their toxicity towards birds (Rivière *et al.*, 1985). Over the past decades, several azoles have been withdrawn from the market because they were identified as endocrine disruptors (Table 2) (Chayawan *et al.*, 2020). The newly developed isopropanol-triazole, mefentrifluconazole, displays a strong antifungal activity, a high specificity for fungal CYP51 over mammalian CYP19 (aromatase) and no endocrine disrupting potential in preliminary studies (Tesh *et al.*, 2019). These characteristics suggest that mefentrifluconazole will be one important active ingredient for STB control in future years, even though cross-resistance to some other azoles has been described.

3.8.3.4. Inhibitors of mitochondrial respiration.

The mitochondrial electron transport chain (ETC) is composed of five protein complexes that couple electron transfer to the establishment of a proton gradient, necessary for ATP synthesis. So far, inhibitors of complexes II and III are used to control *Z. tritici* in the field. This section briefly presents these classes of anti-STB fungicides. For a detailed description of the mitochondrial ETC and its inhibitors, please refer to section 4.

Complex II of the mitochondrial electron transport chain is succinate dehydrogenase (see section 4.2.3.). Succinate dehydrogenase inhibitors (SDHIs) represent the most recent class of anti-STB fungicides, although this mode of action has been used against other fungal diseases since the 1970s. Since the launch of boscalid on the French cereal market in 2007, this class of fungicides has rapidly expanded. Recently developed SDHIs are broad-spectrum fungicides and are now widely used throughout Europe (Sierotzki and Scalliet, 2013).

Complex III of the mitochondrial ETC is the cytochrome *bc*₁ complex (see section 4.2.4.). The major class of complex III inhibitors are called QoIs, for Quinone outside Inhibitors, predominantly composed of the strobilurins (see section 4.2.4.6.1.). Like the use of “azoles” to describe DMIs, QoIs are usually referred to as “strobilurins”.

Fenpicoxamid is a new complex III inhibitor impairing cytochrome *b* function, like QoIs, but with a different binding site (Young *et al.*, 2018) (see section 4.2.4.6.3.). Fenpicoxamid is the active substance this work is based on.

Table 2. Fungicides registered for *Z. tritici* control in France, associated resistance mechanisms and activity range (adapted from Garnault *et al.*, 2019).

Fungicides													
Biochemical mode of action			Chemical structures		Active ingredient	Authorization in France			Activity range	Target site resistance		Non target site resistance	
Main processes	Target site and mode of action	Abbreviation	Main classes	Secondary classes		Registration	First year of use	Removal	Estimate against sensitive WT strain (mg/L)	Target alteration	Target overexpression	Compensation	Enhanced efflux (MDR)
Mitochondrial respiration	Complex II or succinate dehydrogenase. Ubiquinone binding site (subunits SdhB, SdhC and SdhD).	SDHI	Carboxamides	Benzamides	Fluopyram	2016	2017	-	0.2 ¹⁶	SdhB: N225I/T, R265P, H267L, T268A/I SdhC: T79N/I, W80S/A, A84F, N86A/S, P127A, R151M/S/T, H152R, I261S, V166M, T168R SdhD: R47W, I50F, M114F/V, D129G 1, 2, 10, 11,18	(+) SdhC paralog ¹³	(-)	(+) Mfs1 promoter ^{8,9} : insert type I, II or III
				Nicotinamides (syn. Pyridine-carboxamides)	Boscalid	2006	2007	-	0.75 ¹				
				Pyrazole-carboxamides	Benzovindiflupyr	2016	2017	-	0.03				
					Bixafen	2011	2011	-	0.03 ¹⁶				
					Penthiopyrad	2014	2014	-	0.2 ¹				
					Fluxapyroxad	2011	2011	-	0.04 ¹⁶				
	Complex III or cytochrome bc ₁ complex. Ubiquinone Q _o binding site.	QoI	Synthetic strobilurins and analogs	Methoxy-acrylates	Azoxystrobin	1996	1997	-	0.002 ¹⁷	Cytb: F129L, G143A 2, 15, 18	(-)	(+) AOX over-expression	(+) Mfs1 promoter ^{8,9} : insert type I, II or III
					Picoxystrobin	1999	2000	2018					
				Methoxy-carbamates	Pyraclostrobin	2000	2000	-	0.002 ¹⁴				
				Oximino-acetates	Kresoxim-methyl	1996	1997	2013					
					Trifloxystrobin	2001	2002	-					
				Oximino-acetamides	Dimoxystrobin	2009	2010	-					
					Fluoxastrobin	2006	2006	-					
Complex III or cytochrome bc ₁ complex. Ubiquinone Q _i binding site.	QiI	Carboxamides	Picolinamides	Fenpicoxamid	2020	2021	-	0.007 ¹⁷		(-)	(+) AOX over-expression	(+) Mfs1 promoter ^{8,9} : insert type I, II or III	

Biochemical mode of action			Chemical structures		Active ingredient	Authorization in France			Activity range	Target site resistance		Non target site resistance	
Main processes	Target site and mode of action	Abbreviation	Main classes	Secondary classes		Registration	First year of use	Removal	Estimate against sensitive WT strain (mg/L)	Target alteration	Target overexpression	Compensation	Enhanced efflux (MDR)
Sterol biosynthesis	Sterol 14 α -demethylase (syn. CYP51)	DMI	Heterocyclic compounds	Imidazoles	Prochloraz	1980	1980?	-	0.03 ⁴	<p><u>Low resistance:</u> CYP51: one change (e.g. Y137F, Y459C/D/H, G460D/S, Y461H/S, ΔY459/G460) or two changes (V136A+Y461H/S or ΔY459/G460)</p> <p><u>Medium resistance:</u> CYP51: two changes including I381V+Y459S/D/N, Y461H/S or ΔY459/G460)</p> <p><u>High resistance:</u> CYP51: three to six changes including those reported for TriLR and TriMR phenotypes, combined with D107V, D134G and/or S524T.</p> <p>2, 4, 5, 12, 18</p>	<p>(+) <i>Cyp51</i> promoter: 120 bp or 1000 bp inserts</p>	<p>(-)</p>	<p>(+) <i>Mfs1</i> promoter⁹: insert type I, II or III</p>
				Triazoles	Bromuconazole	1994	1995	-					
					Cyproconazole	1987	1987	-					
					Difenoconazole	1988	2011	-					
					Epoxiconazole	1992	1992	2020	0.02 ⁴				
					Fenbuconazole	1991	1992	-					
					Fluquinconazole	1997	1998	2016					
					Flusilazole	1985	1986	2013					
					Flutriafol	1983	1984	2015					
					Hexaconazole	1990	1990	2007					
					Metconazole	1993	1994	-	0.02 ⁴				
					Propiconazole	1980	1980?	2019					
					Tebuconazole	1988	1989	-	0.1 ⁴				
					Tetraconazole	1991	1991?	-					
Triadimenol	1987	1988	2014										
Mefentrifluconazole	2019	2020	-										
Triazolinethiones	Prothioconazole	2006	2006	-	0.05 ⁴								

Biochemical mode of action			Chemical structures		Active ingredient	Authorization in France			Activity range	Target site resistance		Non target site resistance	
Main processes	Target site and mode of action	Abbreviation	Main classes	Secondary classes		Registration	First year of use	Removal	Estimate against sensitive WT strain (mg/L)	Target alteration	Target overexpression	Compensation	Enhanced efflux (MDR)
Micro tubules	β -tubulin	MBC	Benzimidazoles and precursors	Benzimidazoles	Carbendazim	1972	1972?	2009	0.03 ¹⁷	TUB2: E198A/K _{2,3,18}	(-)	(-)	(-)
				Thiophanates	Thiophanate-methyl	1973	1980?	2021					
Multisite activity	Multiple targets	MSI	Dithio-carbamates	Akylene-bis-dithiocarbamates	Mancozeb	1980	1980?	2021		None	None	None	None
					Maneb	1988	1988?	2017					
			Chloronitriles	Phthalonitriles	Chlorothalonil	1980	1980?	2020	0.3				
			Halogenated alkylthio compounds	Phthalamides	Folpet	2003	2003	2009 (re introduced in 2020)					
			Sulfur-based mineral substances	-	Elemental sulfur	1942	1942?	-					

? Approximate date.

References:

- (Dooley *et al.*, 2016)
- (Garnault *et al.*, 2019)
- (Griffin and Fisher, 1985)
- (Leroux and Walker, 2011)
- (Leroux *et al.*, 2007)
- (Lucas *et al.*, 2015)
- (Miguez *et al.*, 2004)
- (Omrane *et al.*, 2017)
- (Omrane *et al.*, 2015)
- (Rehfus *et al.*, 2018)
- (Scalliet *et al.*, 2012)
- (Stammler *et al.*, 2008)
- (Steinhauer *et al.*, 2019)
- (Suemoto *et al.*, 2019)
- (Torriani *et al.*, 2009)
- (Yamashita and Fraaije, 2018)
- (Young *et al.*, 2018)
- <http://www.r4p-inra.fr>

4. THE MITOCHONDRION, CELL POWER PLANT AND FREQUENT TARGET FOR INHIBITORS.

4.1. Mitochondrial origin, characteristics and main functions.

Mitochondria are the result of a 2 billion years old endosymbiosis, when the ancestor of actual eukaryotic cells engulfed an α -proteobacterium (for review, Friedman and Nunnari, 2014). After debate for almost a century, the endosymbiotic origin of mitochondria and other plastids (like chloroplasts) is now accepted. Indeed, the molecular analysis of mitochondrial 16S RNA confirmed its bacterial origin, but its exact source remains difficult to establish. This is due to the complicated genetic history of this endosymbiont, marked by the transfer of most of its genome into its host cell nucleus (Zimorski *et al.*, 2014). The mitochondrion still possesses its own DNA as a unique circular chromosome with a size ranging from 6 kb (*Plasmodium falciparum*) to more than 200 kb (for some flowering plants), as a remnant of its bacterial origin. In fungi, mitochondrial genomes range from approximately 20 to 200 kb, variations being due mostly to the presence of introns and to the length of intergenic sequences (Song *et al.*, 2020). It carries only a few genes, the number of which can also vary from one eukaryotic species to another (for review, Chatre and Ricchetti, 2014). Some genes are highly conserved in the mitochondrial genome such as the cytochrome *b* or cyclooxygenase encoding genes. Different hypotheses have been proposed to explain such gene retention in mitochondria. One hypothesis is that highly hydrophobic proteins are produced inside the mitochondria to avoid transport issues. A second proposal suggests an advantage in having regulation of key gene expression at the organelle level, allowing precise control of energy production. A final hypothesis is that the difference in genetic code and higher GC content in the mitochondrial genome prevented the transfer of some genes to the nucleus (Johnston and Williams, 2016).

Mitochondria are organelles present in all eukaryotic cells, with few exceptions (Chatre and Ricchetti, 2014). The mitochondrion and its host cell are entirely dependent on each other for survival, even though a recent study revealed the presence of cell-free competent mitochondria in human blood, the role of which, however, remains unknown (Al Amir Dache *et al.*, 2020). Mitochondrial shape, number and intracellular distribution can vary depending on the cellular type, the tissue, their energy needs and the organism's physiological situation (stress, disease, excess of nutrients...) (Zimorski *et al.*, 2014). Mitochondria can exist as fragmented puncta which are isolated in the cell, but usually they create a highly dynamic interconnected network. Fusion and fission events allow the switch between both states, and govern the "mitochondrial

life cycle” (Cogliati *et al.*, 2016). The degradation of dysfunctional or excessive mitochondria is a process called mitophagy. Mitochondria destined for mitophagy are recognized through specific mitochondrial receptors, involved in the formation of auto-phagosomes. A different ubiquitin-mediated and lysosome-dependent pathway also exists in mammals (Wei *et al.*, 2015).

Mitochondria have two independent membranes, the outer membrane (OMM) and the inner membrane (IMM). These membranes delineate the inter-membrane space (IMS), and the innermost compartment is the mitochondrial matrix (Fig. 16). The mitochondrial matrix is the site where the tricarboxylic acid (TCA) cycle (or Krebs cycle) takes place (Fig. 16). Two different pathways produce acetyl CoA that fuels the TCA cycle. The first one is glycolysis that takes place in the cytoplasm. Glycolysis is the pathway of carbohydrate assimilation, which catabolizes glucose and ATP to produce pyruvate, with the release of NADH (Nicotinamide Adenine Dinucleotide). Pyruvate enters the mitochondrial matrix where it is finally oxidized to acetyl CoA. The second pathway for acetyl CoA production is fatty acid β -oxidization that takes place in the mitochondrial matrix. The TCA cycle also generates NADH, as well as FADH₂ (Flavin Adenine Dinucleotide), both of which are electron donors involved in the oxidative phosphorylation (OXPHOS) electron transport through complexes I and II (Fig. 16; see section 4.2.1.). The TCA cycle, which also generates certain amino-acid precursors, is an ancient and highly conserved pathway among aerobic eukaryotes (van der Blik *et al.*, 2017). The mitochondrial matrix also contains multiple copies of the mitochondrial chromosome, and all the proteins necessary for its replication, transcription, translation and maintenance (Friedman and Nunnari, 2014).

The IMM is impermeable to ions. It is characteristically invaginated, creating bag-like structures called cristae. Parts of the IMM that are not in the cristae constitute the inner boundary membrane (IBM), which contains the translocases that import proteins from the IMS, to the matrix. Cristae are physically isolated from the IMS by narrow tubular junctions that limit molecule diffusion (Fig. 16). Cristae are the site of the OXPHOS (see section 4.2.1.) as shown by the fact that they contain more than 80% of the cell’s content in OXPHOS complex III, ATP synthase or cytochrome *c* (Fig. 16; see section 4.2.) (Cogliati *et al.*, 2016).

The OMM isolates the mitochondria from cytosol and is permeable to small molecules (< 5 kDa) and ions. It contains embedded translocases which import cytosol-synthesized proteins into the IMS. It is not uniform but contains differentiated regions, some of which are involved in communication with other organelles such as the endoplasmic reticulum (ER) or other

mitochondria. The OMM is also involved in mitochondrial shaping, fusion and fission processes, and in mitochondria-related apoptotic events (Cogliati *et al.*, 2016).

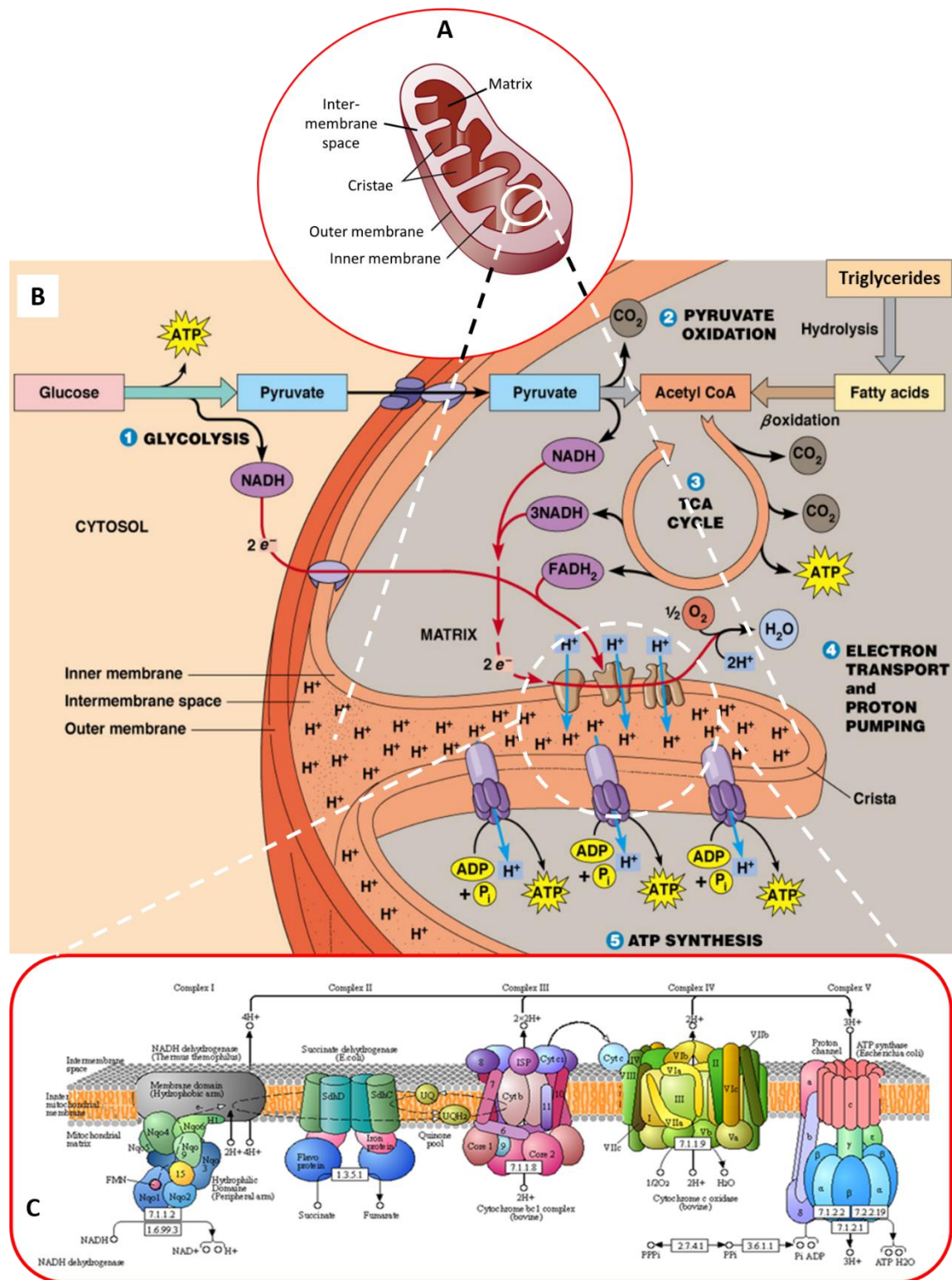


Figure 16. Different levels of mitochondrial organization (adapted from ©2012 Pearson education, Inc. and KEGG, Kyoto University, 2004). **A.** General view of mitochondrial organization. **B.** Organization of a crista and neighboring matrix biological processes. **C.** View of the five OXPHOS protein complexes of the mitochondrial electron transport chain.

Despite their central role as the cell power plant through OXPHOS, mitochondria are also involved in many other cellular processes like fatty acid synthesis, biogenesis of iron-sulfur clusters, heme synthesis, Ca^{2+} homeostasis, signaling through the production of reactive oxygen species (ROS), regulating cell apoptosis or activating ER-stress response (van der Bliëk *et al.*, 2017). They are also stakeholders in human inflammatory and immune responses (Häfner, 2015). Being such key organelles in eukaryotic cells, mitochondria have been extremely important targets for antifungal and anti-parasitic drugs.

4.2. The mitochondrial respiratory chain and its inhibitors.

4.2.1. The ATP production process.

OXPHOS designates the biochemical process that leads to production of ATP, the energy-storing molecule of the cell. OXPHOS consists in a transfer of electrons through four individual redox active protein complexes embedded in the IMM; the OXPHOS complexes I, II, III and IV (Fig. 17). Together these complexes form the mitochondrial respiratory chain, also referred to as the mitochondrial electron transport chain (ETC). Oxygen is the final electron acceptor, and its reduction leads to the production of water molecules. Consequently, oxygen consumption reflects mitochondrial function (van der Bliëk *et al.*, 2017). The mitochondrial ETC also encompasses two mobile interactors. Ubiquinone, a small fat-soluble molecule that moves freely inside and across the IMM. Ubiquinone carries the electrons from complexes I or II, to complex III. The second mobile electron carrier is cytochrome *c*, a protein mainly present in the cristae (see section 4.1.) that transfers electrons from complex III to complex IV (Fig. 17; Rich and Maréchal, 2010).

Electron transport is coupled with proton translocation from the matrix to the crista, creating an electro-chemical gradient (Fig. 17; van der Bliëk *et al.*, 2017). This electrochemical gradient powers an ancient rotary turbine enzyme, the ATP synthase, also known as OXPHOS complex V. ATP synthase produces ATP through ADP phosphorylation (Fig. 16 and 17; Friedman and Nunnari, 2014). ATP synthases are embedded as dimers in the IMM at the end of the cristae. The dimer configuration causes a slight curvature in the IMM, and the presence of long rows of dimers is probably responsible for the shape of the cristae. The presence of ATP synthases at the end of the crista, rapidly consuming protons, creates a source-to-sink proton gradient inside the crista. This gradient optimizes the movement of protons, accelerating ATP production (Cogliati *et al.*, 2016).

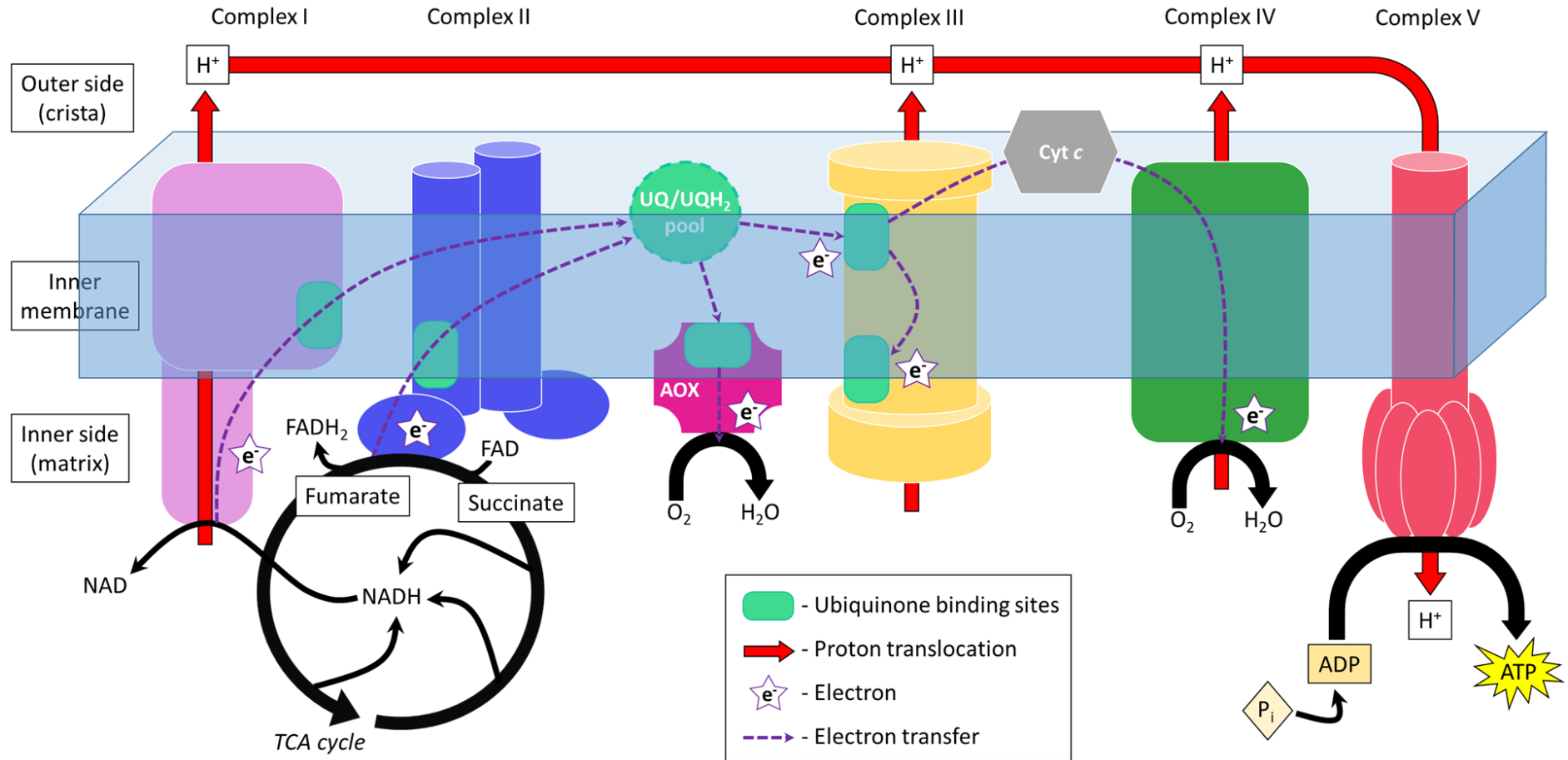


Figure 17. Mitochondrial electron transport chain (adapted from Leroux and Walker, unpublished). UQ/UQH₂ represents the pool of ubiquinone /ubiquinol that carries the electrons. AOX is the alternative oxidase.

OXPPOS is responsible for the production of 38 ATP molecules starting from one molecule of glucose (for review, Rich and Maréchal, 2010).

Crista morphology can change according to the cell's energy needs. When metabolic activity diminishes, the cristae junctions open and the efficiency of the mitochondrial ETC decreases, while protons and cytochrome *c* become free to diffuse inside the IMS away from the OXPPOS complexes. During programmed cell death, crista shape is also modified, releasing cytochrome *c* that diffuses into the cytosol and participates in the apoptosis signaling cascade (Cogliati *et al.*, 2016).

OXPPOS complexes are present in cristae as both isolated complexes, or associated with other complexes forming structures called the supercomplexes (SCs). SCs enhance electron transport and regulate ROS production by maintaining individual complexes close to each other. The main SCs associate complexes I + III, I + III + IV or III + IV. Most complexes I are associated with SCs because it increases their stability. Complexes II, on the other hand, are never found in SCs, as they are mainly localized at the IBM. Complexes V are also never associated with SCs. The shape of the cristae can influence the assembly and stability of SCs, therefore adapting energy production to cellular needs. For these reasons, SCs represent the true functional unit of the mitochondrial ETC (Cogliati *et al.*, 2016).

4.2.2. Complex I: composition, function and inhibitors.

Complex I is also referred to as the NADH:UQ oxidoreductase (UQ designates ubiquinone) because it triggers NADH oxidation and ubiquinone reduction (Fig. 17). It is an “L”-shaped complex (Fig. 16 and 17) composed of many subunits (45 for the bovine complex I), a majority of which are encoded by nuclear DNA. Fourteen subunits compose the catalytic part of the complex, involved in redox reactions and proton translocation (van der Bliëk *et al.*, 2017). In addition, complex I embeds several cofactors like a flavin adenine dinucleotide (FAD) and different iron-sulfur clusters (for review, Lill, 2009). Complex I oxidizes NADH that releases one electron further transferred to ubiquinone. The electron transfer is coupled to the translocation of four protons to the IMS.

Some organisms like *Saccharomyces cerevisiae* lack a real complex I, and instead possess three independent NADH dehydrogenases. One of them locates in the IMM and oxidizes the NADH produced during the TCA cycle. The other two are present in the OMM, oxidizing the NADH produced in the cytosol. However, these oxidation steps are not coupled to proton translocation.

Other organisms like plants or some fungi possess two alternative NADH dehydrogenases in addition to a fully functional complex I (Kerscher *et al.*, 2008).

Complex I inhibitors prevent the electron transfer between iron-sulfur clusters and ubiquinone. They display various biocidal properties, mostly insecticidal and acaricidal. However, pyrimidamines are known for their antifungal activities. Their only authorized representative in agriculture is diflumetorim (Fig. 18; Table 3), with anecdotal use in horticulture in Japan against rusts and mildews (Jeschke *et al.*, 2019). However, a renewed interest in pyrimidamines has recently emerged with the discovery of potent fungicidal compounds, some derived from diflumetorim (Guan *et al.*, 2017; Yang *et al.*, 2020).

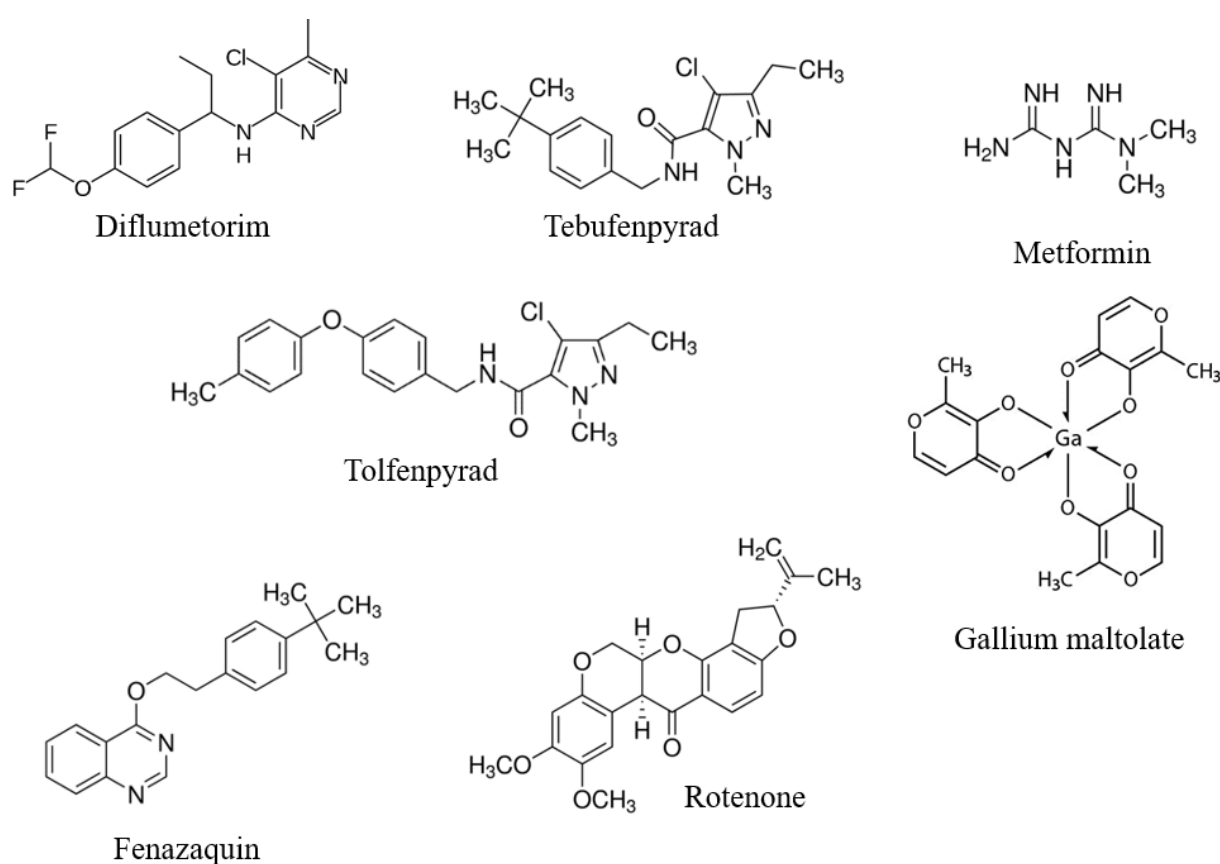


Figure 18. Chemical structures of some major complex I inhibitors.

The next compounds display antifungal properties but are mainly used as miticides. This is the case of the pyrazoles tebufenpyrad and tolfenpyrad (Fig. 18; Table 3). Tebufenpyrad also displays acaricide properties (Meister and Sine, 2012). Recently, tolfenpyrad and some of its derivatives were found to be also very potent against the ruminant parasitic nematode *Haemonchus contortus* (Jiao *et al.*, 2020). The quinazoline fenazaquin (Fig. 18; Table 3) displays fungicidal, acaricidal and insecticidal activities. It is used against mites in various crops

like fruits (grapes, citrus, peaches...), nuts, or in horticulture. The natural toxin rotenone (Fig. 18) is another complex I inhibitor, also known for its broad-spectrum insecticide, but also piscicide properties (Ambrose and Haag, 1936).

Complex I inhibitors are also used in human medicine. For example, metformin (Fig. 18), a biguanide used to treat type 2 diabetes, regulates glucose metabolism through complex I inhibition, among other mechanisms of action (Kopel *et al.*, 2020). Metformin, in combination with another complex I inhibitor, gallium maltolate (Fig. 18), has recently given encouraging data in the treatment of glioblastoma, an aggressive form of brain cancer, in rat (Alhajala *et al.*, 2020).

4.2.3. Complex II: composition, function and inhibitors.

Complex II is called succinate dehydrogenase (SDH) or succinate:UQ oxidoreductase. It is the smallest complex, composed of only four subunits, and a second entry point for electrons in the ETC. It is the only complex whose subunits are all encoded in the nuclear genome in most species, with very few exceptions. Complex II is a component of both the mitochondrial ETC and the TCA cycle (Fig. 16). It is also the only complex that does not translocate protons into the IMS (for review Hägerhäll, 1997), and that does not locate in the cristae but in the IBM, generally as homodimers (Fig. 16 and 17) (Cogliati *et al.*, 2016). Subunits A and B are hydrophilic and oriented to the matrix, while subunits C and D are hydrophobic and anchored in the IMM. Complex II embeds one ubiquinone fixation site, bounded by the subunits B, C and D and close to the inner side of the IMM (Fig. 17). It couples its catalytic function from the TCA cycle (the oxidation of the succinate into fumarate) to ubiquinone reduction through electron transfer (for review Hägerhäll, 1997).

Consequently, complex II can be inhibited either by preventing succinate oxidation or by preventing ubiquinone reduction. Several competitive inhibitors of succinate oxidation are known like some dicarboxylic acids as malonic acid, oxalic acid or oxaloacetic acid (Huang *et al.*, 2006). The anticancer agent lonidamine (Fig. 19) also inhibits fumarate formation by SDH (Guo *et al.*, 2016). However, most inhibitors are preventing ubiquinone reduction. It is the case of some vitamin E analogs, used as anticancer agents (Neuzil *et al.*, 2007) or the natural antibiotics, the atpenins (Fig. 19) (Miyadera *et al.*, 2003).

SDH inhibitors (SDHIs) were mostly developed as antifungal agents. These antifungals share a common structural feature: an amide group (Fig. 19) necessary to create hydrogen bonds with

the ubiquinone fixation site, suggesting a similar binding mode for all SDHIs (Glättli *et al.*, 2011).

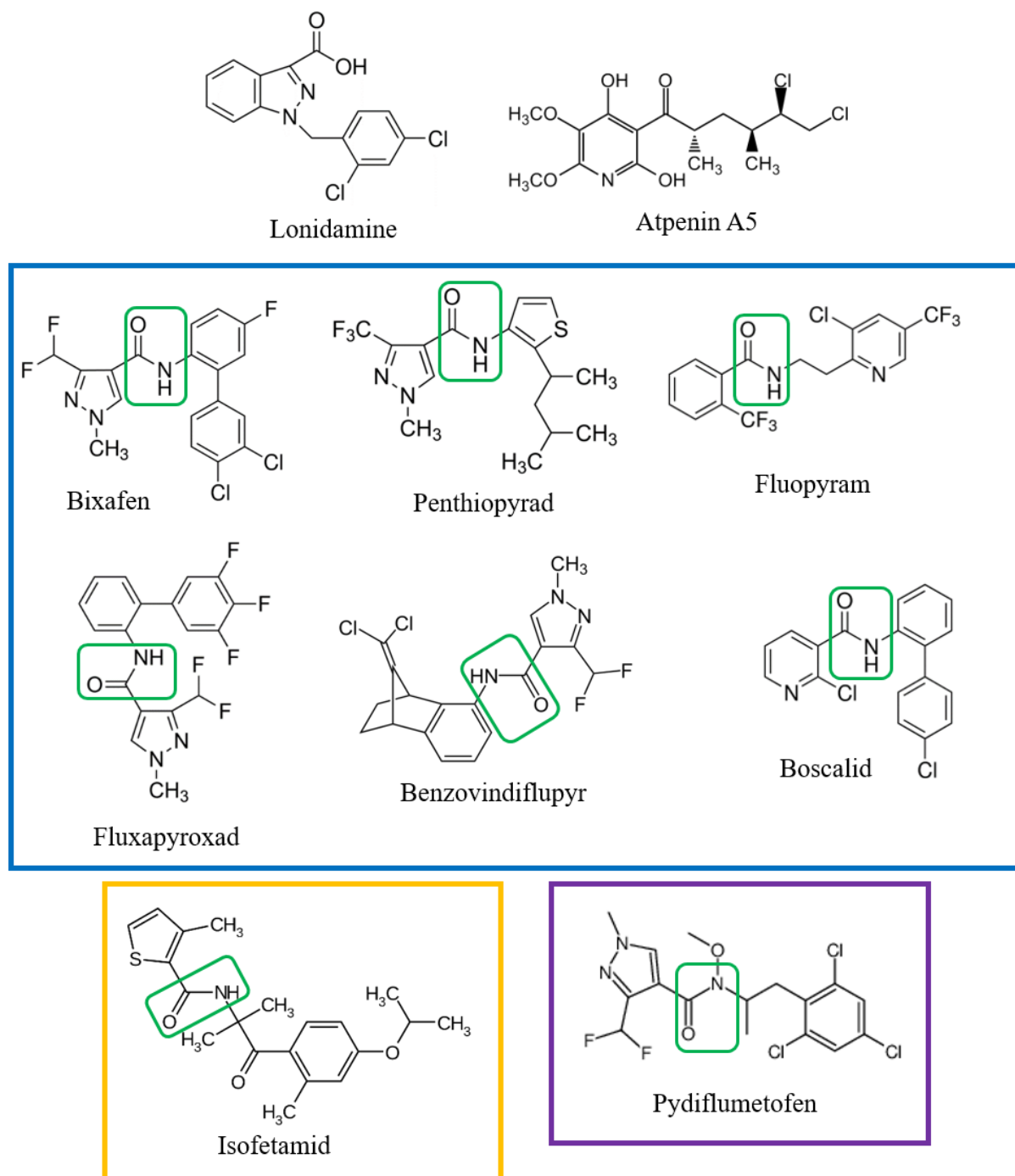


Figure 19. Chemical structures of some major complex II inhibitors. Framed molecules are SDHIs used in agriculture. Blue frame: pyrazole-4-carboxamides; Yellow frame: phenyl-oxo-ethyl-thiophene amide; Purple frame: N-methoxy-(phenyl-ethyl)-pyrazole-carboxamide. Green frame: carboxamide group necessary for SDHIs activity.

These fungicides are regrouped under the general name “carboxamides”. Oxathiin- (e.g. carboxin and oxycarboxin) and benzamide-carboxamides were first developed in the 1960s and had a narrow activity spectrum, mostly limited to basidiomycetes. Broad-spectrum SDHIs were only experimental. Therefore, the first generation SDHIs were mostly used against *Ustilago nuda* on barley, the loose smut agent *U. tritici* and rusts (Table 1). The first broad spectrum SDHI launched on the French market was the pyridine boscalid (Fig. 19) in 2006 (Table 2; Lalève, 2013). Ever since, the SDHI fungicide class has rapidly increased and diversified, in terms of new active ingredients and chemical classes. In particular, the pyrazole-carboxamides (Fig. 19; Table 3) now constitute the most used and expanding chemical class. Currently, the expansion of SDHI class is ongoing. Due to their strong antifungal activity and lack of equally efficient control methods, farmers quickly adopted SDHIs, as shown by their use in more than one field out of two in the UK since 2012 (Sierotzki and Scalliet, 2013).

Among SDHIs are found two pro-acaricides: cyflumetofen and cyenopyrafen. Their activation results in the production of β -cetonitriles displaying structural similarities with carboxamide fungicides (Khalighi *et al.*, 2013).

4.2.4. Complex III: composition, function and inhibitors.

Complex III is referred to as the UQ:cytochrome *c* oxidoreductase or as the cytochrome *bc*₁ complex, often shortened to *bc*₁ complex. Its role is to transfer electrons from ubiquinol to cytochrome *c* while translocating protons to the IMS. It is one of the main ROS producing complexes. It functions as homodimer, each monomer being composed of eleven subunits (Fig. 16). The catalytic core of the complex is made of three proteins: cytochrome *b*, the Rieske protein and cytochrome *c*₁ (Hunte *et al.*, 2000).

4.2.4.1. Cytochrome *b*.

Cytochrome *b* (390 amino-acids, 44 kDa) is the only mitochondrially encoded subunit of complex III. It occupies the central position in the complex and is composed of eight parallel transmembrane helices, its N- and C-terminal domains being in the matrix (Fig. 20). Helices are linked by large loops, some of which harbor one or two helices parallel to the membrane (Fig. 20). It embeds both ubiquinone binding sites of the complex, one closer to the outer side of the IMM, called the Q_o site, and the other one closer to the inner side, the Q_i site (Fig. 17).

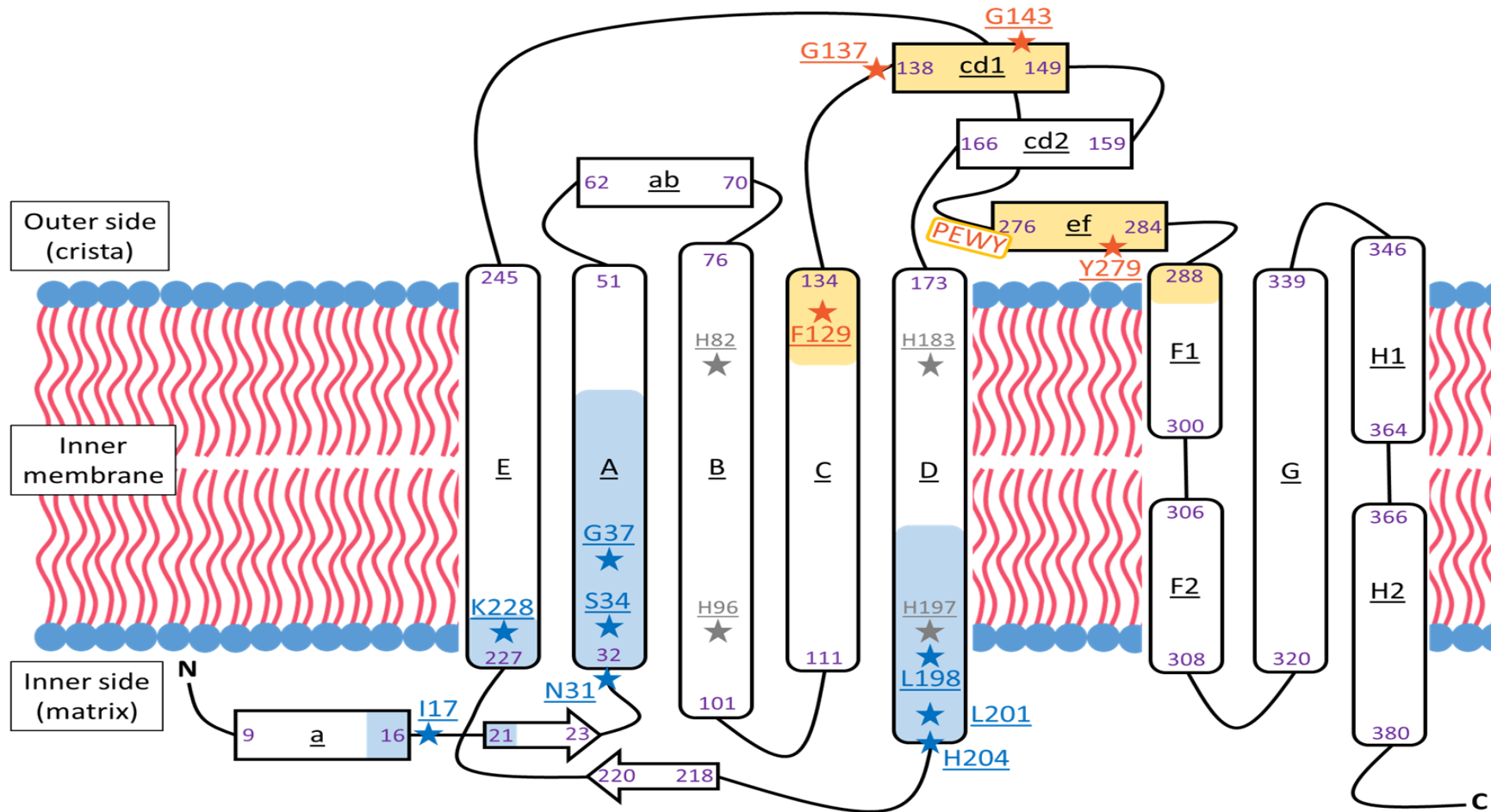


Figure 20. Schematic representation of the cytochrome *b* structure (adapted from Fisher and Meunier, 2008; Mounkoro *et al.*, 2019). Yellow region = Q_o site; Blue region = Q_i site; Grey stars = histidine residues involved in the binding of the heme groups b_L and b_H . Positions of main residues involved in inhibitor resistance in *S. cerevisiae* laboratory-selected mutants are shown: orange stars (Q_o site) = F129, G137, G143 and Y279; blue stars (Q_i site) = I17, N31, S34, G37, L198, L201, H204 and K228.

The Q_o site is the ubiquinol oxidation site, and the Q_i site is the ubiquinone reduction site. Both sites are connected by an electron transfer path, composed of two type *b* heme groups, the heme *b_L* and *b_H* groups (Fig. 21). Helixes A, B, C and D embed both heme groups, which are stabilized by hydrogen bonds. Moreover, the heme central iron atoms are linked to histidine residues belonging to helixes B and D (Fig. 20). Histidines 82 and 183 are linked to the heme *b_L*, while histidines 96 and 197 are linked to the heme *b_H*. Cytochrome *b* also encompasses the proton translocation path to the IMS, and the docking platform of the Rieske protein (Hunte *et al.*, 2000).

4.2.4.2. Cytochrome *c*₁.

Cytochrome *c*₁ belongs permanently to the complex, and differs from cytochrome *c*. Its catalytic domain is hydrophilic and located at the N-terminal region, inside the IMS. It embeds a *c*-type heme group, covalently linked to the protein. The transmembrane domain anchors the protein into the IMM, while the C-terminal region guides the cytochrome *c*₁ membrane insertion. Cytochrome *c*₁ possesses regions interacting with both the Rieske protein and cytochrome *c* (Arnold *et al.*, 1998).

4.2.4.3. The Rieske protein.

The Rieske protein possesses four distinct domains: the catalytic domain located in the IMS, anchored to the transmembrane domain by a flexible linking domain, and the N-terminal domain, located in the matrix. The catalytic domain embeds an iron-sulfur cluster, and can be found close to each of the monomeric cytochrome *c*₁ thanks to the flexible domain (Rieske and Coleman, 1964).

4.2.4.4. Additional subunits.

The other subunits of the OXPHOS complex III can display many different functions. So far, identified roles are as various as protein maturation, protein-protein interactions, complex assembly, proton translocation, dimer assembly and stabilization, iron-sulfur cluster insertion in proteins and cytochrome degradation prevention.

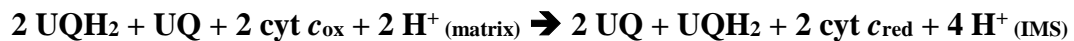
4.2.4.5. Activity of the cytochrome *bc*₁ complex.

4.2.4.5.1. The Q cycle.

The process by which electrons are transferred from ubiquinol to cytochrome *c* is called the Q cycle, divided in two half cycles (Fig. 21).

The first half cycle begins with the binding of one ubiquinol molecule at the Q_o site. As a reminder, ubiquinol comes from the reduction of ubiquinone by either complex I or II. At the Q_o site, ubiquinol oxidation releases two protons that are pumped into the IMS, and two electrons, regenerating a ubiquinone molecule. The first electron is transferred to the iron-sulfur cluster of the Rieske protein, and then to the heme group of cytochrome *c*₁, where it reduces a Fe³⁺ ion into Fe²⁺. Finally, the electron reaches cytochrome *c*, which once in its reduced form can detach from complex III and move towards complex IV. The second electron released by ubiquinol is transferred successively to the *b*_L and *b*_H heme groups of cytochrome *b*. The final electron acceptor is a ubiquinone molecule bound at the Q_i site. The partial reduction of ubiquinone generates a ubisemiquinone radical ion (Fig. 21) (Hunte *et al.*, 2003).

The second half cycle is the same, and begins with the binding of a second ubiquinol molecule at the Q_o site. When the second electron is transferred to the ubisemiquinone radical ion, two protons are pumped from the mitochondrial matrix and used to regenerate one ubiquinol molecule (Fig. 21) (Hunte *et al.*, 2003). The outcome of the Q cycle is:



An incomplete Q cycle would lead to energy loss with only two protons instead of four released in the IMS. Moreover, in the absence of complete ubisemiquinone reduction, this radical may react with oxygen, producing harmful superoxide ions. The configuration (positions and distances) of the different redox centers are probably responsible for the correct distribution and transfer of electrons (Mazat and Ransac, 2010).

The two protons coming from ubiquinol oxidation, like the two electrons, follow different paths. One is transferred to a histidine residue of the Rieske protein and follows the electron transfer, before being rejected into the IMS. The second proton's path is not known yet. However, cardiolipin, a membrane phospholipid specific to the mitochondrial IMM, is supposed to be involved in proton translocation and pumping at both the Q_o and Q_i sites (for review, Hunte *et al.*, 2003).

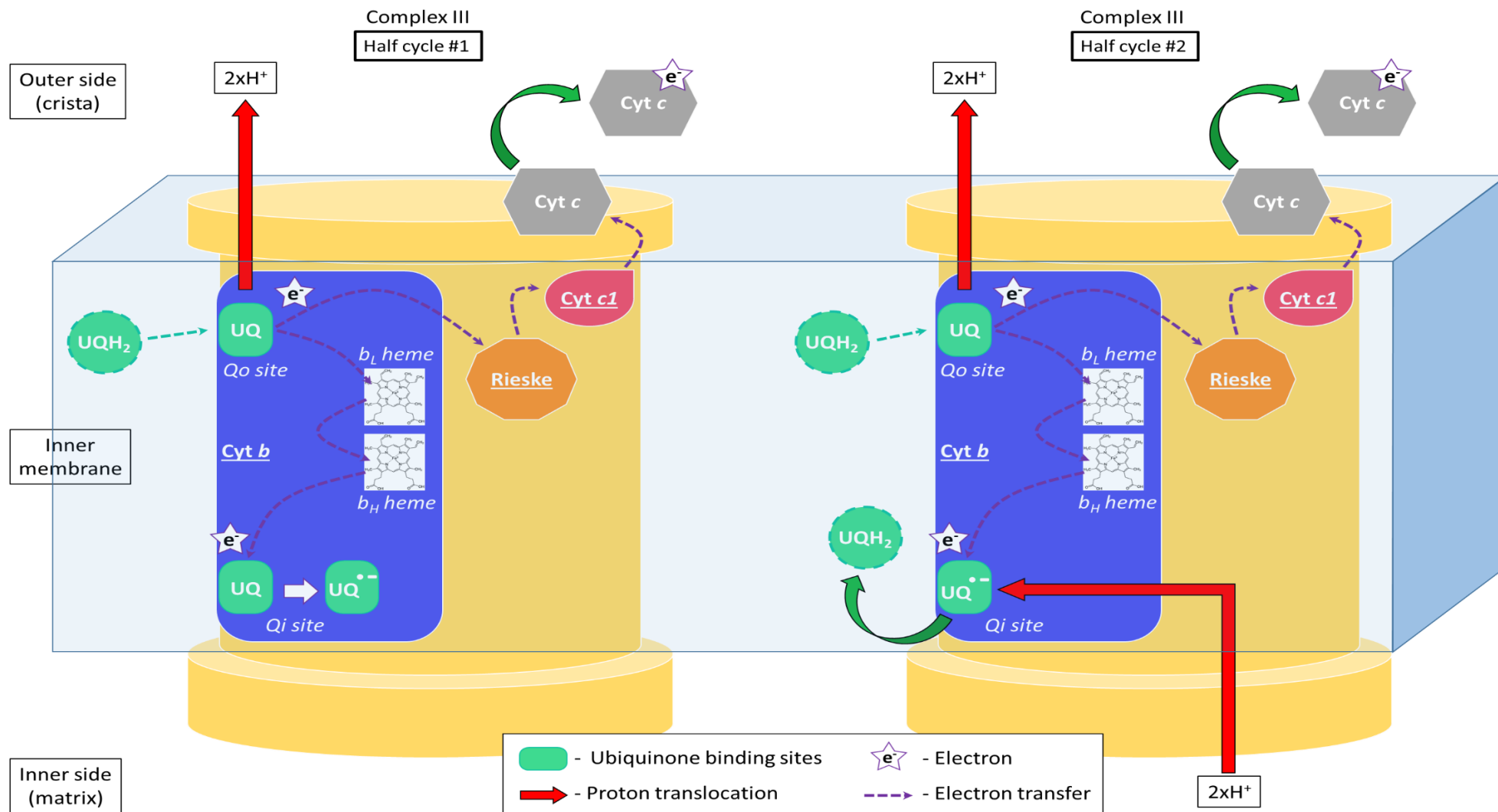


Figure 21. The Q cycle of the mitochondrial ETC complex III (based on Mitchell, 1975). UQ = Ubiquinone; UQH₂ = Ubiquinol; UQ^{•-} = Ubisemiquinone radical ion.

4.2.4.5.2. The ubiquinone outer binding site or Q_o site.

The Q_o site is a large region bounded by the C-terminal region of the transmembrane helix C, the surface helix cd1, the loop containing the PEWY motif and the surface helix ef, and a small part of the F1 transmembrane helix (Fig. 20). The PEWY motif is a highly conserved amino-acid sequence Pro-Glu-Trp-Tyr essential for cytochrome *b* catalytic activity (Fisher and Meunier, 2008). The Q_o site opens up into a hydrophobic pocket where substrates can be exchanged. Complex III dimers embed two hydrophobic cavities, each one being shared by the one monomer's Q_o site and the other monomer's Q_i site (for review, Hunte *et al.*, 2008).

4.2.4.5.3. The ubiquinone inner binding site or Q_i site.

The C-terminal region of surface helix A, the N-terminal region of transmembrane helices A and E, and the C-terminal region of helix D define the ubiquinone reduction site (Fig. 20). Crystallographic data obtained with yeast, bovine and chicken cytochrome *b* with a ubiquinone molecule bound at the Q_i site revealed two important residues. In yeast, histidine 202 and serine 206 create hydrogen bonds with the benzoquinone headgroup of ubiquinone. This interaction allows correct orientation and stabilization of ubiquinone. Moreover, ubiquinone binds close to the *b_H* heme group (3.5 Å), optimizing electron transfer (Fisher and Meunier, 2008).

4.2.4.6. Complex III inhibitors.

Specific inhibitors of the *bc*₁ complex constitute a large class of antimicrobial agents. Their ability to block the mitochondrial ETC with high efficiency, leading to energy depletion and ROS accumulation in microbial cells, have generated a great deal of interest in these molecules. However, the highly conserved structure of cytochrome *b* among species increases the difficulty to find pathogen-specific active ingredients that are safe to non-targeted species. Progress has been made in this area, and over the past decades, the use of *bc*₁ complex inhibitors has largely expanded in both medicine and agriculture. This group is composed of inhibitors specific to the Q_o or Q_i site, and inhibitors that can bind to both sites (Fisher and Meunier, 2008).

4.2.4.6.1. Inhibitors of the Q_o site (QoIs).

At the Q_o site, inhibitors can bind in two distinct positions. However, only one inhibitor at a time can bind as docking positions overlap. Class I inhibitors or QoIs-D bind to the distal part of the Q_o site, close to the Rieske protein binding site. This binding pose is strengthened by a

hydrogen bond between the inhibitor molecule and a histidine residue of the Rieske protein, therefore immobilizing the Rieske protein. Class II inhibitors or QoIs-P bind to the proximal part of the Q_o site, without interacting with the Rieske protein. No cross-resistance is observed between the two inhibitor types (Crofts *et al.*, 1999). Cross-resistance refers to the situation in which resistance to one active ingredient triggers resistance to some other ones (Cândido *et al.*, 2019).

Strobilurins are the largest class of QoIs-P (Class II). Strobilurins got their name from the fungus they were first isolated from, *Strobilurus tenacellus* (Schramm *et al.*, 1978). They are used in agriculture against a broad range of fungal pathogens (Table 3). First strobilurins shared a methoxyacrylate (or derivative) pharmacophore originating from the natural molecule from which they are derived, strobilurin A (Fig. 22). Natural strobilurins were not developed as fungicides because of their toxicity and instability, especially under light. Since the introduction of the first synthetic strobilurin azoxystrobin in the field in 1996, this class has widely expanded, and now encompasses ten chemical classes and at least twenty active ingredients registered (Table 3). Strobilurins display high antifungal and anti-oomycete activities, and are used on extremely various crops from cereals to fruits, nuts, cucurbits, coffee etc... (Bartlett *et al.*, 2002).

Other QoIs were also natural compounds isolated from microorganisms (Fisher and Meunier, 2008). As examples, mucidin (another natural strobilurin) was isolated from the basidiomycete *Oudemansiella mucida*, myxothiazol, a methoxyacrylamide from *Myxococcus fulvus*, and stigmatellin, from *Stigmatella aurantiaca* (Fig. 22). Stigmatellin is the model molecule for the binding of class I inhibitors (QoIs-D), while myxothiazol is the model for class II inhibitors (QoIs-P). Nevertheless, due to their high activity against mammalian cytochrome *b*, these compounds were not developed for further use in medicine or agriculture (Fisher and Meunier, 2008). However, about ten years ago, a new natural QoI called nepeltolide was isolated from a deep-water sponge. Nepeltolide is a macrolide bearing a carbamate that seems to bind in the two positions at the Q_o site, therefore representing a putative novel class of QoIs (QoI-D,P?) (Berry and Huang, 2011). Different modifications of the original chemical structure of nepeltolide gave promising results for the development of a new QoI fungicide class (Zhu *et al.*, 2019).

Aside from strobilurins, other QoI classes are available in agriculture are famoxadone, an oxazolidine-dione, fenamidone, an imidazolinone especially active against downy mildews, and the more recently developed methyltetraprole, a tetrazolone (Fig. 22; Table 3). These

compounds do not display the characteristic chemical features of strobilurins but were identified as QoIs due to their pattern of cross-resistance with strobilurins (Jordan *et al.*, 1999). Famoxadone has a unique binding pose, as it is a class II inhibitor maintaining the Rieske protein in the same position as class I inhibitors, though without any direct interactions (Jordan *et al.*, 1999).

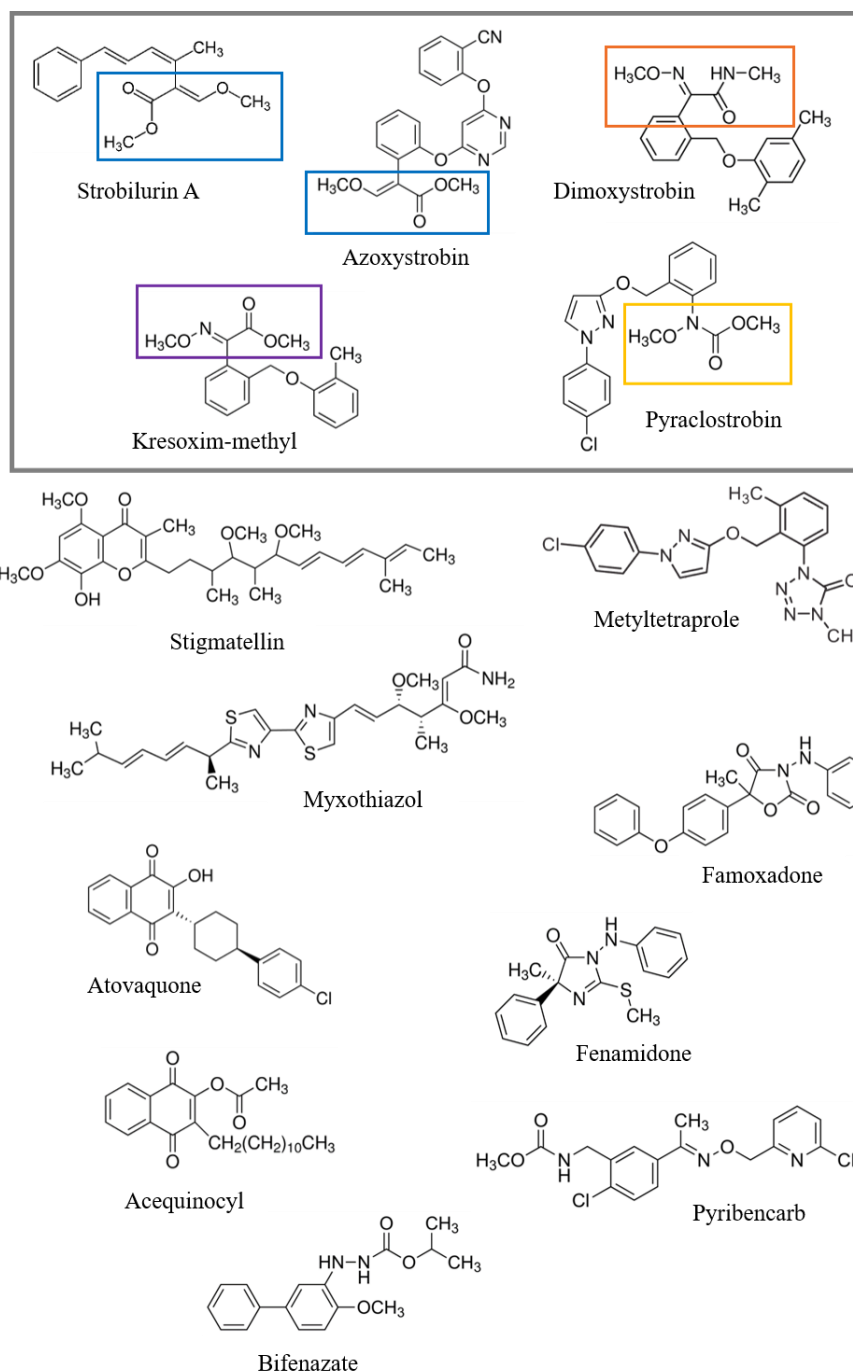


Figure 22. Chemical structures of different QoIs. Grey frame: strobilurins encompassing different chemical classes: methoxy-acrylates (blue frame), oximino-acetamide (orange frame), oximino-acetate

(purple frame) and methoxy-carbamate (yellow frame). Metyltetraprole displays a methyl-tetrazolone moiety but the rest of its structure is almost the same as pyraclostrobin.

Two other antifungal QoIs used in agriculture are pyribencarb, a benzylcarbamate developed in 2010 (Kataoka *et al.*, 2010), and the recently authorized metyltetraprole (Suemoto *et al.*, 2019), structurally similar to pyraclostrobin, but without cross-resistance to other QoIs (Fig. 22; Table 2). Today, QoIs are authorized on more than eighty different crops and on turf grass, in more than 70 countries, which corresponds to more than 400 pathosystems potentially controlled by QoIs (Bartlett *et al.*, 2002).

Among QoIs are found other anti-parasitic drugs, such as atovaquone (Fig. 22). Atovaquone is a class I hydroxynaphthoquinone inhibitor used against the malaria agent *Plasmodium falciparum*, but also efficient against the yeast-like fungus *Pneumocystis jirovecii*, or the toxoplasmosis agent, *Toxoplasma gondii* (Fisher and Meunier, 2008). Against *P. falciparum*, some phthalimide derivatives acting as QoIs display activity and selectivity good enough for further development as new antimalarial drugs (Okada-Junior *et al.*, 2018). Bifenazate and acequinocyl (Fig. 22) are two QoIs used for their acaricide and insecticide properties, especially against parasitic mites (Fotoukkaia *et al.*, 2020).

4.2.4.6.2. Inhibitors of the Q_i site (QiIs).

When a QiI binds to only one cytochrome *b* of the dimer, electrons that cannot be transferred from the *b_H* heme group to the ubisemiquinone, are able to go back to the *b_L* heme group and operate a *b_L* - *b_L* transition. Using this path, electrons can be transferred to the *b_H* heme group and to the ubisemiquinone of the second monomeric cytochrome *b*. This step is not limiting and almost as fast as the “normal” pathway. Therefore, no inhibition is observed until the QiI binds to both monomers (Ransac and Mazat, 2010).

Some natural QiIs have been known for a long time. Examples are funiculosin (Fig. 23), isolated from *Penicillium funiculosum*, which is known from the 1970s but never led to drug development, and diuron (Fig. 23), which is a weak *bc₁* complex inhibitor but a very powerful inhibitor of photosynthesis, therefore used as an herbicide.

Illicolin H (Fig. 23) is another natural QiI first described in the early 2000s, interestingly displaying a 40 times higher inhibitory activity on the yeast enzyme than on the bovine enzyme. Indeed, such a difference in activity might be promising for further antifungal drug development (Fisher and Meunier, 2008).

Only two QiI fungicides are used in agriculture so far, the sulfonamides cyazofamid and amisulbrom (Fig. 22; Table 3). They were both launched on the market in the early 2000s and display strictly anti-oomycete activity (Mitani *et al.*, 2001).

In human medicine, several quinolones acting against *P. falciparum* were also found to be QiIs. Examples are different endochin-like quinolones (ELQ) (Doggett *et al.*, 2012), and the powerful inhibitor 1-hydroxy-2-dodecyl-4(1H)quinolone (HDQ) (Fig. 22; Eschemann *et al.*, 2005). Among QiIs, the pro-insecticide hydramethylnon is used in the form of baits for cockroaches and ants.

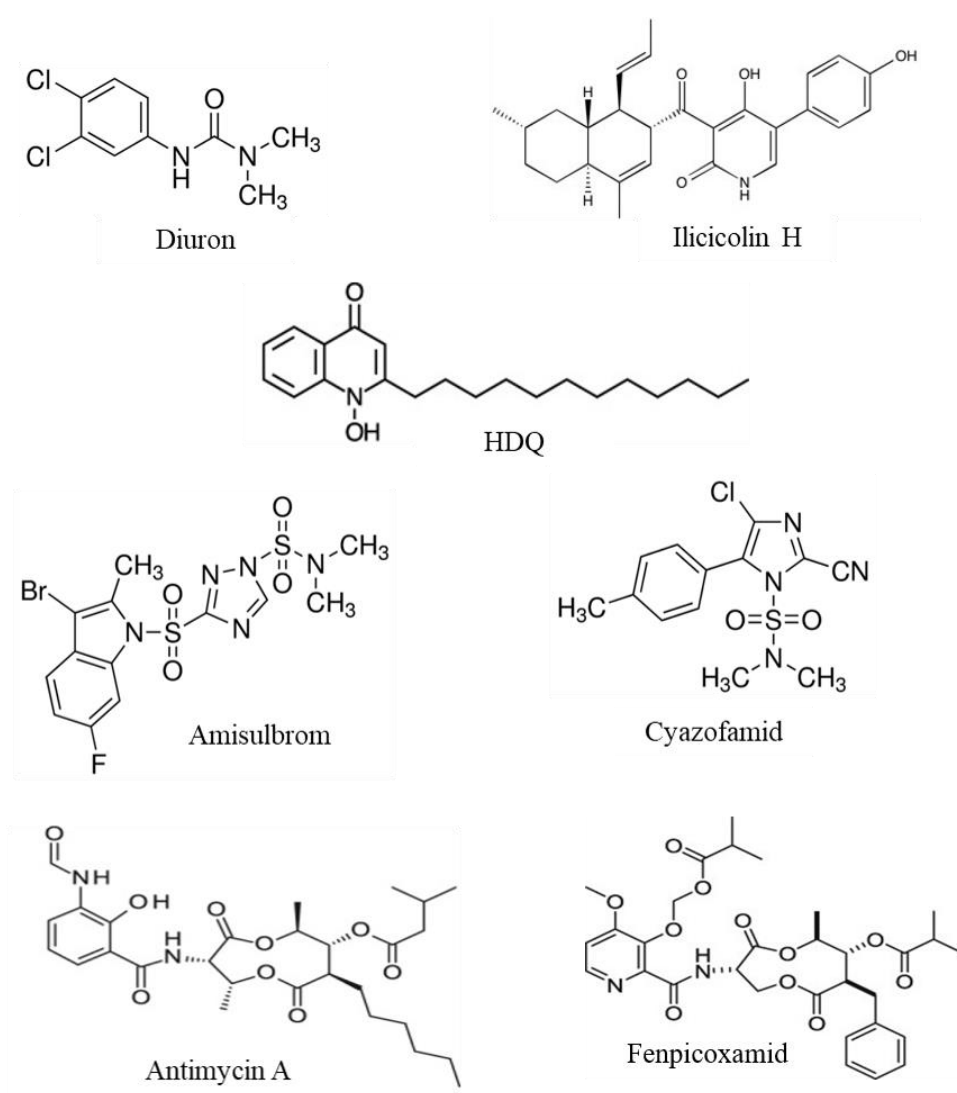


Figure 23. Chemical structures of different QiIs.

Antimycin A (Fig. 23) is a natural salicylamide compound, produced by some *Streptomyces* species. It is a potent QiI able to inhibit yeast, chicken, bovine and other mammalian bc_1 complexes, therefore displaying cytotoxicity to a broad range of cells and organisms (Ueki *et al.*, 1996). Its docking site overlaps that of ubiquinone, and its binding relies mostly on hydrophobic interactions (Fisher and Meunier, 2008). Three highly conserved residues stabilize its binding, aspartic acid 228 through direct hydrogen bonding, as well as lysine 227 and histidine 201 through water-bridged hydrogen bonds. Antimycin displays tight binding but is a slow inhibitor, likely because it needs to undergo a conformational change to be able to bind, therefore delaying noticeable inhibition (Li *et al.*, 2014). Due to its lack of specificity, antimycin cannot be used in medicine or agriculture.

4.2.4.6.3. Fepicoxamid, a new anti-STB for wheat crops.

The natural antifungal compound UK-2A (Fig. 24) is secreted by the soil-borne bacterium *Streptomyces sp.* 517-02 (Owen *et al.*, 2017; Ueki *et al.*, 1996). It has been studied for its strong antifungal properties since the 1990s, especially against filamentous fungi like *A. fumigatus* or *Sclerotinia sclerotiorum*, and some yeast like *Candida spp* (Ueki *et al.*, 1996). It is a picolinamide (Fig. 24) QiI, also displaying activity against STB, yellow rust, brown rust and tan spot diseases (Table 1). However, in spite of its high intrinsic activity and favorable ecotoxicological profile, UK-2A is unsuitable for a direct use in agriculture due its high instability under UV light (Owen *et al.*, 2017). In order to circumvent this issue, Corteva™ Agriscience, in collaboration with Meiji Seika Pharma Co. Ltd., developed fepicoxamid. The etherification of UK-2A (Fig. 24) confers fepicoxamid with a much higher stability under UV light, making possible its use in the field. Moreover, once it penetrates inside the fungal cells or the wheat cells, fepicoxamid is rapidly metabolized back into UK-2A, which is the active metabolite (Owen *et al.*, 2017; Young *et al.*, 2018).

UK-2A is structurally similar to antimycin which is a salicylamide, not a picolinamide (Fig. 24). It has been demonstrated that the 3-formylamino group is essential for antimycin activity. Its substitution by a 4-methoxy group in UK-2A, as well as the replacement of the salicylic acid moiety by a pyridine ring might explain the differences in their cytotoxic activity. Indeed, antimycin displays cytotoxicity to a broad range of organisms, while UK-2A is mainly fungitoxic (Ueki *et al.*, 1996).

The 9-membered bislactone ring (Fig. 24) in both compounds is not essential for respiratory inhibition. However, its high hydrophobicity allows a better cell penetration and a tighter

binding at the cytochrome *b* Q_i site (Usuki *et al.*, 2006). More recent work showed that the picolinamide ring of UK-2A is not essential for its activity either (Owen *et al.*, 2019). However, the pyridyl nitrogen seems to be very important for UK-2A binding (Fig. 25).

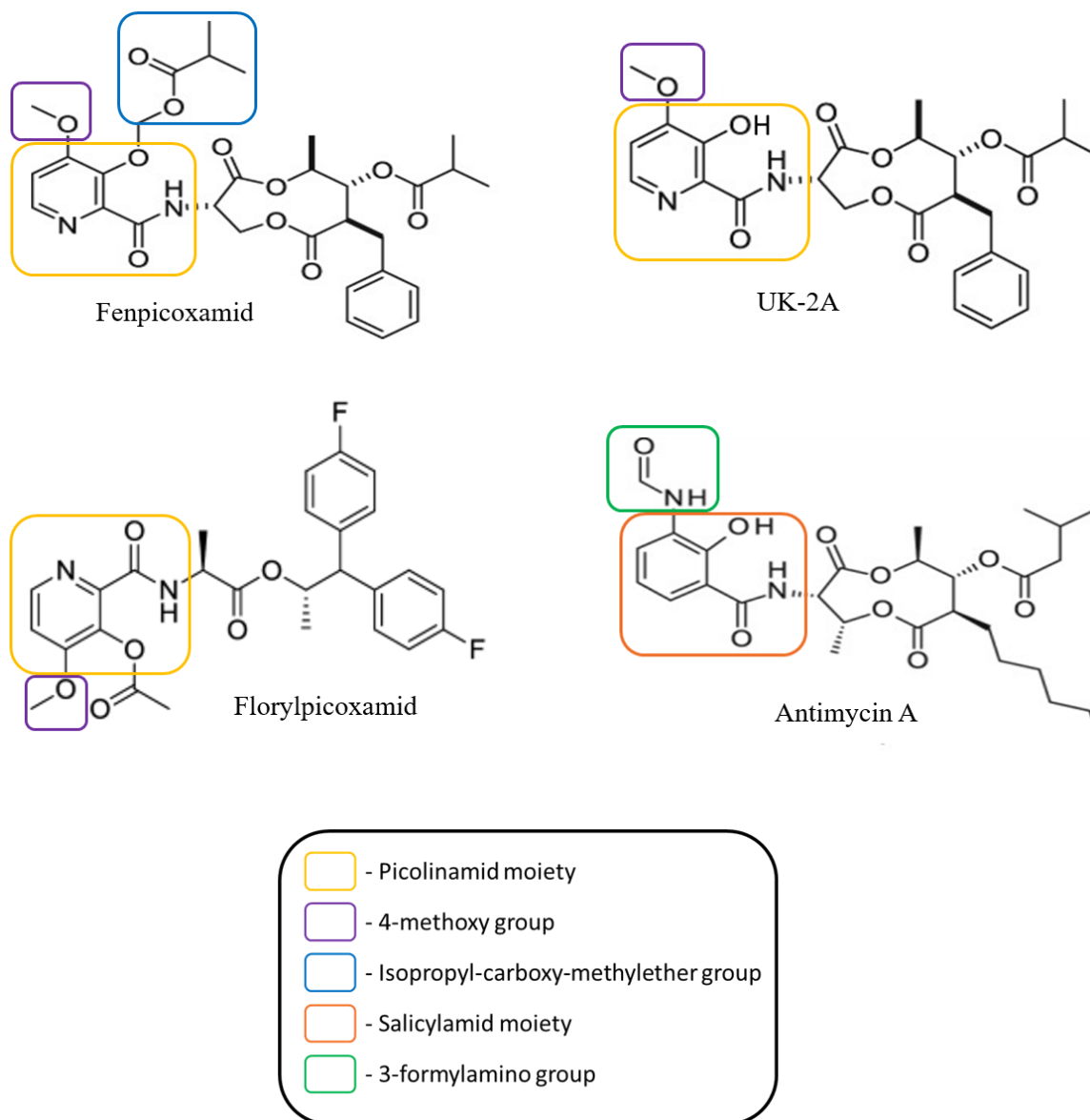


Figure 24. Comparison of chemical structures of fempicoxamid, UK-2A, antimycin A and florylpicoxamid. UK-2A and antimycin A are both natural compounds. Fempicoxamid derives from UK-2A by the addition of an isopropyl-carboxy-methylether group. Florylpicoxamid is another picolinamide Q_iI with a strong anti-ascomycete activity.

When UK-2A binds to the Q_i site, its pyridine head rotates by 180 degrees, and the nitrogen, positively charged, forms a salt bridge with the aspartate in position 229 (D229), reinforcing the binding (Young *et al.*, 2018). In comparison, the nitrogen of the formylamino group in antimycin forms hydrogen bonds with the lysine 228 (K228) and the asparagine 31 (N31). D229 and N31 are close to each other in the folded protein, making them two key residues for UK-2A binding (Fig. 20; Fig. 25). Two other important residues, regarding their steric hindrance, are glycine 37 (G37), which is close to the bislactone ring and the exocyclic methyl group, and leucine 198 (L198), which is also close to the bislactone ring (Fig. 25) (Young *et al.*, 2018).

UK-2A shows strong inhibition of the complex III enzyme activity of *Z. tritici*, as well as abolishing mitochondrial membrane potential. Its activity (IC_{50} [5-8] nM) is comparable to that of antimycin (IC_{50} [6-8] nM) and pyraclostrobin, (IC_{50} [3-4] nM), two of the most powerful known cytochrome *b* inhibitors (Young *et al.*, 2018). It is a potent inhibitor of spore germination and mycelial growth. Fenpicoxamid can therefore be used preventively or curatively, but gives better disease control when applied as a protectant treatment. Inatreq™ (commercial name of fenpicoxamid) shows good persistence on leaf surface (90% persistence after 8 days) and progressive plant penetration (up to 5% after 48h). Once inside the plant tissues, fenpicoxamid is converted back into UK-2A, which redistributes acropetally in the plant's vascular system, and shows translaminar migration. In the field, first trials performed in France with Inatreq™ in association with a triazole (prothioconazole) or a SDHI (benzovindiflupyr) exhibited disease control and yield gains similar to those of standard treatments already on the market (Arvalis, 2019).

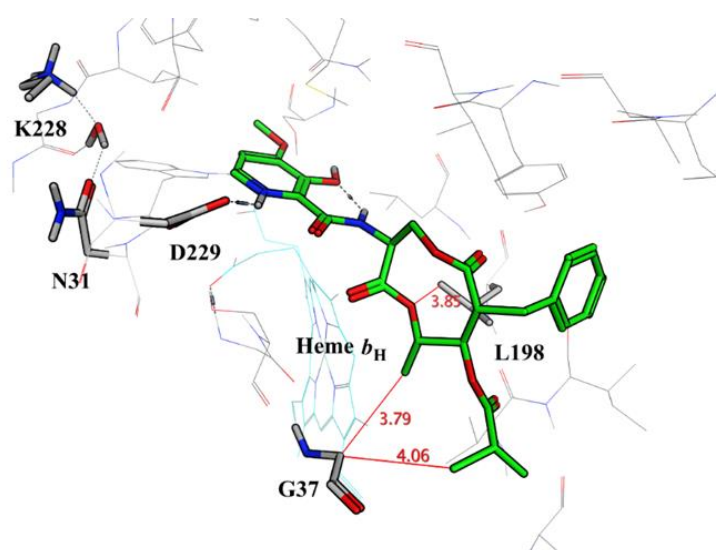


Figure 25. Binding of UK-2A at the Q_i site of *S. cerevisiae* cytochrome *b* (Young *et al.*, 2018).

Fenpicoxamid showed no toxicity towards crops, mammals or other terrestrial species. However, it is highly toxic to fish and aquatic invertebrates. In the environment, Inatreq™ is rapidly degraded to UK-2A which in turn is rapidly degraded to inactive compounds through microbial degradation, hydrolysis and photolysis. For these reasons, even if Inatreq™ is strongly adsorbed by soil, it displays very low contaminating potential towards groundwater (Inatreq™ global technical bulletin, Corteva, 2020). Inatreq™ was officially registered for use in the French wheat fungicide market in 2020, and should be first applied in the fields during season 2021.

4.2.4.6.4. Inhibitor of Q_o and Q_i sites (QioI).

A recently developed fungicide, ametoctradin binds at both the Q_o and the Q_i sites of cytochrome *b* (Fig. 26; Table 3). First identified as a QoI with a stigmatellin-like binding pose (QoI-D = class I) (Fehr *et al.*, 2016), recent studies have demonstrated that ametoctradin can also bind at the Q_i site, with a preference for the reduced cytochrome *b* (Dreinert *et al.*, 2018). Indeed, the presence of a point mutation in the Q_i site leading to ametoctradin resistance confirmed its preferential binding to the Q_i site. In fact, ametoctradin activity relies mainly on its binding at the Q_i site rather than at the Q_o site. Ametoctradin is an anti-oomycete, however its binding at the Q_i site is different from that of cyazofamid, amisulbrom and antimycin (Dreinert *et al.*, 2018; Mounkoro *et al.*, 2019).

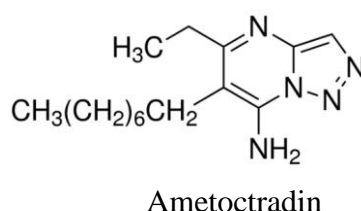


Figure 26. Chemical structure of the QioI ametoctradin.

Among ELQs tested in human medicine (see 4.2.4.6.2.), some would be also QioIs as described in Song *et al.*, 2018. The active form of tebufloquin, an agricultural pro-fungicide, is also an ELQ, but with a currently unknown binding site, therefore classified as a QxI.

Ascochlorin is a natural QioI antibiotic produced by a phytopathogenic fungus, *Ascochyta viciae* (Berry *et al.*, 2010). Interestingly, one of its structural analog, ascofuranone, does not inhibit complex III but reveals to be a strong inhibitor of the AOX (Ebiloma *et al.*, 2019).

4.2.5. Complex IV: composition, function and inhibitors.

Complex IV is also known as the cytochrome *c* oxidase. It is embedded in the cristae IMM as homodimers. For each monomer, 13 subunits have been identified so far (Fig. 16). Electron transfer in complex IV leads to the oxidation of cytochrome *c* and the reduction of molecular oxygen to water (Fig. 17). Complex IV embeds three copper atoms, organized in two distinct copper centers, and two heme molecules ($a + a_3$) which are responsible for electron transfer. To perform an entire cycle, complex IV requires four reduced cytochrome *c* molecules, one oxygen molecule and four protons pumped from the matrix. The complete cycle releases one water molecule in the matrix and four protons into the IMS, contributing to the electrochemical gradient (Wikstrom, 1977).

To date, no complex IV inhibitors are used as treatments. However, some inhibitors have been known for a long time like carbon monoxide, azide, phosphine, aluminum phosphide or cyanides. The cyanide ion (CN^-), in particular, is a potent poison for complex IV, and is used in laboratory experiments to block cytochrome *c* oxidation; for example, the measurement of cytochrome *c* reduction rate by complex III (Lalève, 2013). Porphyrin photosensitizers and N-(4-Hydroxyphenyl)-retinamide, all inhibiting complex IV, have reached advanced phases of clinical trials as anticancer agents (Rohlena *et al.*, 2011).

4.2.6. Complex V: composition, function and inhibitors.

Complex V, better known as ATP synthase, is sometimes referred to as the F_0F_1 -ATP synthase, regarding its structure. The F_0 part is embedded in the IMM and is called the proton channel or the rotor. The F_1 part, called the stator, is in the matrix and responsible for ATP synthesis. F_0 can rotate along an axis generated by the configuration of F_1 subunits. It encompasses from ten to fifteen subunits organized as a crown-shaped structure (Fig. 16; Fig. 17), and characterized by the presence of negatively charged amino-acids in the channel. F_1 is the static part of the complex. It encompasses seven subunits (Fig. 16), and shares two subunits with F_0 (Fig. 16; for review, Nakanishi-Matsui *et al.*, 2010).

When protons enter the F_0 channel, negative charges are neutralized which triggers the rotation of the F_0 region. This rotation generates a conformational change of the F_1 β subunits (Fig. 16). These catalytic subunits alternate between three different conformations: “open” is the conformation allowing ADP and P_i to bind, and ATP to be released in the matrix; “tense” or

“tight” is the conformation that allows ATP synthesis; “loose” is the intermediate conformation (Neupane *et al.*, 2019).

Complex V inhibitors can be classified into three different groups. The first group are the uncouplers of OXPHOS which inhibit ATP synthesis indirectly. These compounds are able to translocate protons back into the matrix, neutralizing the electrochemical gradient and therefore stopping ATP synthesis. Among these, fluazinam (Fig. 27; Table 3), a 2,6-dinitro-aniline broad-spectrum fungicide, active against grey mold, downy mildews and oomycetes, among other diseases (Guo *et al.*, 1991). Two dinitrophenyl crotonates, meptyl dinocap and binapacryl (Fig. 27; Table 3) also belong to this group. They are mostly active against powdery mildew and display acaricide properties. Among uncouplers, the carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Fig. 27) is used in the laboratory to abolish the mitochondrial electrochemical gradient, especially during oxygen consumption experiments. Chlorfenapyr, a pro-acaricide and pro-insecticide, is also an uncoupler of OXPHOS.

The second group of complex V inhibitors encompasses the compounds that directly inhibit ATP synthase. To date, more than 250 inhibitors of ATP synthase have been reported (reviewed in Neupane *et al.*, 2019). As examples, diarylquinolines like bedaquiline are used to treat tuberculosis. Bedaquiline is even active against multidrug resistant tuberculosis. They impair enzyme function by binding to the F_0 subunits. Melittin is a polypeptide present in honeybee venom that inhibits ATP synthase in both mitochondria and chloroplasts. Other inhibitors are listed in Table 3.

Organotin compounds also belong to this group. These fungicides were largely used in agriculture for their activity against many phytopathogenic ascomycetes and oomycetes. They also display bactericidal, insecticidal, acaricidal and herbicidal activities (Crowe, 1987). They all contain a tin atom linked to carbon chains sometimes cyclic. Fentin derivatives (acetate, chloride and hydroxide) (Fig. 27; Table 3) were introduced in the 1960s before being forbidden in Europe in the 2000s because of their high toxicity towards aquatic organisms (Okoro *et al.*, 2014). In this group of complex V inhibitors are also found some organosulphur compounds like tetradifon, or the thiourea pro-insecticide diafenthiuron, the desulfuration of which leads to the formation of a toxic carbodiimide.

The third group of inhibitors comprises compounds that inhibit adenine nucleotide (ADP/ATP) transporters, which translocate ATP from mitochondria to the cytoplasm (Joseph-Horne *et al.*, 2000). Therefore, they do not inhibit complex V activity directly, but prevent ATP from being

used by the cell. This group includes only one fungicide used in agriculture, silthiofam (Fig. 27; Table 3). Silthiofam is a carboxamide highly active against the straw cereal take-all agent, *Gaeumannomyces graminis var. tritici*. Since this fungus is a soil-borne pathogen, silthiofam is used as a seed treatment (Yun *et al.*, 2012).

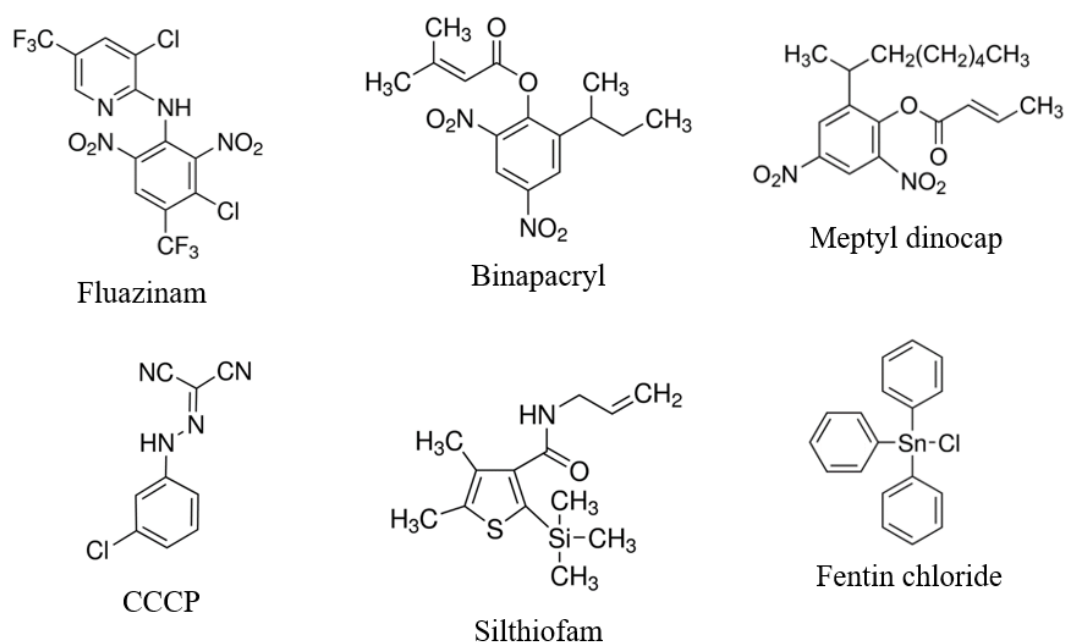


Figure 27. Chemical structures of different complex V inhibitors.

4.2.7. The alternative oxidase (AOX).

Most fungi, higher plants and protists possess in their mitochondrial ETC an additional ubiquinol terminal oxidase, the AOX (Fig. 17). The AOX is able to perform ubiquinol oxidation directly coupled to molecular oxygen reduction into water. However, AOX does not trigger proton translocation into the IMS, therefore does not contribute to the electrochemical gradient. As a result alternative respiration is about 60% less efficient in terms of energy production. AOX is transcriptionally up-regulated in the case of disruption of the mitochondrial ETC or an oxidative stress, suggesting that its primary function is to protect the cell against oxidative damages (Wood and Hollomon, 2003). However, in fungi, AOX seems also to be involved in other important processes like vegetative development, respiration or virulence (Lin *et al.*, 2019).

It has been observed that efficacy of strobilurins strongly depends on the time of infection when they are applied. Indeed, when applied before spore germination (or zoospore penetration for

oomycetes) or before symptoms' appearance, strobilurins are highly efficient. However, at later stages, their efficiency is limited. These observations led to the hypothesis that not all fungal developmental stages require as much energy, and some might be rescued by the AOX (Wood and Hollomon, 2003).

Miguez and colleagues conducted a study on field strains obtained from leaf lesions in strobilurin treated fields. These strains showed stable resistance to strobilurins in the lab, but a susceptible phenotype was restored by the addition of an AOX inhibitor. The interesting finding was that these strains were unable to cause symptoms when they were inoculated on QoI pre-treated plants, pycnidiospores displaying incapacity to germinate (Miguez *et al.*, 2004). Therefore, knowing that early stages of infection require high level of energy production, one hypothesis could be that the AOX pathway is not sufficient to sustain fungal development on its own during these stages in *Z. tritici*.

4.2.8. Summary: fungicides targeting mitochondria used in agriculture.

Mitochondria perform certain roles in fungi that are not found as frequently in other organisms. A proper mitochondrial function is necessary for survival, especially during quiescent periods, but also for virulence and pathogenicity. Many phytopathogenic species like *Cryphonectria parasitica*, *Ustilago maydis* or *Fusarium oxysporum* display mitochondria-dependent virulence levels (Chatre and Ricchetti, 2014). In fungi, mitochondria are also involved in drug tolerance through different pathways such as activation of drug efflux, as in the pathogenic yeast *Candida glabrata* (Chatre and Ricchetti, 2014; Shingu-Vazquez and Traven, 2011). For all these reasons, and given its central role in controlling the life and death cycle of the eukaryotic cell, the mitochondrion has been an important target for fungicide development against phytopathogenic fungi and oomycetes. These compounds create energy depletion in the fungal cell, and sometimes trigger ROS accumulation which damages cellular redox-sensitive components (Fernandez-Ortuno *et al.*, 2010). Energy deficiency will affect fungal development, especially during stages with high energy requirements, like spore germination for ascomycetes and basidiomycetes, or zoospore motility for oomycetes (Leinhos *et al.*, 1997). However, their influence on mycelial growth is limited, and so is their curative activity. Consequently, these compounds give better results when used preventively. Table 3 summarizes the main registered fungicides for agricultural use that target the mitochondrial ETC, along with other inhibitors mentioned in the previous section.

Table 3. Active ingredients affecting respiration processes and their principal properties (source: <http://www.r4p-inra.fr>; <https://www.frac.info/>). Bold = fungicides used in agriculture; No R4P code = medical or experimental drugs; F = fungicide, A = acaricide, I = insecticide, K = anticancer drug, B = bactericide, P = parasiticide, M = mycobactericide, R = rodenticide, V = antiviral.

Target	Abbreviation	R4P Code	FRAC code	Chemical class	Active ingredients
Complex I Ubiquinone binding site	NDHIs	A1a	-	Benzopyranones	Rotenone (A, I)
		A1b	C1	Pyrimidines	Diflometorim (F)
				Pyrazoles	Tolfenpyrad (F, I, A) Tebufenpyrad (I, A)
				Quinazolines	Fenazaquin (F, I, A)
				Biguanides	Metformin (K)
		-	-	Coordination complexes	Gallium maltolate (K)
Complex II Ubiquinone binding site, B, C and D subunits	SDHIs	A2a	C2	Benzamides	Benodanil, flutolanil, mepronil, fluopyram (F)
				Thiophene-carboxamides	Isofetamid (F)
				Furan-carboxamides	Fenfuram (F)
				Oxathiin- carboxamides	Carboxin, oxycarboxin (F)
				Thiazole- carboxamides	Thifluzamide (F)
				Pyrazole-carboxamides	Benzovindiflupyr, bixafen, fluxapyroxad, penflufen, furametpyr, penthiopyrad, isopyrazam, sedaxane, fluindapyr, inpyrfluxam (F)
		Nicotinamides	Boscalid (F)		
		Pyrazine-carboxamides	Pyraziflumid (F)		
		-	-	Dicarboxylic acids	Malonic, oxalic and oxaloacetic acids Lonidamine (K)
		-	-	α -tocopherols	Vitamin E analogs (K)
-	-	Pyridinones	Atpenins (B)		

Target	Abbreviation	R4P Code	FRAC Code	Chemical class	Active ingredients		
Complex III Q _o site close to heme <i>b_L</i>	QoIs-P	A5	C3	Methoxy-acrylates	Azoxystrobin, coumoxystrobin... (F)		
				Methoxy-acetamides	Mandestrobin (F)		
				Methoxy-carbamates	Pyraclostrobin, pyrametostrobin... (F)		
				Methoxy-imino-acetates	Kresoxim-methyl, trifloxystrobin (F)		
				Methoxy-imino-acetamides	Dimoxystrobin, fenaminstrobin... (F)		
				Oxazolidinediones	Famoxadone (F)		
				Dihydro-dioxazines	Fluoxastrobin (F)		
				Imidazolinones	Fenamidone (F)		
				Benzyl-carbamates	Pyribencarb (F)		
				-	-	Enoate esters	Mucidin (F)
Complex III Q _o site close to the rieske protein	QoIs-D	A4a	-	Dienamides	Myxothiazole (F, I)		
				Macrolides	Neopeltolide (B, F)		
				Carbazates	Bifenazate (A, I)		
				-	-	Naphtoquinones	Acequinocyl (A, I) Atovaquone (P)
				-	-	Hydroxychromone	Stigmatellin (B, F)
				-	-	Phtalimides	N-phenyl substituted phtalimides (P, M)
				-	-	Imidazoles	Cyazofamid (F)
Complex III Q _i site	QiIs	A3b	C4	Triazoles	Amisulbrom (F)		
				Picolinamides	Fenpicoxamid (UK-2A), florylpicoxamid (F)		
				-	-	Salicylamides	Antimycin A (F)
				B2a	-	Phenylureas	Diuron (H)
				-	-	Pyridones	Funiculosin (V, F) Ilicicolin H (F)
				-	-	Quinolones	Endochin-like (P) HDQ (P)
				Complex III Q _o and Q _i sites	QioI (or QoSI)	A6	C8

Target	Abbreviation	R4P Code	FRAC Code	Chemical class	Active ingredients
Complex IV	COXI	A8a	-	Cyanide-based mineral substances	Hydrocyanic acid, cyanide salts (I)
		A8b	-	Phosphide-based mineral substances	Aluminium phosphide (I, R)
		-	-	Gas	Carbon monoxide, phosphine
		-	-	Ions	Cyanide, azide
		-	-	Heterocyclic macrocycle organic compounds	Porphyrins (K)
		-	-	Retinoids	N-(4-Hydroxyphenyl)-retinamide (K)
Complex V Uncouplers of OXPHOS	-	A10b	C5	Dinitrophenols	Binapacryl, metyldinocap, dinocap (F)
		A10a		Dinitroaniline	Fluazinam (F)
		-	-	Hydrazone	CCCP
		A9c	C6	Triphenyl tin compounds	Fentin acetate, chloride, hydroxide (F)
Complex V Inhibitors of ATP synthase	-	-	-	Diarylquinolines	Bedaquiline (M)
		-	-	Polypeptides	Melittin (P, V, I)
		-	-	Macrolides	Efrapeptins (F, I)
		-	-	Macrolides	Oligomycin (B)
		-	-	Phenolic compounds	Quercetin (B, V, K) , resveratrol (B, F, K), genistein (B, V, K)
Complex V Inhibitors of ATP transport	-	A11	C7	Thiophene-carboxamides	Silthiofam (F)

4.3. Biology and genetics of fungal mitochondria.

4.3.1. A much-conserved mitochondrial DNA.

The *Z. tritici* mitochondrial genome is about 40 kb, and approximately 30% encodes proteins (Fig. 28). It encompasses fourteen genes encoding OXPHOS-related proteins, including cytochrome *b* (*CYTb*), three ATPase subunits (*ATP6*, *ATP8*, *ATP9*), three complex IV subunits (*COX1*, *COX2*, *COX3*) and seven complex I subunits (*NAD 1-6*, *NAD4L*). It also encompasses DNA transcription related genes: one RNA polymerase encoding gene, 2 ribosomal RNA genes and 27 tRNAs, clustered in an order highly conserved among ascomycetes. It displays an AT enrichment very common among fungi, but no introns (Torriani *et al.*, 2008).

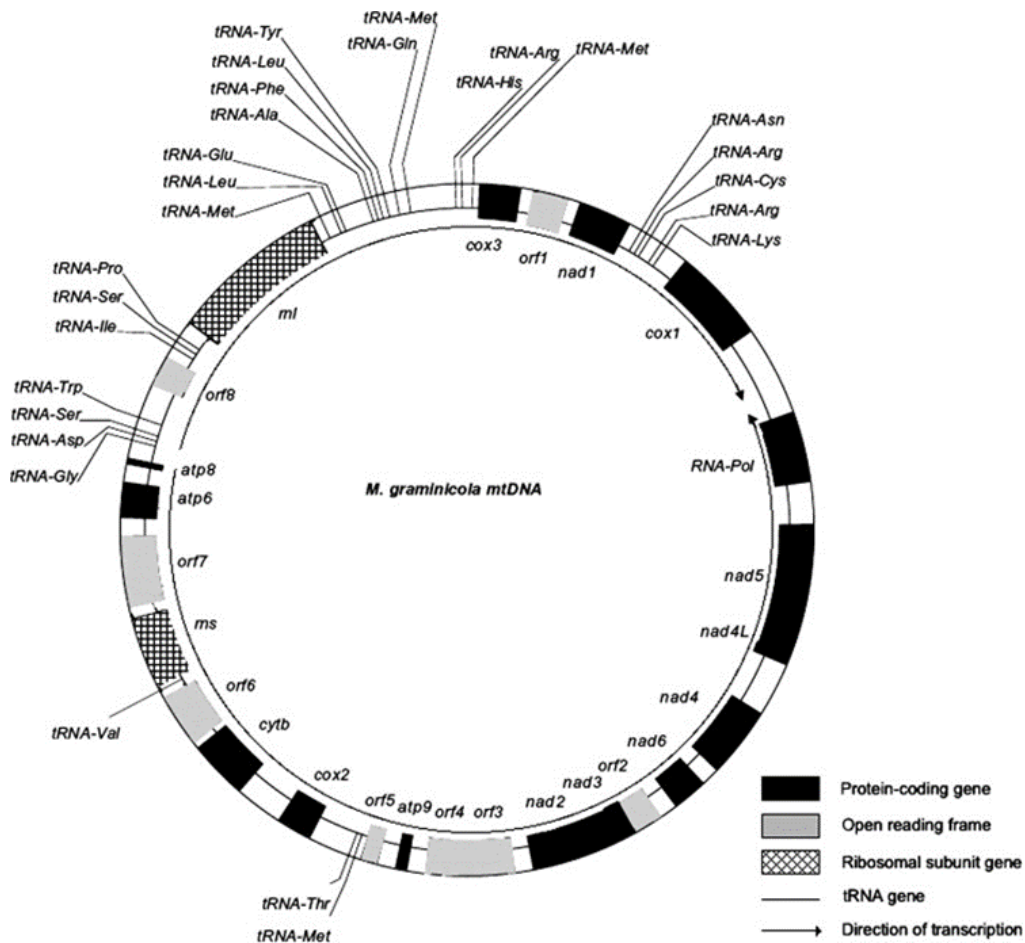


Figure 28. Circular map of the *Z. tritici* mitochondrial genome (Torriani *et al.*, 2008).

Contrary to the highly polymorphic nuclear genome, the mitochondrial DNA in *Z. tritici* is extremely conserved. The sequencing of more than 1600 field isolates revealed only seven

different mitochondrial haplotypes, the two most common representing more than 90% of all samples. The polymorphisms were mainly due to insertion/deletion events but gene content remained the same (Zhan *et al.*, 2003). In another study based on 37 field isolates, the estimated nucleotide diversity in the mitochondrial genome ranged from 0.16 to 0.22% (less than 30 SNPs). This diversity is one of the lowest ever published, and the lowest among fungi (Torriani *et al.*, 2008). Such a low diversity could be explained by the fact that mutations may be more deleterious in these essential mitochondrial genes than in nuclear genes. Consequently, mutants would be more frequently eliminated (Christie *et al.*, 2015). A second hypothesis could be the existence of a lower mutation rate in the mitochondria than in the nucleus, contrary to what we observe in mammals (Torriani *et al.*, 2008).

4.3.2. A maternal inheritance influenced by selection pressure.

In species displaying a differentiation between male and female individuals, produced gametes have their own specific characteristics (Allen, 1996). The male gamete is commonly small and mobile, while the female gamete is bigger and static. In this system, the transmission of cytoplasm and contained organelles, like mitochondria, is maternal. Behind this observation lies a process in which the male gamete mitochondria are needed for mobility that requires a high level of energy production. Consequently, these mitochondria are exposed to high levels of ROS production, suffer irreversible DNA damage and are therefore eliminated during fecundation. In contrast, female gamete mitochondria are in a quiescent stage called “promitochondria”, which differentiate into mitochondria only when energy production is required (Allen, 1996). Despite this long-accepted dogma, recently some proof of paternal transmission of mitochondrial DNA in humans has been reported. However this situation remains exceptional, and is probably the consequence of impaired elimination process of male mitochondria (Luo *et al.*, 2018). Moreover, paternal transmission of mitochondria and persistence of heteroplasmy are not such rare phenomena, and have been found in many different species belonging to different animal classes (reviewed in Mendoza *et al.*, 2020).

In fungi, sexual reproduction happens through cell-cell fusion involving two isogametes or two mycelia (Ni *et al.*, 2011). Homothallic species are self-fertile, while heterothallic species need two individuals with compatible mating types to fuse. Genes belonging to the mating-type locus, called *MAT*, determine the mating types. The cell-cell fusion results in a dikaryotic state that precedes nuclear fusion and meiosis. Most of the time, the two nuclei migrate and fuse while cytoplasm containing organelles is left behind. Following this step, one of the two

populations of organelles, hence mitochondria, is eliminated leading to uniparental inheritance just like in plants or mammals. Some species display biparental inheritance of mitochondria, but quickly cells undergo rearrangements to retain only one mitochondrial genotype, inherited from one of the two parents or being a recombinant. Genes located at the *MAT* locus are required for proper uniparental inheritance, but their identification, exact role and regulation remain to be elucidated (Ni *et al.*, 2011).

In 2005, a study quantifying the proportion of individuals resistant to QoIs among a *Z. tritici* ascospore population revealed that it increased rapidly from 35 to 80% after a QoI field application (Fraaije *et al.*, 2005). This fast increase of the QoI mitochondrion-based resistance (due to a mutation in the *CYTb* gene) prompted further studies of the sexual behavior of field strains when exposed to selection pressures. In 2018, Kema and colleagues studied the outcome of sexual reproduction between two compatible strains, with contrasting sensitivity towards different selection pressures. The susceptible strain under selection pressure was found to always behave as the “male” parent, meaning that the resistant one always transmitted its mitochondria. A similar observation was made whether the selection pressure was imposed by a resistant wheat cultivar, or by a fungicide application (Kema *et al.*, 2018). This observation means that environmental conditions can influence the propagation rate of some mitochondrial alleles among field populations.

4.3.3. Heteroplasmy in fungi.

Heteroplasmy refers to a cellular state where at least two different mitochondrial populations (also called mitotypes) coexist in the same cell, even for a transient period (Mendoza *et al.*, 2020). In several fungi, heteroplasmy can be observed after sexual reproduction, nuclear fusion and meiosis. However, shortly after cell-cell fusion, the homoplasmic state is recovered. Mitotypes are either distributed in different daughter cells, as in *S. cerevisiae* or *Microbotryum violaceum*, or one of them is eliminated as in *Ustilago maydis* or *Cryptococcus neoformans* (reviewed in Mendoza *et al.*, 2020).

Selection against heteroplasmy is meant to prevent dysfunctional mitochondria with deleterious mutations from surviving and multiplying inside the cell (Christie *et al.*, 2015). Moreover, the existence of a fitness penalty in cells maintaining a heteroplasmic state had long been suspected, before it was demonstrated in mice (Sharpley *et al.*, 2012). Nevertheless, some species seem to display heteroplasmy in a more frequent and stable way. For example, heteroplasmy has been found in field isolates of *B. cinerea*, where QoI resistance is conferred by one mitochondrial

allele that persists even after removal of the selection pressure (Hashimoto *et al.*, 2015). Different QoI resistance levels based on heteroplasmy have also been reported in *Fusicladium carpophilum* (Luo *et al.*, 2013), *Venturia inaequalis* (Villani and Cox, 2014), *Podosphaera xanthii* (Vielba-Fernández *et al.*, 2018), *Corynespora cassiicola* and *Mycovellosiella natrassii* (Ishii *et al.*, 2007), and *Leveillula taurica* (Mosquera *et al.*, 2019). In these species, heteroplasmy seems stable, and the proportion of both alleles is variable depending on the conditions. Even when the homoplasmic state is reached, the situation is reversible and the other allele can still be selected again. A study conducted in the human pathogenic yeast *Candida glabrata* demonstrated such characteristics of heteroplasmy (Zhou *et al.*, 2010). Heteroplasmy has also been found associated with bifenazate resistance in the parasitic mite *Tetranychus urticae* (Van Leeuwen *et al.*, 2008) or described for chloroplast inheritance in *Senecio vulgaris* (Frey *et al.*, 2005). However, the underlying mechanisms of heteroplasmy tolerance in phytopathogenic fungi have not yet been deciphered (Sierotzki, 2015). To my knowledge, a stable and persistent heteroplasmic state has never been described in *Z. tritici*.

4.3.4. Yeast as a model to study mitochondrial inhibition and resistance.

Having a good model for detailed studies is required due to the difficulty in growing certain fungi in the laboratory, obtaining enough active mitochondrial membrane preparation, or performing functional validation in mitochondrial DNA. Baker's yeast, *Saccharomyces cerevisiae*, is this organism of choice. First, *S. cerevisiae* presents the advantage of not being pathogenic so it can be easily manipulated. Second, yeast *CYTb* gene sequence is very similar to that of most phytopathogenic fungi, and the transformation of its mitochondrial genome is possible, using a biolistic method. Indeed, mutations can be introduced in yeast mitochondrial genes in order to obtain the exact same allele as the one in the studied pathogen. Moreover, the protocol to obtain highly purified yeast mitochondria has been known for a long time, is now optimized and easy to set up. Finally, yeast is also able to grow on fermentable substrates even in the absence of a functional mitochondrial ETC, and yeast grows quickly, compared to phytopathogenic fungi. All these reasons make *S. cerevisiae* the ideal organism to study the mitochondrial ETC of phytopathogenic fungi, including *Z. tritici*, and especially to study the activity and specificity of complex III inhibitors (Fisher and Meunier, 2008).

5. RESISTANCE AS AN ADAPTIVE PROCESS TO FUNGICIDE SELECTION PRESSURE.

5.1. Acquired or natural biological resistance and resistance in the field.

Acquired resistance, developed in response to fungicide selection pressure, is to be distinguished from natural (or intrinsic) resistance. Intrinsic resistance designates the fact that some species are naturally insensitive to some active ingredients, defining the activity (or action) spectrum of a fungicide. Most of the time, this absence of susceptibility is due to molecular differences in targeted proteins, or to the absence of the targeted gene. However, other mechanisms can be involved like additional copies of the encoding gene, absence of fungicide penetration, increased efflux, sequestration, detoxification... This type of resistance is the result of ancient evolutionary processes, not related to fungicide introduction (reviewed in Lucas *et al.*, 2015; Oliver and Hewitt, 2014).

As mentioned above, the evolutionary potential of fungi allows them to adapt rapidly to a new environment, hence to new selection pressures. In modern agriculture, intensive and regular fungicide use from the 1970s, especially single-site fungicides allowing good disease control, exerted important selection pressure on fungal pathogen populations, influencing their evolution (Brent, 2012; Stukenbrock and McDonald, 2008). Indeed, this situation led to the selection of isolates with reduced fungicide sensitivity among sensitive populations, a phenotype that is commonly referred to as “resistant”. From an evolutionary point of view, resistance can be defined as a phenotypic optimization, which is an adaptive response to natural selection and can have different origins. Biological resistance relies on intraspecific polymorphism by which one or several genetic changes (SNPs, insertions, deletions, TE mobilization...) confer on one individual reduced sensitivity to a fungicide, a trait that is advantageous in the specific condition where the fungicide is applied (reviewed in Lucas *et al.*, 2015). Under a selecting environment, the frequency of the beneficial allele increases. Interestingly, the outcome of the selection under different fungicide spray programs, even on short time scales, can vary widely in both intensity and quality, and in time and space. For these reasons, fungicide resistance is a good model for evolutionary biology studies.

At the population scale, field resistance refers to a decrease in fungicide efficacy, when applied at the maximum permitted rate and frequency, in optimal spray conditions, as a result of biological resistance selection. Field resistance depends on the level of resistance of individuals (generally moderate to high depending on the pathogen and the concerned fungicide), and their

frequency in the fungal population (Oliver and Hewitt, 2014). Consequently, biological resistance does not systematically lead to observable field resistance.

5.2. Origin, emergence and evolution of resistance in natural populations.

Two evolutionary hypotheses may explain resistance (Fig. 20). Firstly, a resistant allele may emerge spontaneously from a mutation. In this context of *de novo* mutations, the resistant allele is not present in the population prior to pesticide introduction. The advantageous mutation occurs (once or multiple times independently) under pesticide selection and spreads. In this situation, mutation and selection are the evolutionary forces. Second, resistance can emerge from standing variation. In this case, resistance is already present in the population at low frequency, as a monogenic or polygenic trait. After pesticide introduction, this trait becomes advantageous and therefore increases in frequency in populations. In this situation, selection is the evolutionary force (Hawkins *et al.*, 2019). In order to characterize the origin of resistance, it is possible to search for genomic selective signatures, and to use comparison with unselected populations and/or phylogenetic dating. Most of the time, resistance to herbicides comes from standing variation, like resistance to acetyl CoA carboxylase inhibitors in *Lolium rigidum* (Neve and Powles, 2005) or glyphosate resistance in *Ipomoea purpurea* (Kuester *et al.*, 2015). Insecticide resistance generally originates from a combination of standing variation and selection of *de novo* mutations, as for organophosphate resistance in two Australian blowfly species (Hartley *et al.*, 2006; Rose *et al.*, 2011). For its part, fungicide resistance seems mostly linked to the selection of *de novo* mutations, like QoI resistance in *Plasmopara viticola* (Chen *et al.*, 2007) or *Z. tritici* (Torriani *et al.*, 2009), or azole resistance in *Z. tritici* (Cools and Fraaije, 2013) (reviewed in Hawkins *et al.*, 2019).

The emergence of *de novo* mutations first depends on the mutational supply, which results from the mutation rate and the population size. Mutation rate in fungi is estimated to be around 10^{-10} changes per site per cell division (Ene *et al.*, 2018; Kasuga *et al.*, 2002; Long *et al.*, 2016). Due to their short generation time and large population size, mutational supply is high in pathogenic fungi, and even rare mutations can emerge. Once a resistance mutation has appeared in a population, its spread depends upon its fitness, upon migration rate, directly depending on the biology of dissemination propagules, and upon the intensity of the selection pressure. For example, ascospores that can be air-dispersed over very long distances are an efficient vector to spread resistance genes rapidly (Hawkins *et al.*, 2019).

Following its selection, a resistant allele will tend to increase in frequency in a population under fungicide selection pressure (Fig. 29). The rate of this increase results from an equilibrium between its associated fitness cost and the intensity of the selection pressure (see section 5.4., Fig. 31). With increasing frequency, the resistant allele reaches several different thresholds that will affect the management of fungicides in the field. First, the resistant allele becomes detectable by monitoring programs, a threshold that marks the limit between prevention and management, based on proper resistance characteristics. Second, when the local frequency of the resistant allele reaches a specific level, a decrease in the fungicide's field efficacy can be observed, defining the beginning of "field resistance" (see previous section). Finally, generalization corresponds to the phase where the resistant allele's frequency reaches (almost) 100%. When the fungicide is not used anymore (lack of selection pressure), either the resistant allele's frequency remains stable, then resistance is fixed, or decreases, then resistance regresses (Fig. 29), due to a fitness penalty.

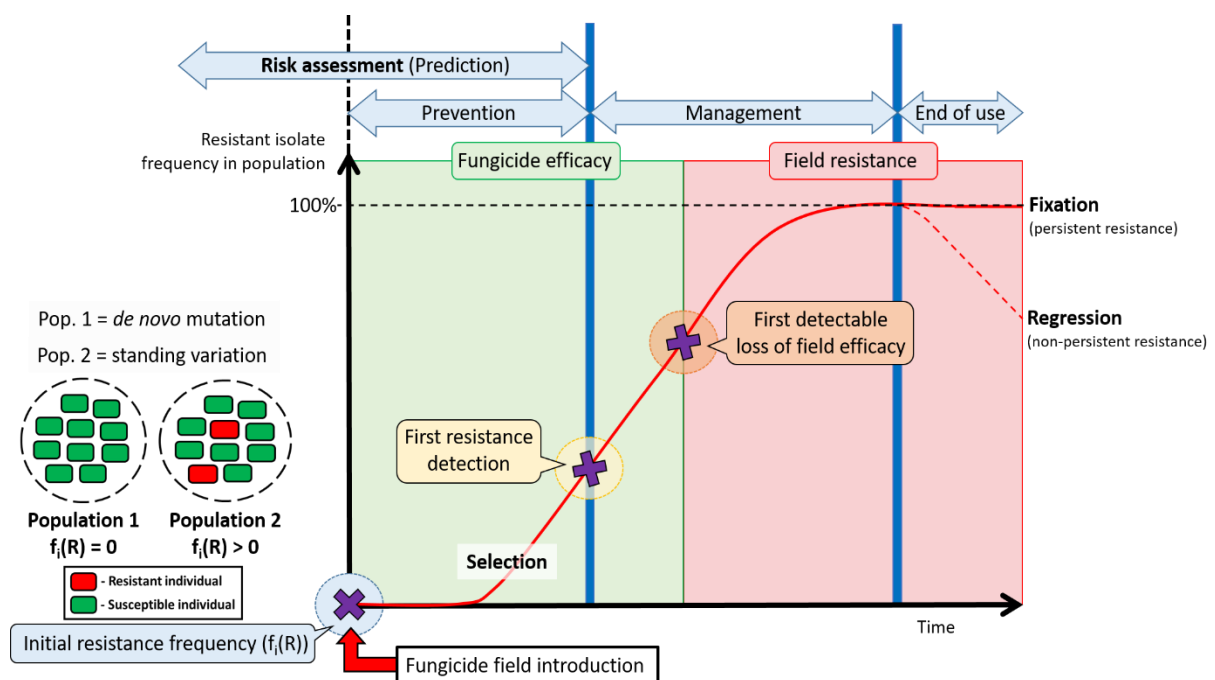


Figure 29. Evolution of a resistant allele's frequency in a population and its impact on the fate of fungicides (source: <http://www.r4p-inra.fr>).

Consequently, the early prediction of resistance emergence and evolution, even before the use of a fungicide in the field, would be of great interest in order to optimize prevention and management.

5.3. Quantifying resistance levels.

Assessing resistance level in the laboratory implies the determination of some parameters quantifying the loss of sensitivity of the resistant strains. Among the most common indicators, the half-maximal effective concentration (EC_{50}) (Fig. 30) is the fungicide concentration inhibiting 50% of a cellular process (growth, enzyme activity...) compared to an untreated control (Oliver and Hewitt, 2014). Sometimes, EC_{50} is called IC_{50} or I_{50} , standing for the half-maximal inhibitory concentration. The minimal inhibitory concentration (MIC) (Fig. 30), which is the concentration that inhibits entirely the considered cellular process, therefore corresponding to the EC_{100} , is also a frequently used parameter, mainly in the clinics. Assayed biological traits should be adapted to the fungicide mode of action like spore germination for respiration inhibitors, or germ tube length for benzimidazoles. Assessment of these traits is directly depending on the conditions (medium, day of measurement, temperature, humidity...) which makes data comparison sometimes difficult.

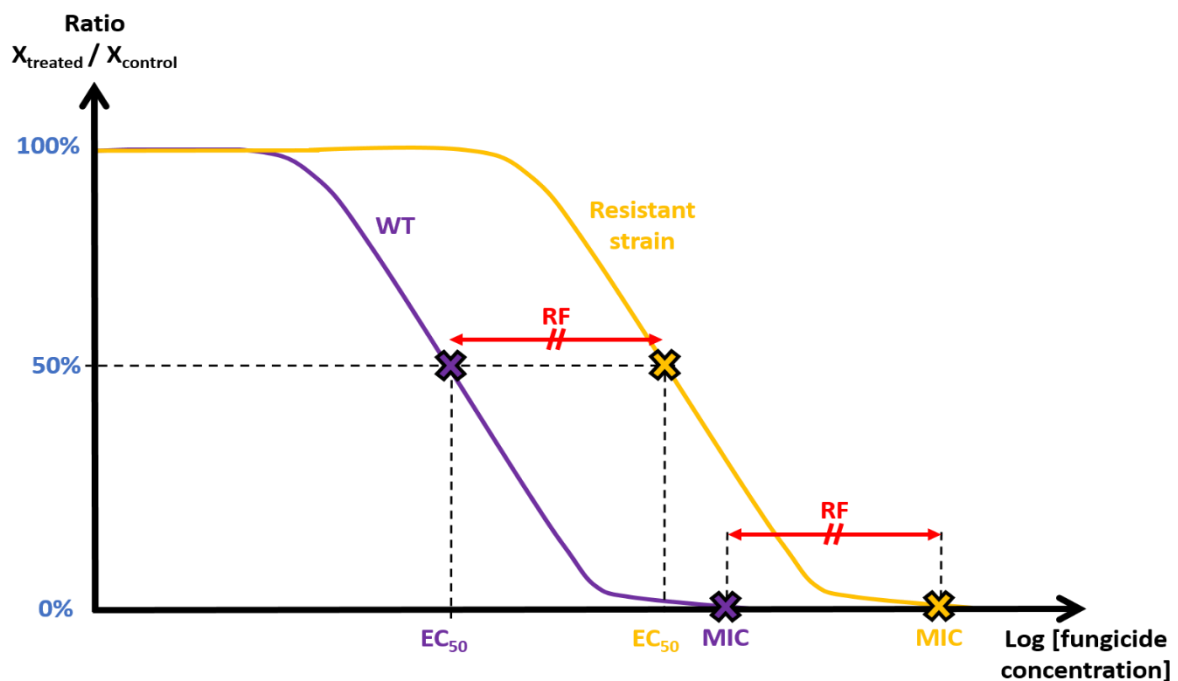


Figure 30. Dose response curve. X is the value of a considered biological trait. Purple curve: WT; Yellow curve: resistant strain; RF = resistant factor.

The resistance factor (RF) is the ratio between the EC_{50} values (or the MIC values) of the resistant strain compared to a relevant sensitive strain, in the same conditions. Main issues in

RF measurement are sources of variability, like the biological differences between isolates, the technical limitations of the assay, and sometimes operator-dependent scoring variability. To circumvent this variability, comparing EC₅₀s of isogenic strains when possible is ideal, especially in different genetic backgrounds. Otherwise, comparing the EC₅₀ of a resistant strain to the average EC₅₀s of a set of representative WT strains is preferable, especially strains representing natural variability before the fungicide introduction (Oliver and Hewitt, 2014).

5.4. The cost of resistance influences the selection.

In the case of phytopathogenic fungi, fitness represents the ability of a strain to reproduce and to cause disease from year to year in the absence of fungicide, and in competition with other phenotypes (Oliver and Hewitt, 2014). Resistance is an advantageous phenotypic trait in the context of the selection pressure exerted by the fungicide (Oliver and Hewitt, 2014). However, its emergence, spread and stabilization in populations is the result of a balance between this selectively advantageous trait, and any negative physiological effect(s) potentially associated with this new genotype. These putative “side effects” are commonly referred to as the “cost” of the resistance, or its “fitness penalty” (Fig. 31). Fitness cost can affect different biological traits during the life cycle, and can sometimes be compensated.

In natural populations, fitness penalty is often invoked as the main reason why resistance takes longer to emerge or does not emerge at all (Fig. 31), or why a resistant allele persists or not after fungicide selection pressure is removed (Fig. 29) (Hawkins and Fraaije, 2018). From a practical point of view, the concept of fitness penalty seems straightforward. For example, if we consider that evolution naturally selects the optimal version of a protein, any mutation should trigger at least a slight decrease in its biological activity along with fungicide resistance. Another situation would be resistance through an energy-requiring process, which would oblige the fungus to reallocate energy supplies to address resistance, at the expense of other cellular processes such as mycelial growth, spore production or pathogenicity (Hawkins *et al.*, 2019). In both situations, the cost of resistance would be easy to characterize and to evaluate. However, it turns out that the concept of fitness penalty is not so obvious, and assessing it is even less so. Indeed, a mutation itself must not be considered simply as beneficial, neutral or deleterious, as its visible impact depends on the environment, the genetic background (pleiotropic effects), and the other competitive alleles in the population.

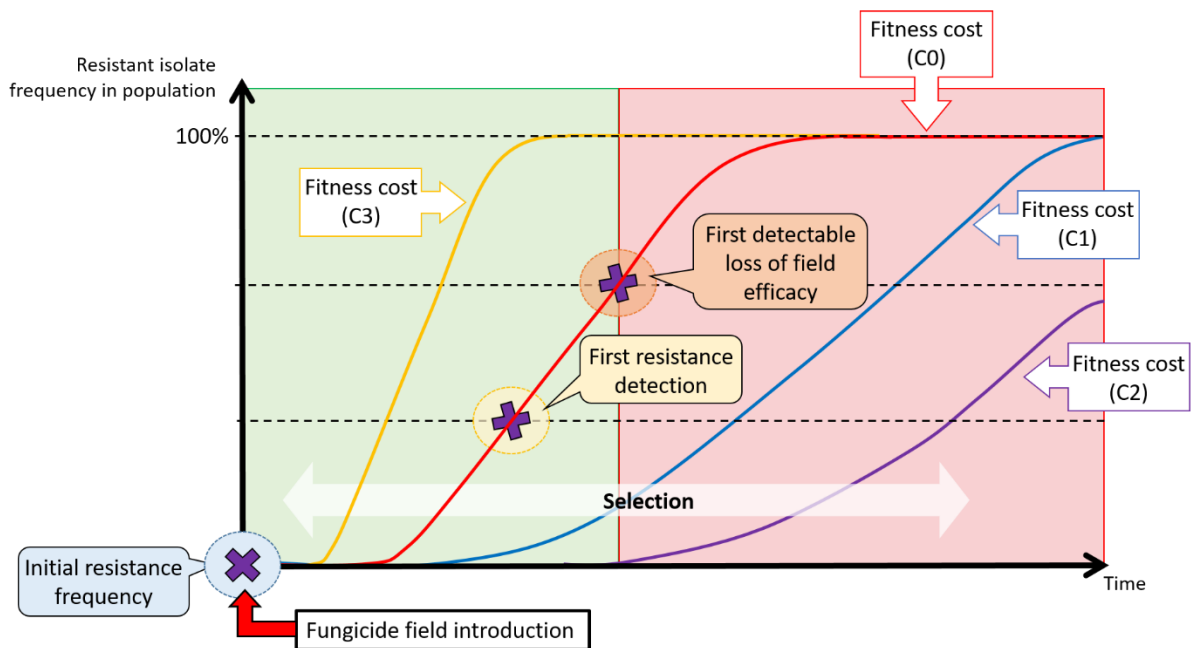


Figure 31. Evolution of different resistant alleles in a population (source: <http://www.r4p-inra.fr>). Each allele is associated with a different fitness cost, with $C2 > C1 > C0 > C3$. $C0$ is the fitness of the WT. $C1$ and $C2$ represent strains with reduced fitness compared to the WT. $C3$ represents a strain with improved fitness. In all cases, the cost of resistance modulates selection.

Moreover, the assessment of absolute fitness is a difficult matter as it can vary widely depending on whether it is based on one or more traits, some of which can be mitigated by compensatory phenomena. This leads to the conclusion that there is not “a” resistance cost, but an infinity of them. In this context, precisely assessing a resistance cost seems a difficult task. The choice of a proper environment and a sufficiently well-adapted sensitive strain for comparison, are parameters that can modify the absolute “value” of the cost, and even its nature (“positive” or negative cost) (Fig. 31) (Lenormand *et al.*, 2018).

The main difficulty is to find a way to assess absolute fitness of an individual. The ideal fitness measurement would be in the field where absolute fitness can be assessed globally. In the field, environment is highly variable, different host genotypes can be present, and resistant strains would have to compete with susceptible strains and a wide range of other microorganisms. Moreover, in the field, all stages of the fungal life cycle are challenged, including sexual reproduction that is difficult to observe under laboratory conditions for most pathogenic fungi, and survival during the interepidemic season. In the absence of fungicide selection pressure, the evolution of resistant strain frequency in populations from year to year may be a good indicator of the fitness cost of resistance as it would reflect the ability of the strain to

competitively cause infection, reproduce, spread and survive (Mikaberidze and McDonald, 2015). This kind of study has already been conducted with French populations of *Oculimacula aciformis* and *Oculimacula yallundae*, among which prochloraz resistance remained stable for years after fungicide removal, suggesting the absence of a fitness cost (Leroux *et al.*, 2013). In contrast, propiconazole resistance in *Z. tritici* populations in Oregon was shown to decline in the absence of fungicide selection pressure, suggesting the existence of a fitness penalty (Hagerty *et al.*, 2017). However, frequency may also vary according to other unrelated parameters such as susceptible isolate immigration, selection for other traits or genetic drift (Mikaberidze and McDonald, 2015).

Another approach is to estimate the absolute fitness by measuring various traits, reflecting the entire life cycle of the fungus as closely as possible. Many different traits of the fungal life cycle can be used, such as spore production, pathogenicity, mycelial growth etc... It is important to keep in mind that each type of study comes with its own bias and limitations. For example, laboratory studies often use a single isolate that is not relevant regarding the heterogeneity of field populations, but allows comparison of resistant and sensitive alleles within similar genetic backgrounds (Hawkins and Fraaije, 2018). Studying an insufficient number of individuals may also lead to confusion between a real fitness penalty and simple genetic variability (Walker A.-S., personal communication). Although assessing fitness cost under laboratory conditions remains informative, usually it does not allow accurate prediction of what will happen with field mutants, primarily because in the field, compensatory mutations are more likely to appear (Lalève *et al.*, 2014a; Mikaberidze and McDonald, 2015).

To conclude, there is no ideal and consensual way to assess the fitness cost of a genetic modification that triggers fungicide resistance, because the concept of fitness itself depends on specific conditions. However, comparing the fitness of isogenic strains differing only in the resistance allele, if possible in different genetic backgrounds, is less biased than comparing field ones, especially if the comparison is made for many different traits and using several different genotypes. In these types of study, fitness differences can be assigned to the resistance-conferring mutation only. Consequently, gathering data at various levels (enzyme, organism, populations...) and in different conditions (*in vitro*, *in planta*, single isolates, competitions...) may give a good estimate of the resistance fitness cost. With such data, although no certain and general conclusion can be drawn, results may identify a trend or most likely scenario.

5.5. Tolerance, an ignored phenomenon in resistance management in agriculture.

In the field of human medicine, tolerance has drawn attention after the finding of an important discrepancy between the failure of treatments for human fungal diseases, and the low proportion of isolated resistant individuals (reviewed in Berman and Krysan, 2020). Consequently, knowledge of this phenomenon has increased significantly in the last few years, while it remains largely ignored in resistance studies concerning agricultural fungicides. Moreover, in agriculture, the term “tolerance” is often misused to refer to strains with partial resistance or displaying low RFs. Tolerance and resistance both designate a phenotype of an individual able to grow in the presence of an antimicrobial drug. However, there is a clear, yet subtle, difference between these two phenomena, as distinguished in the medical area. Resistance refers to a genetic-based decrease in sensitivity, which is heritable and shared by all resistant individuals with the same genotype (“biological resistance”, as previously introduced). Tolerance, on the other hand, refers to the ability of susceptible (non-resistant) individuals to grow in the presence of a drug, even at concentrations higher than the MIC. This phenomenon is the result of a combination of alleles (probably several hundreds), but also of physiological responses that can be heterogeneous, even in a population presumed to be clonal. Genetic determinants of tolerance are not yet known, and the metabolic pathways involved just starting to be deciphered. So far, chaperone activity, calcium homeostasis, iron metabolism, uptake and efflux regulation, several central stress response pathways, cell wall chitin increase, or vacuole trafficking are among the many metabolic processes involved, at least partially, in drug tolerance. Key differences between resistant and tolerant isolates are that tolerant isolates display slow growth in the presence of the drug, regardless of the concentration, and their MIC values remain the same. In contrast, resistant isolates grow at a normal rate in the presence of the drug and their MIC values are increased. Therefore, the word “tolerance” should be used with great caution in agriculture. In addition, the contribution of tolerance in resistance selection should not be underestimated. Since tolerant individuals are still able to divide under fungicide selection pressure, they increase the population size, hence the likelihood of resistance mutation(s) to emerge.

5.6. Main mechanisms of resistance towards fungicides.

The main resistance mechanisms developed by human and plant pathogenic fungi are represented in figure 32. They are commonly classified in two categories:

- **Mechanisms related to the target protein, or target site resistance (TSR):** target modification and target overproduction.
- **Mechanisms not related to the target protein or non-target site resistance (NTSR),** including mechanisms related to fungicide transport (increased efflux and reduced penetration), mechanisms related to fungicide metabolism (detoxification and default in activation) and bypass resistance due to the activation of alternative metabolic pathways.

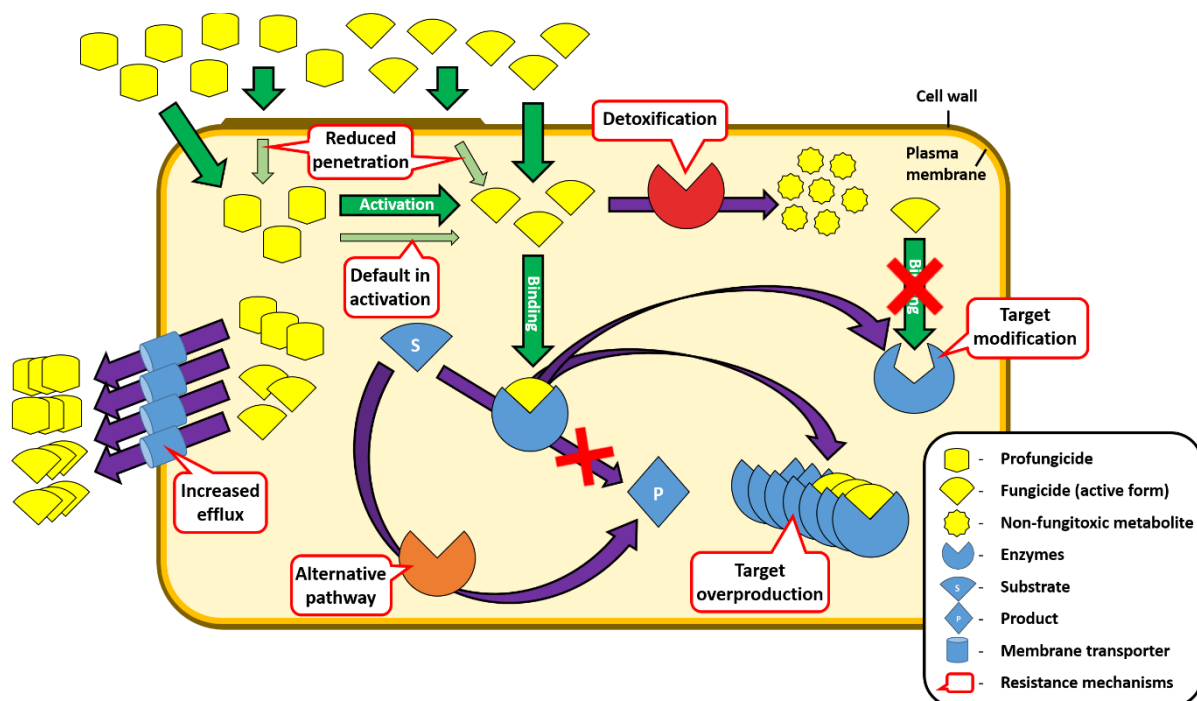


Figure 32. Main resistance mechanisms to fungicides (source: <http://www.r4p-inra.fr>).

5.6.1. Resistance mechanisms linked to the target.

5.6.1.1. Target modification.

Target protein alteration, or target protein modification, is a common resistance mechanism developed by phytopathogenic fungi, which affects almost all unisite modes of action (Lucas *et al.*, 2015).

This mechanism relies on the selection of one or several SNPs in the DNA sequence, resulting in amino-acid substitution(s). The introduced amino-acid generally displays different physicochemical properties from the original residue, such as side chains with higher steric hindrance, different electrical charge or a different hydrophobic/hydrophilic state, modifying

the 3D structure of the protein. The substitution usually takes place near or inside the substrate binding pocket, decreasing enzyme affinity for the fungicide while maintaining its affinity (or restoring a higher affinity) for its natural substrate. In fewer cases, the modification of the protein sequence can also be a deletion of one or several amino-acids, like in the *Z. tritici* CYP51 protein (Brunner *et al.*, 2008; Leroux *et al.*, 2007), or an addition of amino-acids as in *Plasmopara viticola* CYTb (Cherrad *et al.*, 2018).

Regarding *Z. tritici*, the main substitutions identified as leading to resistance to different modes of action are listed in table 2. The substitutions triggering QoI resistance in diverse pathogens were reviewed in Leroux and Walker, 2015, and the ones triggering QiI resistance were reviewed in Mounkoro *et al.*, 2019.

5.6.1.2. Target overexpression.

Overexpression of the target protein-encoding gene has also been described in several phytopathogenic species, particularly for azole resistance. This mechanism does not directly reduce fungicide activity, but allows fungicide inhibition to be overcome through an increased production of the target enzyme. Overexpression can have different origins.

The most common origin is an insertion in the promoter region of the target gene, which triggers constitutive overexpression. This kind of insertion in the promoter of the *CYP51* gene has been reported, among other species, in *Pseudocercospora fijensis* (Diaz-Trujillo *et al.*, 2018), *Z. tritici*, *Cercospora beticola*, *Monilinia fructicola*, *Venturia inaequalis* and the human pathogenic yeast, *Candida albicans* (reviewed in Hawkins and Fraaije, 2018). The molecular mechanisms leading to overexpression have yet to be deciphered in most species. The most common hypotheses are that the insertions either disrupt the binding of a transcription repressor, or encompass binding sequences of activating transcription factors. For example, in *V. inaequalis* or *P. fijensis*, the presence of repeated elements inserted in the *CYP51* promoter region have been shown to act as transcriptional enhancer (Diaz-Trujillo *et al.*, 2018; Villani *et al.*, 2016).

A second origin of the overexpression can be mutations in a transcription factor regulating the target gene's activity. In *C. albicans*, a correlation between the overexpression of *CYP51* and, at least three different gain-of-function mutations in the transcription factor-encoding gene *Upc2*, was clearly established (Heilmann *et al.*, 2010; Hoot *et al.*, 2011).

Aneuploidy, which designates the presence of an abnormal number of chromosomes (or chromosome arms) in a cell, can also increase the number of gene copies. This phenomenon is frequently observed in pathogenic yeasts, especially under fungicide selection pressure. For example, the presence of extra copies of the left arm of chromosome 5 in *C. albicans* bearing the *CYP51* gene, has been shown to lead to overexpression and to azole resistance (Selmecki *et al.*, 2008). Similar mechanisms have also been described in other pathogenic yeast species, *Candida glabrata* and *Cryptococcus neoformans* (reviewed in Kwon-Chung and Chang, 2012).

Finally, the overexpression of the target-encoding gene can be due to the presence of one or several extra copies or paralog(s) in the genome. The presence of a *CYP51* paralog named *CYP51A*, bearing mutations and displaying inducible expression, has been found in *Rhynchosporium commune* (Hawkins and Fraaije, 2018), *Colletotrichum spp.* (Chen *et al.*, 2020), *Fusarium spp.* (Pasquali *et al.*, 2020) and some *Aspergillus spp.* (Osheroov *et al.*, 2001). In all these species, the extra copy of *CYP51* has been linked to overproduction of CYP51 and azole resistance.

5.6.2. Resistance mechanisms not linked to the target.

5.6.2.1. Mechanisms linked to fungicide transport.

5.6.2.1.1. Reduced penetration.

Reduced penetration is not a widespread resistance mechanism in phytopathogenic fungi. Indeed, most fungicides are small molecules that easily diffuse into fungal cells without the need of a specific transporter. However, one documented example is polyoxin resistance in the pear pathogenic species, *Alternaria kikuchiana* (Misato *et al.*, 1977). Resistant strains of *A. kikuchiana* showed the ability to trap polyoxins at their surface, preventing uptake into the cytoplasm.

In contrast to phytopathogens, in human pathogenic species, cell-wall modifications are frequently observed, and have been linked to fungicide resistance in many cases. *Candida spp.* and *Aspergillus spp.*, in particular, have shown abilities to modify their cell-wall composition and/or thickness to sequester fungicide molecules and/or prevent them from reaching the plasma membrane (Lima *et al.*, 2019).

5.6.2.1.2. Increased efflux.

Increasing efflux, which reduces intracellular concentration of a drug is a ubiquitous resistance mechanism found in fungi, bacteria and even cancer cells. This mechanism is not specific, hence potentially affects the fungus' susceptibility to a range of unisite fungicides, depending on the nature of the transporter involved. It is therefore responsible for a phenomenon called multi-drug resistance (MDR). In agriculture, resistance factors triggered by MDR range from low to medium, while in human pathogens, especially in *C. albicans*, increased efflux causes major treatment failure (Nishimoto *et al.*, 2019). This mechanism relies on the overexpression of membrane transporters, either ATP-binding cassette (ABC) transporters, or major facilitator superfamily (MFS) transporters. ABC transporters require ATP hydrolysis to translocate molecules across membranes and therefore require energy (Jones and George, 2004). MFS transporters, on the other hand, are proton antiporters, *i.e.* they use a proton gradient across the membrane to translocate xenobiotics (Pao *et al.*, 1998). All fungal species are particularly rich in both categories of transporter genes (Coleman and Mylonakis, 2009). Overexpressed transporters affect antifungal compounds differently therefore leading to a specific spectrum of MDR. This phenomenon should not be confused with multiple resistance, which is the accumulation of specific resistance mechanisms (Walker A.-S., personal communication).

Among phytopathogenic oomycetes and fungi, MDR has been reported in *Phytophthora infestans*, *Botrytis cinerea*, *V. inaequalis*, *Z. tritici*, *Colletotrichum spp.* and many other species. (de Waard *et al.*, 2006). Just like target genes, transporter-encoding genes can be overexpressed following an insertion in the promoter region, or because of a mutation in a transcription factor-encoding gene. Such mechanisms have been reported in *B. cinerea* (Kretschmer *et al.*, 2009; Leroux and Walker, 2013), *Sclerotinia homoeocarpa* (Sang *et al.*, 2018) and *C. albicans* (Nishimoto *et al.*, 2019), three species in which both MFS and ABC transporters are involved in MDR. Transporter overproduction is constitutive and does not seem to trigger any fitness penalty.

5.6.2.2. Activation of alternative pathways.

In some situations, fungi can activate an alternative metabolic pathway in order to compensate for inhibition of the one that is affected by the fungicide. The most common alternative pathway is the mitochondrial alternative oxidase (AOX)-mediated respiratory pathway (see section 4.2.7.). AOX allows ATP production bypassing complexes III and IV, therefore constituting a

respiratory pathway insensitive to all complex III inhibitors used in agriculture, *i.e.* QoI-D, QoI-P, QiI and QioI.

Cases of AOX overproduction linked to QoI reduced sensitivity have been documented in the ascomycetes *B. cinerea* (Tamura *et al.*, 1999), *V. inaequalis* (Steinfeld *et al.*, 2001), *Microdochium nivale* and *Microdochium majus* (Walker *et al.*, 2009), *Gaeumannomyces graminis* var. *tritici* (Joseph-Horne *et al.*, 1998) and *Z. tritici* (Miguez *et al.*, 2004; Wood and Hollomon, 2003). However, no reduced field efficacy of QoIs due to the AOX has been reported, except for *B. cinerea* (Ishii *et al.*, 2009), which is not a target organism of QoIs in French vineyards. Indeed, in *B. cinerea*, like in *Fusarium graminearum* (Kaneko and Ishii, 2009), the AOX contributes to natural resistance to complex III inhibitors, as it can be rapidly induced. In most species, the AOX pathway is not efficient enough in energy production to sustain high energy requiring developmental stages, especially during infection where plant flavonoids inhibit its activity. The reason why *B. cinerea* and *F. graminearum* are exceptions is not yet clear, but higher amounts of ROS production during infection leading to increased AOX production, is one mentioned possibility (Tamura *et al.*, 1999).

The situation in oomycetes with respect to QoI, QioI and QiI resistance is quite different. In French *Plasmopara viticola* populations, the proportion of individuals overproducing AOX has been shown to increase within a single season of fungicide use and continues to increase on a yearly basis, meaning that the AOX pathway is not limiting in these organisms. Moreover, resistance factors to ametoctradin, cyazofamid and amisulbrom measured in these strains are high, up to 300, and trigger efficacy losses in the field (Fehr *et al.*, 2016; Fontaine *et al.*, 2019). Moreover, in both oomycetes and fungi, overproduction of AOX is a mechanism that allows for longer survival under fungicide selection pressure, and is therefore suspected to favor selection of target site resistance.

Another interesting example of an alternative pathway is the alternative sterol biosynthesis pathway in *C. albicans* (Nishimoto *et al.*, 2019). Indeed, some *C. albicans* isolates that present a defect in the C-5 sterol desaturase are able to use an alternative sterol biosynthesis pathway, avoiding C-14 demethylation, hence being insensitive to azoles. These isolates are able to integrate different sterols into their plasma membrane that replace ergosterol, and are therefore resistant to azoles. The alternative sterol biosynthesis pathway remains a rare, yet important, azole resistance mechanism in *C. albicans* (Nishimoto *et al.*, 2019).

5.6.2.3. Resistance mechanisms based on fungicide metabolism.

5.6.2.3.1. Detoxification.

Detoxification designates the ability of a microorganism to transform enzymatically a pesticide into inactive metabolites. This kind of mechanism is frequently encountered for insecticide or herbicide resistance, but seems to remain anecdotal in fungi, as it is rarely explored. Only few cases have been documented. One example is the natural resistance of *Botrytis pseudocinerea*, a cryptic species of *B. cinerea*, to the hydroxyanilide fenhexamid (Billard *et al.*, 2011). Fenhexamid selection pressure triggers the overexpression of a gene encoding a cytochrome P450 enzyme in *B. pseudocinerea*, but not in the sensitive *B. cinerea*. Deletion of this gene partially abolishes the resistance in *B. pseudocinerea*, confirming that metabolism plays a key role in resistance (Billard *et al.*, 2011).

A well-documented example of fungicide resistance through detoxification is *Sclerotinia homoeocarpa* (Sang *et al.*, 2018). The MDR strains of *S. homoeocarpa* display overexpression of genes encoding membrane transporters, but also genes encoding cytochrome P450 monooxygenases. These monooxygenases are metabolizing enzymes and, in *S. homoeocarpa*, the overexpression of the genes *CYP561*, *CYP65* and *CYP68* leads to resistance to propiconazole (DMI), flurprimidol (plant growth regulator), boscalid (SDHI) and iprodione (inhibitor of osmotic signal transduction) (Sang *et al.*, 2018).

Detoxification is also suspected to happen in *B. cinerea* concerning resistance to fluazinam (Leroux *et al.*, 2002), and in *V. inaequalis* concerning kresoxim-methyl resistance, but metabolizing enzymes have not been identified yet (reviewed in Billard, 2011).

5.6.2.3.2. Default in activation.

Some pesticides cannot be applied directly in their active form, mainly for stability, bioavailability, or toxicity issues. Sometimes, these active ingredients can be chemically modified into a compound called a propesticide, which may display either a higher stability against abiotic factors like UV, a better selectivity (hence a lower toxicity for the host or the environment), or a greater bioavailability (for the host or the target organism). The propesticide then undergoes metabolism to the active form, a process that is called “activation” (Fig. 32). Activation is an enzymatic process due to hydrolases or esterases most of the time,

but sometimes involves cytochrome P450 oxidases. In agriculture, some fungicides need to be activated by the fungal or plant cells, like prothioconazole or fenpicoxamid.

Failure of activation as resistance mechanism is not common in phytopathogenic fungi. It has been described regarding phosphorothiolate resistance in *Magnaporthe oryzae*. In this pathogen, the deficiency in a cytochrome P450 oxidase results in a decrease in the production of dephosphorylated toxic metabolites, hence leading to a lower fungal susceptibility (Sugiura *et al.*, 1993). A case of natural resistance due to a default in metabolization has been reported in *B. cinerea* regarding cymoxanil susceptibility (Tellier *et al.*, 2009), even though cymoxanil is mostly used as an anti-oomycete. One metabolic pathway of cymoxanil leads to the production of *N*-acetylcyanoglycine, which is a fungitoxic metabolite. Strains displaying absence or dysfunction of this metabolic pathway turned out to be resistant to cymoxanil (Tellier *et al.*, 2009). A default in metabolic activation is also a known resistance mechanism against the human antifungal drug 5-flucytosine (Vermes *et al.*, 2000).

5.7. Resistance dynamics in populations.

Resistance dynamics in populations can be of two kinds. In the first scenario, the population is composed of two phenotypic groups, present at varying frequencies, one being resistant and the other susceptible. Under fungicide selection pressure, the resistance-conferring mutation is selected and its frequency increases. In this case, the population goes from mainly susceptible to mainly resistant over time, while the fungicide efficacy decreases (Fig. 33). This type of resistance is called qualitative, and is a very common scenario encountered for resistance to single-site fungicides (Walker A.-S., personal communication). Qualitative resistance can be triggered by one mutation (monoallelic), or several independent mutations (polyallelic). It is always monogenic but mutations can affect the protein-encoding gene, or a non-related gene, like those involved in increased efflux or detoxification for example (Fig. 33).

In the second situation, the population is composed of individuals with varying levels of resistance. Population is therefore characterized by a continuous spectrum of resistance levels, with a progressive shift towards the most resistant individuals under fungicide selection pressure (Fig. 33) (Lucas *et al.*, 2015). In that scenario, the resistance is called quantitative, as each allele and/or each allele combination confers a different level of resistance. Fungicides efficacy also decreases when resistance is quantitative but there still can be some efficacy depending on the population composition, *i.e.* on the nature of the alleles present and their respective frequencies (Walker A.-S., personal communication). Resistance is triggered by the

accumulation of mutations in the target gene, and/or other genes also contributing to the resistance level (Fig. 33). If mutations only occurs in the targeted gene, then resistance is monogenic, yet multiallelic. If other genes are also mutated, then resistance is multigenic, and can be mono- or multiallelic (Fig. 33).

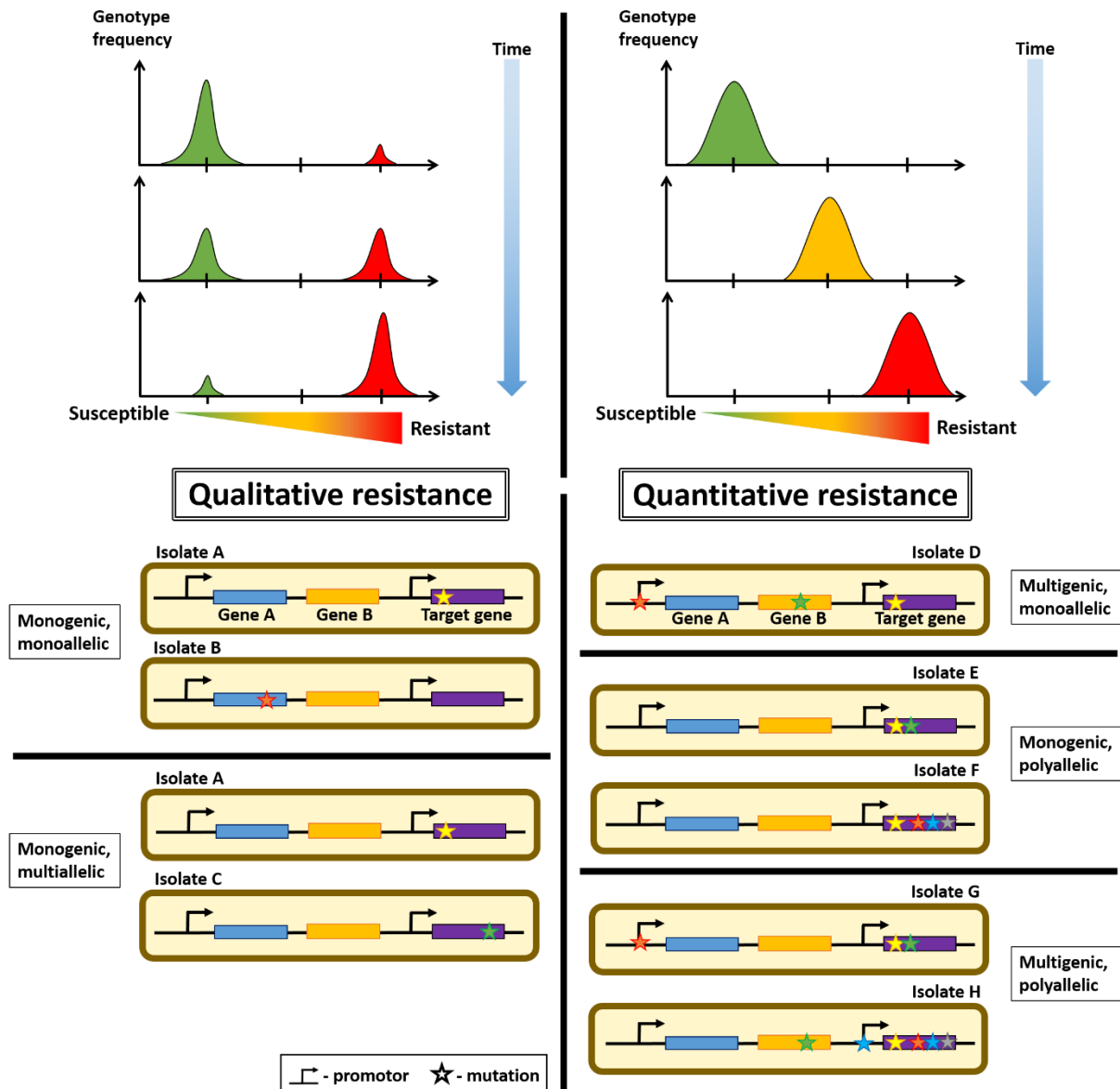


Figure 33. Dynamics in population and genetic origin of qualitative and quantitative resistance (Walker A.-S., unpublished). Top: Dynamics of qualitative resistance (left) and quantitative resistance (right) in populations. It shows the evolution of susceptible and resistant phenotypes in a population under fungicide selection pressure over time. Bottom: genetics underlying both types of resistance. Black lines separate populations.

5.8. Managing resistance.

Managing resistance aims at delaying the selection of resistance, hence slowing down this evolution. To this end, different fungicide-based strategies have been developed to modulate the selection pressure on populations in order to delay adaptation. The most common strategies are the use of fungicide mixtures, fungicide alternation, spatial mosaic or modulation of the fungicide dose. Spatial mosaic, mixture and alternation require the use of different modes of action, or fungicides with the same mode of action but with different resistance profiles as described for azoles (Heick *et al.*, 2017). Fungicide mixtures involve the simultaneous use of at least two active ingredients, triggering a more heterogenous selection pressure. In the field, mixed partners are used at the maximum permitted dose, and not at the equivalent efficiency of both partners, meaning that the selection pressure exerted by the mixture is always higher than the one of both partners used individually. Alternation consists in the use of different active ingredients one after another. In this case, selection pressure becomes heterogenous over time. Spatial mosaic consist in a geographically heterogenous selection pressure, while modulation of the fungicide dose allows having a selection pressure heterogenous in terms of intensity.

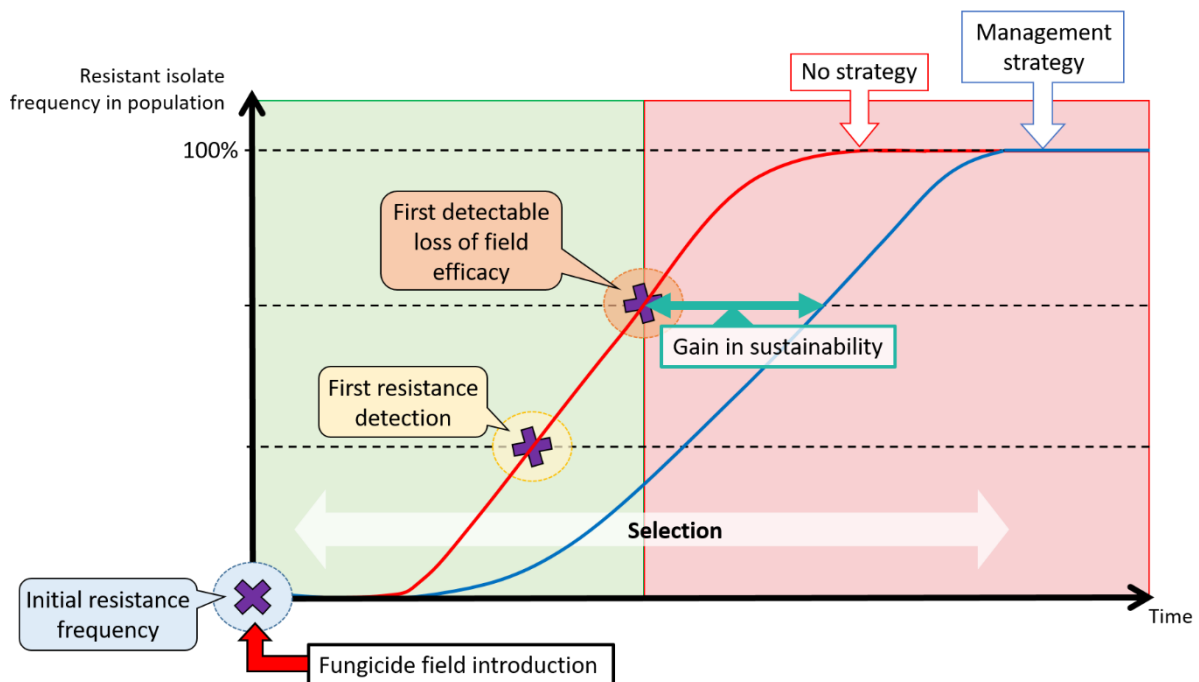


Figure 34. Gain in fungicide sustainability achieved through management strategy. The earlier the strategy can be established, the greater the gain in sustainability. Being able to predict resistance selection and evolution is crucial to design efficient anti-resistance strategies, even prior to fungicide use in the field.

Modeling studies and usual recommendations often designate mixtures as the most efficient strategies to delay resistance selection (Hobbelen *et al.*, 2013). However, recent *in vitro* evolution experiments have shown that efficacy of mixture and alternation are not so easy to distinguish (Ballu *et al.*, unpublished). Several parameters can influence the success of a strategy, like the cost of resistance, or the risk of selecting resistance associated with each active ingredient.

Experimental, or directed, evolution consists in evolving strains, or cell lines, under drug exposure but without mutagenic agents, to accelerate resistance selection in miniaturized conditions (for review, Kawecki *et al.*, 2012). It proved to be a useful tool to study or predict resistance selection in many different organisms like cancer cells (Kern and Weisenthal, 1990), bacteria (Jahn *et al.*, 2017; Orenca *et al.*, 2001), nematodes (Lopes *et al.*, 2008) or fungi (Cowen *et al.*, 2001; Gutiérrez-Alonso *et al.*, 2017; Schoustra *et al.*, 2006). It allows evolving *Z. tritici* populations under different selection pressures: concurrent use, mixture or alternation, using different fungicide partners. Therefore, it can be used to try predicting the better resistance management strategies (Fig. 35), especially for active ingredients that are not on the market yet.

5.9. Resistance status of *Zymoseptoria tritici* field populations in Europe.

Z. tritici has developed resistance towards all available single-site fungicide modes of action developed since the 1970s: the benzimidazoles, the QoIs, the DMIs and the SDHIs (Table 3), displaying various resistance mechanisms (Garnault *et al.*, 2019).

5.9.1. Resistance to benzimidazoles.

Z. tritici is a useful textbook example when it comes to target alteration-based resistance. Indeed, benzimidazole resistance is due to the selection of a mutation with a strong phenotypic effect and is therefore typically qualitative, as well as multiallelic (Fig. 33). Resistance to benzimidazoles developed in the mid-1980s in Western Europe (Lucas *et al.*, 2015). The changes responsible for the resistance are the E198A/K changes in β -tubulin (TUB2) (Fig. 7; Table 2) (Griffin and Fisher, 1985). The E198A change is the most common and has been reported in 20 different fungal pathogen species. Other changes are less frequent and observed occasionally in other fungal species (Hawkins and Fraaije, 2016). Mutations at codon 198 confer resistance to all registered benzimidazoles. Since its emergence, resistance has spread in *Z. tritici* populations to reach a stable frequency, which is today about 90% in France and the UK (Fig. 34; Garnault *et al.*, 2019; Lucas *et al.*, 2015). Benzimidazole use is now marginalized

on wheat and only aim at controlling *Fusarium* head blight and rusts, however maintaining a little selection pressure on *Z. tritici* populations (Garnault *et al.*, 2019). The stabilization of resistant alleles probably also reflects the absence of a detectable fitness penalty linked to the E198A/K changes, in field conditions (see section 5.4) (Hawkins and Fraaije, 2018).

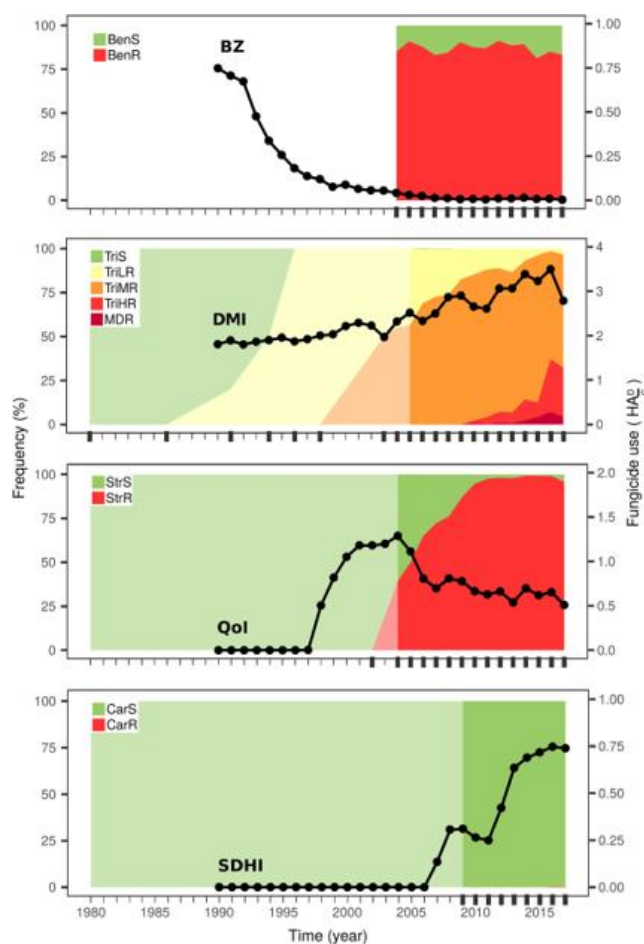


Figure 35. Evolution of *Z. tritici* resistant phenotypes frequencies (background colors) in French populations and fungicide use (blacklines) (Garnault *et al.*, 2019). BZ: benzimidazoles; DMIs: sterol demethylation inhibitors; QoIs: quinone outside inhibitors; SDHI: succinate dehydrogenase inhibitors.

5.9.2. Resistance to QoIs.

As for benzimidazoles, QoI resistance is qualitative, though mainly monoallelic (Fig. 24). In *Z. tritici*, a mutation in the mitochondrial *CYTb* gene leading to the G143A change is responsible for high resistance levels to QoIs, as in most species concerned by this resistance (Hawkins and Fraaije, 2021). In some isolates, the F129L change has also been detected. In *Z. tritici* populations, first resistant isolates were selected only five years after the introduction of

strobilurins in the fields (Fig. 34; Garnault *et al.*, 2019), and resistance emerged at least four times independently in European *Z. tritici* populations (Torriani *et al.*, 2009). QoI resistance has spread much faster than resistance to some other MoAs, as exemplified by its increasing frequency in French populations higher than that of other fungicide classes (Garnault *et al.*, 2019). In *Z. tritici*, strains that are not under selection pressure always behave as the maternal parent, transmitting their mitochondria (see section 4.3.2.). Consequently, spread of the maternally inherited QoI resistance has been favored by continuous fungicide selection pressure (Kema *et al.*, 2018). Moreover, the management of strobilurins in the field has been poor, as they were applied multiple times, using multiple different active ingredients, and often without a mixing partner fungicide (Bartlett *et al.*, 2002). Consequently, due to rapid resistance spreading, QoI use in the field started to decrease progressively as early as 2004 (Fig. 34). In spite of that, the frequency of cytochrome *b* G143A remains high (more than 90%) and stable (Fig. 34; Garnault *et al.*, 2019; Kildea *et al.*, 2019), suggesting that the cytochrome *b* G143A change does not trigger any detectable fitness penalty under field conditions. Moreover, the remaining use of strobilurins to control other wheat fungal diseases probably maintain some selection pressure on *Z. tritici* populations (Garnault *et al.*, 2019).

5.9.3. Resistance to DMIs.

The resistance status towards DMIs has evolved for decades and constitutes a good example of quantitative resistance. Indeed, the accumulation of mutations in the *CYP51* gene due to long-lasting exposure to DMIs is the main resistance mechanism developed by *Z. tritici* (Huf *et al.*, 2018). In France, ten years after the first azoles were introduced, first resistant mutants were detected (Fig. 34; Garnault *et al.*, 2019). Each azole molecule displays a similar binding pose but stabilizes its binding through interactions with different protein residues (see section 3.8.3.3.) (Sheng *et al.*, 2009). The selection pressure exerted on different amino-acids led to the continuous evolution of the *CYP51* gene through the selection and combination of at least thirty different mutations and/or deletions (Table 2) (reviewed in Huf *et al.*, 2018). First reported changes to confer resistance were the Y137F change, and substitutions or deletions between amino-acids 459 and 461. Today, the changes D134G, V136A/C/G, A379G, I381V, S524T, and Δ Y459/G460 are described as the ones conferring the highest resistant factors to DMIs, but with various impacts on the susceptibility to different azoles. Huf *et al.* studied a collection of strains from 2016 sampled all over Europe, and identified 33 different *CYP51* haplotypes, nine of which represented 85%. Interestingly, haplotypes with only the I381V and Y461H changes

that dominated European populations before 2010 were still found in Eastern Europe in 2016, but not in the UK or Ireland. In contrast, 50% of haplotypes originating in Western Europe harbored the S524T substitution (Huf *et al.*, 2018), suspected to decrease sensitivity to most DMIs (Cools and Fraaije, 2013). This situation shows the ongoing evolution of the *CYP51* gene, whose adaptation started in the UK and Ireland where the selection pressure is the highest, due to large wheat production areas and higher number of fungicide applications (Brunner *et al.*, 2008). The evolution of *CYP51* haplotypes then spreads from Western to Eastern Europe (Heick *et al.*, 2020). Changes L50S, S188N and N513K, on the other hand, do not trigger any shift in azole sensitivity (Cools and Fraaije, 2013; Leroux *et al.*, 2007). These changes either may compensate for other changes to avoid a decrease in enzyme activity, or could simply result from genetic variability.

The most common European *CYP51* haplotypes display different sensitivity profiles to azoles, with a particular cross-resistance pattern against epoxiconazole and prothioconazole on the one hand, and to tebuconazole and difenoconazole on the other hand, the four most widely used azoles (Huf *et al.*, 2018; Jørgensen *et al.*, 2018). The result is a variable efficacy of azoles even sometimes at small spatial scales, depending on population structure. However, strategies using mixture or alternation of azoles with different resistance profiles have been shown to provide better resistance control (Heick *et al.*, 2017). As such, the proper management of the different azoles appears to be an important issue in the fight against resistance for the future. Especially because the evolutionary process of *CYP51* is still ongoing and is predicted to continue as new azoles are currently developed and released on the market, like mefentrifluconazole (see section 3.8.3.3.) (Cools and Fraaije, 2013).

In addition to target modification, MDR also plays a role in resistance to DMIs. In *Z. tritici*, MDR is the consequence of the overexpression of the *MFS1* gene (Omrane *et al.*, 2015). *MFS1* overexpression results from insertions in the promoter region. So far, four different insertions have been characterized, but only three trigger overexpression (Mäe *et al.*, 2020; Omrane *et al.*, 2017). MDR causes low to moderate resistance factors to azoles, when not associated with another resistance mechanism (Leroux and Walker, 2011; Omrane *et al.*, 2017). However, in the field, MDR is usually associated with modifications of *CYP51*, and in such situations, RFs are widely increased. In conclusion, the risk associated to MDR is insidious as it is indirect (Walker A.-S. and Gazeau G., unpublished).

Strains overexpressing *CYP51* have also been described in *Z. tritici* (Cools *et al.*, 2012). A 120 bp insertion in the region of the *CYP51* promotor triggers constitutive overexpression of the

gene. Corresponding isolates displayed reduced sensitivity to all tested azoles compared to isolates with the same *CYP51* haplotype but without the promotor insertion (Cools *et al.*, 2012).

With the evolution of the *CYP51* gene combined with MDR and/or target overexpression, the landscape of DMI resistance in European field populations is a continuous spectrum of resistance profiles. It ranges from susceptible individuals, commonly referred to as TriS, to low, medium and highly resistant strains called respectively TriLR, TriMR and TriHR (Fig. 34; Garnault *et al.*, 2019; Huf *et al.*, 2018). This continuous spectrum of resistant phenotypes is characteristic of poly-allelic resistance, and the shift towards the most resistant individuals is currently noticeable as their proportion increases yearly in European populations (Garnault *et al.*, 2019).

5.9.4. Resistance to SDHIs.

SDHI resistance was first detected in France in 2012, and a few years before in Ireland & the UK (Garnault *et al.*, 2019). So far, changes in subunits B, C and D (see section 4.2.3.) have been identified as SDHI resistance-conferring changes (Rehfus *et al.*, 2018). C-T79N and C-N86S are the most common changes in European populations. These changes confer moderate resistance to all SDHIs, but when present in high frequencies, they can cause a decrease in fungicide field efficacy. B-T268I, B-N225I, B-T268A, C-T79I and C-N86A have been found in some isolates but remain rare. Resistance factors linked to these changes are also moderate (Rehfus *et al.*, 2018). The most preoccupying change is C-H152R that confers higher resistance levels to all the major SDHIs. Since its first detection in Ireland in 2015, in this case associated with the D-R47W change (Dooley *et al.*, 2016), this haplotype is now regularly found in French, German and Dutch populations, but remains at low frequency in Western Europe. As for azoles, it seems that the adaptation to SDHIs also started in the UK and Ireland and spread throughout a Western-Eastern gradient. The fact that C-H152R has been linked to a decrease in enzyme activity that may explain why it did not spread faster (Scalliet *et al.*, 2012). However, it is very likely that the selection pressure exerted by the intensive use of SDHIs in the field will eventually favor the spreading of haplotypes with high resistance factors (Dooley *et al.*, 2016). Indeed, from year to year, the proportion of SDHI resistant isolates increases in European populations, including isolates bearing the C-H152R change, as shown by recent monitoring programs (Fig. 34; Garnault *et al.*, 2019).

More recently, a second resistance mechanism to SDHIs has been discovered. A paralog of *SDH3*, the *SDHC*-encoding gene in *Z. tritici* (Fig. 7), has been discovered in some field isolates

and found to be involved in the resistance to a sub-class of SDHIs, including pydiflumetofen, isofetamid and fluopyram, but with no detectable decrease in field efficacy (Steinhauer *et al.*, 2019). The paralogous gene is found in almost one individual out of three in European populations. It is constitutively expressed but with different expression levels among isolates. Moreover, splicing efficiency of the paralogous mRNA seems to be involved in the level of resistance (Steinhauer *et al.*, 2019).

5.9.5. Antiresistance strategies applied to *Zymoseptoria tritici* in wheat fields.

The challenge faced by growers to manage STB in the field is becoming more and more difficult, as fewer modes of action become available, because of the regulatory pressure on the one hand. On the other hand, as shown by the previous sections, the important evolutionary potential of *Z. tritici* enabled it to adapt to every new fungicidal mode of action. The recommended strategies in France consist in limiting fungicide applications to the lowest possible, meaning two applications maximum per season. When applying fungicides, growers should try using one of the remaining multisite fungicides when possible, should apply only one SDHI, one QiI and/or one QoI (seeds and foliar treatments), and should apply only one DMI from the different cross-resistance groups.

6. PHD CHALLENGES, QUESTIONS AND OBJECTIVES.

By far, wheat is the economically most important crop in Europe. The biggest threat for wheat production is the fungal pathogen, *Zymoseptoria tritici*, the causal agent of STB, which has visible economic consequences in terms of both yield losses and the cost of disease control. Despite a clear desire to reduce fungicide use in the fields for environmental and human health issues, chemical control is still the most reliable way to control STB. Due to its important genetic plasticity, *Z. tritici* has the potential to adapt very quickly to any changing situation, including the introduction of new fungicidal modes of action. These genetic characteristics, sometimes coupled with a non-optimal management of (new) active ingredients, led to the emergence and the generalization of resistance to almost all known unisite modes of action in field populations in the past decades. The remaining modes of action for efficient STB control are limited. Consequently, there is a clear need for new modes of action, and for a smarter management of active ingredients, meaning a tailor-made management, taking into account their specific risk components. This especially applies to fenpicoxamid.

Like all previously developed unisite modes of action, QiIs can be affected by target alterations. The mitochondrial target (CYTb) makes any target alteration a threat that needs to be closely monitored because of the fast transmission of mitochondrially encoded resistance (see section 4.3.2.). A better management of such a resistance is a challenge regarding the situation of QoIs. Indeed, the maternal inheritance of cytochrome *b* (target of QoIs and QiIs) may have amplified QoI resistance spread in the early 2000s. However, the presence of a prior alteration in cytochrome *b* (G143A) in about 90% of field strains could influence the selection of other changes in the protein, even at the Q_i site. It is also likely that fenpicoxamid and QiIs' efficacy may be affected by MDR, as it is a non-specific resistance mechanism. The existence of an alternative respiratory pathway in *Z. tritici* through the AOX may also represent a threat for QiIs. However, so far no AOX-based resistant strains have been found to reduce field efficacy of complex III inhibitors in *Z. tritici*. Like MDR, AOX could allow longer survival of strains under fungicide exposure, which increases the risk of resistance mutation selection. Concerning the requirement for activation of fenpicoxamid, a default in conversion to UK-2A could also lead to resistance. Indeed, UK-2A, the active metabolite, has high target site activity, while fenpicoxamid is almost inactive. Although this resistance mechanism has rarely been described in fungi, it could affect fenpicoxamid's efficacy. For all these reasons, the outcome of this pathogen's evolution under selection pressure from complex III inhibitors, in general, remains unpredictable.

In this context, the agronomic challenge of my PhD is to preserve the efficacy of efficient modes of action, and especially of the newly introduced QiI fenpicoxamid, in a situation of high resistance selection risk and increasing social pressure for practices in keeping with agroecology. The scientific challenge is to predict the evolution of resistance to complex III inhibitors, in order to anticipate smart resistance management at the outset.

To contribute to a better understanding of this evolution, my project addresses two main questions.

1 – What is the risk of resistance selection under fenpicoxamid selection pressure in Western European *Z. tritici* populations?

In the end, the risk of resistance will determine the outcome of evolution. It results from the balance between positive selection, expressed as the emergence and establishment of resistant strains, and negative selection, expressed as the fitness cost associated with this resistance. Answering this question therefore requires studying both aspects of selection. My first objective

was to characterize QiI resistance in *Z. tritici*. This meant, first, determining how fast resistance would be selected in populations. Afterward, characterize the resistant strains to identify which resistance mechanisms were most likely to be developed, in particular with respect to the impact on respiration. I achieved this goal by selecting fenpicoxamid-resistant strains using experimental evolution, before phenotyping them for their putative resistance mechanism(s) and further characterization through genetic and biochemical analyses. By these means, we characterized not only the modification linked to inhibition, but also cellular respiration as important component of fungal physiology and fitness. My second objective was to evaluate the possible fitness cost associated with observed fenpicoxamid resistance. This implies a thorough phenotypical characterization of the resistant strains both *in vitro* and *in planta*, in order to address their competitiveness in field conditions.

2 – How to prevent the selection of QiI resistance in *Z. tritici* populations?

The objective here was to identify the best fungicide-based strategy or strategies suited to QiI management by comparing the components of their sustainability. To do this, I decided to use experimental evolution again to compare the evolution of populations subjected to different selection regimes including fenpicoxamid. I wanted to estimate how each regime influenced resistance emergence and generalization, and to compare how the fungicide application timing and the fungicide partner influenced the outcome of the selection.

Chapter 1.

**Predicting resistance mechanisms
triggering fenpicoxamid-resistance
in *Zymoseptoria tritici*.**

INTRODUCTION.

In this chapter, we studied the adaptation of *Z. tritici* to fenpicoxamid selection pressure. To do so, we have selected fenpicoxamid-resistant strains of *Z. tritici* using experimental (or directed) evolution. Experimental evolution allows mimicking “field conditions”, meaning that resistance only results from the “natural” evolution under fungicide selection pressure as close as possible as the one that will occur in nature. This method avoids using mutagenic agents, whether they are chemical agents, such as manganese chloride or sulfate, or physical agents, such as UV light, to prevent mutations not directly related to fungicide selection. In the case of fenpicoxamid-resistance, directed mutagenesis was excluded as the targeted *CYTb* gene belongs to the mitochondrial genome and is hardly transformable with molecular genetics in *Z. tritici*. Moreover, mutagenesis allows only studying target site resistance, while directed evolution could select any putative resistance mechanism. Even though directed evolution has already been used to study resistance to drugs (Gutiérrez-Alonso *et al.*, 2017; Jahn *et al.*, 2017; Kern and Weisenthal, 1990), it is the first time, to our knowledge, it is used to try predicting resistance selection by a drug, prior to its registration.

In the following article, we have first characterized the resistance mechanisms of the selected isolates. We found that target site resistance was the main resistance mechanism in these isolates, consistent with the fact that it is a frequent mechanism encountered for fungicide resistance in phytopathogenic fungi (Lucas *et al.*, 2015). We then assessed the level of fenpicoxamid resistance of these isolates at the cellular level, using microtiter plate fungitoxicity assays, as well as their susceptibility to other cytochrome *b* inhibitors with a different binding site (QoIs). Using biochemical spectrophotometric assays, we validated resistance at the enzyme level, before searching for putative consequences of mutations on cytochrome *b* activity.

Our study also gave some insights about the influence of the Q_o site change, G143A, on QiI resistance selection, and QiI susceptibility. In fact, we have demonstrated the existence of a mutual influence between the Q_o and Q_i site changes on resistance to inhibitors of respective opposite site, as well as on cytochrome *b* activity. Previous work already showed that the presence of changes at the Q_i site, or the binding of QiIs could affect the molecular dialog (*i.e.* the electron transfer) between the Q_o and Q_i sites (Cooley *et al.*, 2006), corroborating our findings.

To conclude, this work allowed us to select and characterize fenpicoxamid-resistant strains without using any mutagenic agent, enabling the possibility to assess the practical risk of resistance in field conditions, in the next chapter. Our findings, consistent with existing literature, place directed evolution as a useful tool for resistance prediction, hence for resistance management. This work was accepted for publication in the journal *Environmental Microbiology*, under the following reference:

Directed evolution predicts cytochrome b G37V target site modification as probable adaptive mechanism towards the Qil fungicide fenpicoxamid in Zymoseptoria tritici.

Guillaume Fouché, Thomas Michel, Anaïs Lalève, Nick X Wang, David H Young, Brigitte Meunier, Danièle Debieu, Sabine Fillinger and Anne-Sophie Walker.

Directed evolution predicts cytochrome *b* G37V target site modification as probable adaptive mechanism towards the QiI fungicide fenpicoxamid in *Zymoseptoria tritici*.

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SUMMARY.

Acquired resistance is a threat for antifungal efficacy in medicine and agriculture. The diversity of possible resistance mechanisms, as well as the highly adaptive traits of pathogens make it difficult to predict evolutionary outcomes of treatments, especially prior to their use. We used directed evolution as an approach to assess the risk of resistance to the new fungicide fenpicoxamid in the wheat pathogenic fungus *Zymoseptoria tritici*. Fenpicoxamid targets the cytochrome *b* ubiquinone reduction site (Q_i site), unlike the widely used strobilurins that bind to the cytochrome *b* ubiquinol oxidation site (Q_o site). We identified the cytochrome *b* G37V change, within the cytochrome *b* Q_i site, as the most likely resistance mechanism to be selected in *Z. tritici*. This change triggered high fenpicoxamid resistance and halved the enzymatic activity of cytochrome *b*, despite no significant penalty for *in vitro* growth. In addition, we identified a negative cross-resistance between isolates harboring the cytochrome *b* G37V change or G143A, a Q_o site change previously selected by strobilurins. Moreover, double mutants were less resistant to both QiIs and QoIs compared to single mutants. This work is a proof of concept that experimental evolution can be used to predict adaptation to fungicides, and provides new perspectives for the management of QiIs.

Keywords.

Fungicide resistance; antifungal; *Septoria tritici* blotch; mitochondria; respiratory inhibitors; mitochondrial complex III; target site resistance; parallel evolution.

INTRODUCTION.

Acquired resistance is a phenotypic adaptation, mainly in pathogens, pests, weeds or cancer cells, in response to drug selection pressure (Hawkins *et al.*, 2019). In both agriculture and medicine, it is responsible for major economic losses and public health concerns (Ahmad and Khan, 2019; Maragakis *et al.*, 2008). Therefore, predicting the adaptation to new active ingredients, or new modes of action, is a critical challenge, complicated by the diversity of resistance mechanisms found in nature, and the particular interactions between active ingredients and targeted organisms. Nevertheless, early prediction and detection of resistance is a prerequisite for sound and sustainable fungicide use as resistance alleles are more likely to be lost by chance when they are maintained at low frequency in populations through an optimized management strategy. Resistance selection in the laboratory can be achieved by two different approaches. The classical method involves random mutagenesis, and searching for the resistance-conferring mutation(s). This approach has proven effective in medicine to understand resistance mechanisms in cancer cells (Azam *et al.*, 2003) or pathogenic yeasts (Rawal *et al.*, 2013), and in agriculture to explore insecticide (McKenzie and Batterham, 1998) or fungicide resistance (Hawkins and Fraaije, 2016; Scalliet *et al.*, 2012). However, random mutagenesis achieved using UV or chemical exposure may cause multiple mutations throughout the genome and make it difficult to correlate the resistance phenotype with a specific mutation. Moreover, some mutations recovered *in vitro* may never occur in nature under selection pressure from agrochemicals or drugs (Hawkins and Fraaije, 2016). The use of molecular techniques to perform site-directed mutagenesis avoids additional mutations, but focuses only on single mutations involving the biochemical target, with no information on other possible mechanisms. Moreover, these techniques cannot be applied to mitochondrial genes in most species. A second approach is directed evolution. This consists in allowing strains or cell lines to evolve under drug exposure, but without mutagenic agents, to accelerate resistance selection in miniaturized conditions (for review, Kawecki *et al.*, 2012). This method mimics natural selection, and may select any putative resistance mechanism. In particular, it has been used with cancer cells (Kern and Weisenthal, 1990), bacteria (Jahn *et al.*, 2017; Orenca *et al.*, 2001), nematodes (Lopes *et al.*, 2008) and fungi (Cowen *et al.*, 2001; Gutiérrez-Alonso *et al.*, 2017; Schoustra *et al.*, 2006).

In agriculture, fungal pathogens represent the biggest threat for crop production (Savary *et al.*, 2019), and there is a continual need for new fungicides due to resistance. As key organelles, mitochondria represent a popular target for fungicide development, and seven different

fungicidal modes of action used in agriculture target the mitochondrial respiratory chain (<https://www.r4p-inra.fr/fr>). Although known mainly for their role in energy production, mitochondria also participate in many other metabolic processes and govern the life and death cycle of eukaryotic cells (reviewed in van der Bliëk *et al.*, 2017). Mitochondrial respiration is a cellular process that provides energy to eukaryotic cells through ATP production by the oxidative phosphorylation (OXPHOS) system. Five multi-subunit protein complexes embedded in the inner mitochondrial membrane (IMM) form the OXPHOS system. Complexes I to IV transfer electrons to oxygen and produce energy, in the form of a transmembrane electrochemical gradient of protons, that is used by complex V (or ATP synthase) for the production of ATP (reviewed in van der Bliëk *et al.*, 2017). Some organisms also possess alternative oxidases (AOX), which allow ATP production while bypassing complexes III and IV (reviewed in Day *et al.*, 1995). Complex II inhibitors, known as SDHIs (succinate dehydrogenase inhibitors) and complex III quinone outside inhibitors (QoIs, or strobilurins), are the most widely used respiratory inhibitor fungicides in agriculture. SDHI resistance has been reported in many fungi, including *Aspergillus flavus* (Masiello *et al.*, 2020), *Botrytis cinerea* (Fernández-Ortuño *et al.*, 2017), *Sclerotinia sclerotiorum* (Peng *et al.*, 2020), *Alternaria alternata* (Fan *et al.*, 2015) and *Zymoseptoria tritici* (Dooley *et al.*, 2016; Steinhauer *et al.*, 2019). Prior to the commercial use of SDHIs to control *Z. tritici*, mutagenesis studies in the laboratory identified some mutations which later occurred in the field, but also produced mutations which have not yet been found in nature (Fraaije *et al.*, 2012; Rehfus *et al.*, 2018; Scalliet *et al.*, 2012). In the case of QoIs, their repeated and unrestricted use in the field (Bartlett *et al.*, 2002) quickly triggered the emergence of target site resistance in many fungal pathogens. This rapid emergence and spread occurred in multiple species (Hawkins and Fraaije, 2021), and had not been predicted. The most common resistance mutation leads to the cytochrome *b* G143A change, and has been selected in *Z. tritici* (Fraaije *et al.*, 2005) and repeatedly in many other pathogens (Hawkins and Fraaije, 2021). In *Z. tritici*, resistance is now generalized in populations from Western Europe (Garnault *et al.*, 2019; Kildea *et al.*, 2019).

Complex III (or cytochrome *bc*₁ complex) catalyzes the transfer of electrons from ubiquinol to cytochrome *c*, and couples this electron transfer to the translocation of protons across the IMM. The complex operates as a dimer. Its monomeric unit consists of 10 or 11 different polypeptides. Three subunits, cytochrome *b*, cytochrome *c*₁ and the Rieske iron-sulphur protein, form the catalytic core of the enzyme. Cytochrome *b* is encoded by the mitochondrial genome in all eukaryotes, and the other subunits are encoded by nuclear genes. The complex possesses

two distinct ubiquinone-binding sites situated on opposite sides of the IMM; the ubiquinol oxidation site (Q_o site) located towards the IMM outer side, and the ubiquinone reduction site (Q_i site) located towards the inner, or matrix, side. The Q_o and Q_i sites are both in the membrane-spanning cytochrome *b* protein (Hunte *et al.*, 2000). Fungicide inhibitors of complex III bind to either the Q_o site (QoIs) or the Q_i site (QiIs), although the Oomycete fungicide ametoctradin appears to bind at both sites (reviewed in Fisher *et al.*, 2020). Two QiIs, cyazofamid and amisulbrom, are currently used to control Oomycete diseases. The picolinamide compound fenpicoxamid (Inatreq™ Active) is the first QiI fungicide active against ascomycete pathogens and will provide a new mode of action in the cereal market to control *Z. tritici* and other diseases (Owen *et al.*, 2017). Derived from the natural antifungal compound UK-2A (Ueki *et al.*, 1996), fenpicoxamid is rapidly metabolized in fungal or wheat cells back into UK-2A, which is responsible for its fungicidal activity (Owen *et al.*, 2017).

Z. tritici is the causal agent of wheat *Septoria tritici* blotch (STB). STB is the economically most important wheat disease in Europe, in terms of potential yield losses and cost of disease control (Fones and Gurr, 2015; Savary *et al.*, 2019). Due to its high genomic plasticity, ability to perform sexual reproduction and to spread over long distances through ascospore production, *Z. tritici* populations have developed resistance towards all currently used unisite modes of action (Garnault *et al.*, 2019), highlighting the resistance risk for new fungicides like fenpicoxamid.

As QiI resistance has been described for cyazofamid and amisulbrom in oomycetes, and as respiration inhibitors are generally at risk, we anticipate that resistance might also affect fenpicoxamid. Different resistance mechanisms can be envisaged in *Z. tritici* (Fig. 1.1.). Firstly, QiIs are subject to resistance risk due to target modification as reported in *Plasmopara viticola* (Fontaine *et al.*, 2019), and in several human protozoan parasites (reviewed in Mounkoro *et al.*, 2019). Mutations conferring resistance to UK-2A and the structurally related natural product antimycin A were also identified in the model organism *Saccharomyces cerevisiae*. In addition, the selection pressure-driven maternal behavior of *Z. tritici* field isolates might accelerate the spread of any resistance based on a mutated mitochondrial gene, as has been reported for QoIs (Kema *et al.*, 2018).

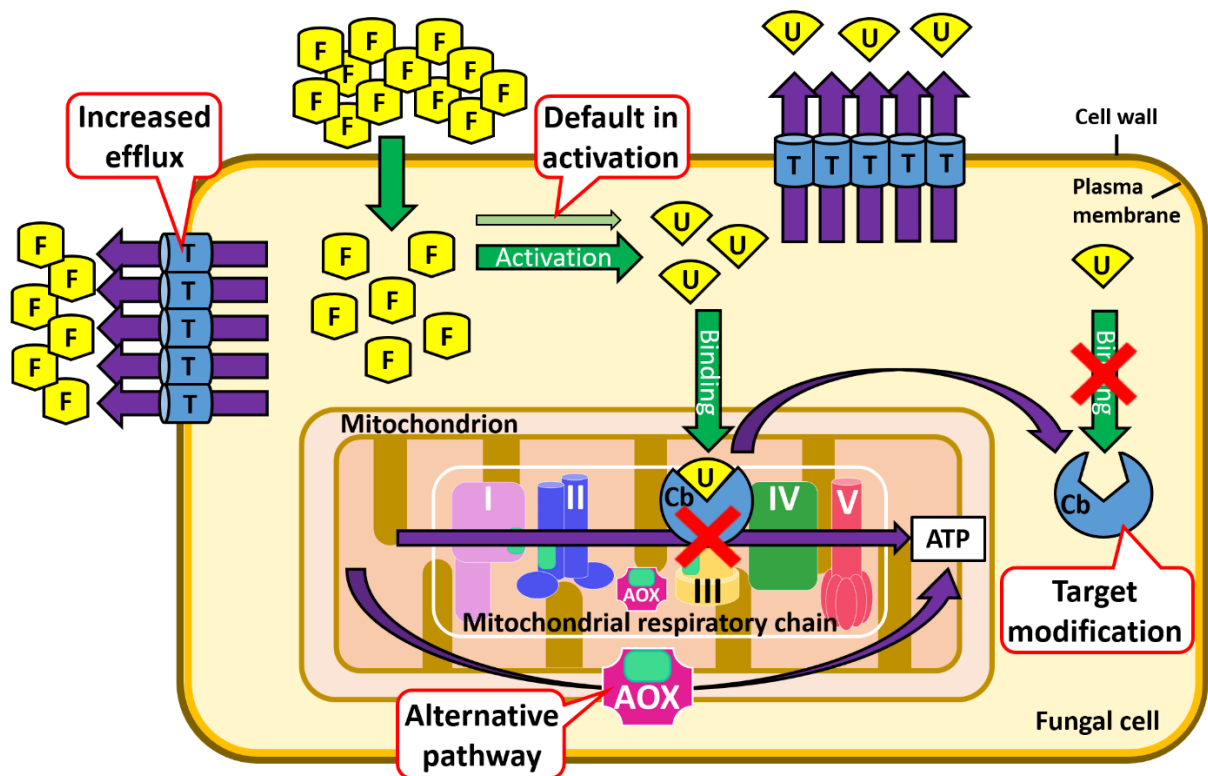


Figure 1.1. Resistance mechanisms that might reduce susceptibility of *Z. tritici* field isolates to fenpicoxamid: target modification, increased efflux, alternative pathway and default in activation. F: fenpicoxamid; U: UK-2A; Cb: cytochrome *b*; AOX: alternative oxidase; T: membrane transporter.

Increased efflux in *Z. tritici* can lead to reduced sensitivity to almost all unisite fungicides, including mitochondrial respiration inhibitors (Leroux and Walker, 2011; Omrane *et al.*, 2017). This phenotype, called MDR for Multi-Drug Resistance, is associated with overexpression of the membrane transporter gene *MFS1* (Omrane *et al.*, 2015). Another potential resistance mechanism, applicable to both QiIs and QoIs, is activation of the AOX pathway. This has been observed in some *P. viticola* strains which are resistant to amisulbrom and ametoctradin but contain no mutation in the *CYTb* gene (Fontaine *et al.*, 2019). Finally, a default in the activation process by which fenpicoxamid is converted to UK-2A could contribute to reduced sensitivity.

Because of its biology, and ease of handling in liquid culture due to its yeast-like form (Francisco *et al.*, 2019; Steinberg, 2015), *Z. tritici* is a convenient organism to predict adaptation to a fungicide. The goal of our study is to assess the risk of resistance to fenpicoxamid by using experimental evolution to produce resistant mutants in *Z. tritici*. Putative resistance mechanisms in isolated mutants were evaluated, revealing a target site modification, which was analyzed for

its effect on complex III activity and growth rate as indicators of fitness, and on binding of UK-2A using molecular docking analysis.

RESULTS.

Impact of enhanced efflux on *Z. tritici* sensitivity to fenpicoxamid. Because increased efflux causes low to moderate resistance to current respiration inhibitors (*i.e.* SDHIs and QoIs; Leroux and Walker, 2011) and other fungicides in *Z. tritici*, we assumed that this resistance mechanism might affect fenpicoxamid. Moreover, MDR strains have been detected in Western European populations at increasing frequencies each year, and represent roughly 40% of the French population (Garnault *et al.*, 2019). We first evaluated the impact of enhanced efflux on fenpicoxamid susceptibility by determining EC₅₀ values for inhibition of apical germ-tube elongation in solid medium, and *in vitro* growth in liquid cultures (Table 1.1.), for representative MDR isolates.

Table 1.1. Impact of increased efflux on sensitivity to fenpicoxamid in *Z. tritici*.

Assay	Isolate	<i>MFS1</i> -genotype ^a	Cytochrome <i>b</i>	EC ₅₀ ^b (µg/mL)	RF
Germ-tube elongation (48h)	IPO323	WT	WT	0.004 ± 0.002	-
	14-H-K3	WT	G143A	0.008 ± 0.002	-
	09-ASA-3a-PZ	MDR ^{Type I}	G143A	0.027 ± 0.006	3 ^c
	09-CB1	MDR ^{Type I}	G143A	0.054 ± 0.023	7 ^c
<i>In vitro</i> growth (3 days)	IPO323	WT	WT	0.004 ± 0.002	-
	35F22	WT	WT	0.003 ± 0.001	-
	35F6	WT	G143A	0.0020 ± 0.0007	-
	35G5	WT	G143A	0.0014 ± 0.0006	-
	37-15	WT	G143A	0.0018 ± 0.0004	-
	37-16	WT	G143A	0.0009 ± 0.0002	-
	11183	MDR ^{Type I}	WT	0.0293 ± 0.0006	8 ^d
	35C3	MDR ^{Type I}	G143A	0.017 ± 0.004	11 ^c
	35C4	MDR ^{Type I}	G143A	0.019 ± 0.003	12 ^c
37-59	MDR ^{Type I}	G143A	0.019 ± 0.005	12 ^c	

^a*MFS1* genotype nomenclature according to Omrane *et al.*, 2017.

^bEC₅₀ is the fungicide concentration inhibiting 50% of apical germ tube elongation or growth, compared to untreated control. Each EC₅₀ value is the average of at least three independent assays.

^cRF is the ratio between the EC₅₀ of the MDR isolate and the EC₅₀ value or the average of EC₅₀ values from the cytochrome *b* G143A isolates.

^dRF is the ratio between the EC₅₀ of the MDR isolate and the average of EC₅₀ values from the WT isolates.

RFs for fenpicoxamid conferred by the *MFS1* allele type I (MDR^{Type I}, Table 1.1.) ranged from 3 to 12, in both the germination and growth assays. Therefore, increased efflux reduces sensitivity to fenpicoxamid resistance, but the effect is relatively weak.

Selection of fenpicoxamid-resistant mutants. Experimental evolution mimics natural evolution while speeding up resistance selection under miniaturized laboratory conditions. Resistance to fenpicoxamid was selected using directed evolution in eleven *Z. tritici* ancestral isolates exhibiting different genetic backgrounds. Isolates used were the reference strain IPO323 (Goodwin *et al.*, 2011), as well as MDR, QoI-resistant (StrR) and MDR+StrR strains. These last three genotypes are the most frequent ones in European populations (Garnault *et al.*, 2019). Briefly, strains were cultivated in liquid medium amended with fenpicoxamid at the minimal inhibitory concentration (MIC) or at 25 times the MIC (25MIC), over eight seven-day cycles. At the end of each cycle, spores were plated on selection medium (containing fenpicoxamid at its respective selection concentration) to isolate resistant colonies. The experiments allowed the selection of almost 400 isolates which produced colony-like growth on fenpicoxamid-containing medium (Fig. 1.2.). However, after subculturing even once on fenpicoxamid-free medium and/or storage, 88% of these isolates lost fenpicoxamid resistance. This transient resistance phenomenon was observed for isolates derived from all ancestral strains, regardless of the genetic background (Fig. 1.2.). Stable resistant isolates were only derived from IPO323 (WT) and 37-16 (StrR).

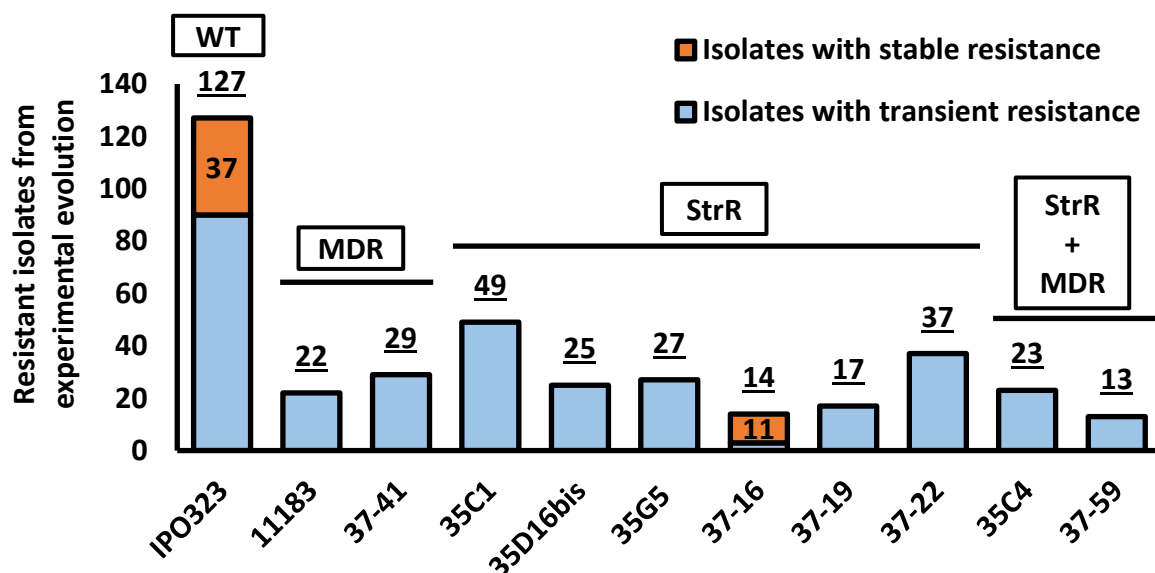


Figure 1.2. Number of fenpicoxamid-resistant isolates generated by experimental evolution for each ancestral strain. Total number of isolates is underlined. Phenotypes of ancestral strains are specified in boxes.

The sensitive IPO323 ancestral strain generated 127 resistant isolates in total, among which 37 (29%) were stable. Individuals with stable resistance were isolated in 50% of the independently evolved IPO323 lines. However, only one isolate with stable resistance was selected in lines evolving at 25MIC, while all others were selected at the MIC. MDR strains, regardless of their cytochrome *b* genotype, never provided stable resistant isolates. Resistance to fenpicoxamid was also explored for six different StrR strains; only one strain (37-16) yielded isolates with stable resistance, representing one line out of 25 (4%).

Characterization of fenpicoxamid-resistant mutants. Resistant isolates were characterized for their putative resistance mechanisms using a droplet test on fungicide-amended YPD medium (Fig. 1.3.). Growth on fenpicoxamid and UK-2A were both assessed, in order to detect a potential default in fenpicoxamid activation in the resistant isolates. Azoxystrobin sensitivity was tested to identify possible cross-resistance between QoIs and QiIs, which would be expected from overexpression of AOX. Growth on fenpicoxamid in the presence of AOX inhibitors, SHAM or propyl gallate, was also assessed to screen for this resistance mechanism, and provided a means of evaluating this mechanism in the StrR isolates where assessment of cross-resistance to azoxystrobin was not applicable. Finally, tolnaftate sensitivity was used to discriminate isolates exhibiting enhanced efflux (Leroux and Walker, 2011).

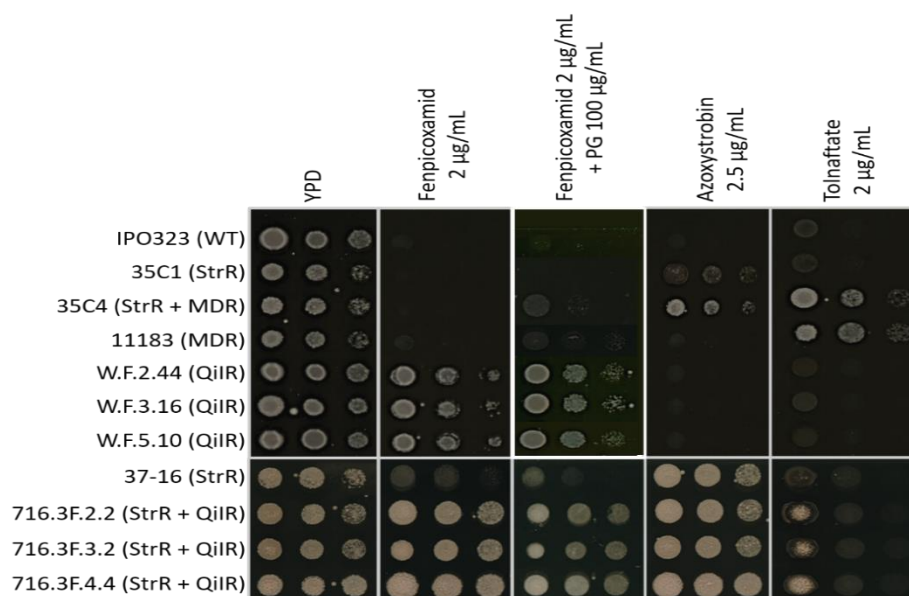


Figure 1.3. Phenotype of some representative resistant isolates obtained by experimental evolution, in comparison to their ancestral strains. The isolates W.F.2.44, W.F.3.16 and W.F.5.10 were derived from IPO323, while isolates 716.3F.2.2, 716.3F.3.2 and 716.3F.4.4 were derived from 37-16 (StrR).

All evolved isolates displayed growth on fenpicoxamid at 2 µg/mL (its approximate solubility limit in aqueous media), a concentration which prevented growth of all ancestral strains (Fig. 1.3.). Those isolates also grew on 0.5 µg/mL UK-2A-amended YPD (data not shown), excluding a defect in fenpicoxamid activation as the resistance mechanism. As expected, IPO323-derived isolates remained susceptible to the QoI azoxystrobin, while isolates derived from 37-16, bearing the cytochrome *b* G143A change, were resistant (Fig. 1.3.). In addition, growth on fenpicoxamid was not prevented by the addition of 100 µg/mL of the AOX inhibitors propyl gallate (Fig. 1.3.) or SHAM (data not shown), for any of the resistant isolates, demonstrating that AOX overexpression was not involved in resistance. None of these isolates was able to grow on 2 µg/mL tolnaftate (Fig. 1.3.), rejecting increased efflux as a resistance mechanism in the selected isolates.

After phenotypic analysis, the cytochrome *b* encoding gene (*CYTb*) was sequenced for 29 isolates. All sequences exhibited as sole *CYTb* mutation the same G to T substitution at codon 37, leading to replacement of the glycine residue by valine. This supports target site modification as the most plausible resistance mechanism towards fenpicoxamid.

Consequences of the cytochrome *b* G37V change on QiI and QoI susceptibility during *in vitro* growth. We first assessed the resistance to fenpicoxamid associated with target modification at the fungal cell level. Isolates bearing only the cytochrome *b* G37V change were highly resistant to UK-2A (Table 1.2.), with EC₅₀ values ranging from 0.8 to 1.6 µg/mL, and RFs higher than 200. The double mutants, displaying both the cytochrome *b* G37V and the G143A changes, were also highly resistant to UK-2A, with EC₅₀ values ranging from 0.28 to 1.28 µg/mL, and RFs ranging from 79 to 356 when compared to IPO323. Relative to G143A ancestral isolates, RFs for the cytochrome *b* G37V+G143A double mutants ranged from 183 to 828.

Resistance to QoIs was also measured for double mutants, using azoxystrobin. RFs ranged from 10 to 138 when compared to IPO323. However, RFs of the cytochrome *b* G37V+G143A double mutants when compared to cytochrome *b* G143A single mutants were below 1, demonstrating that their resistance towards QoIs was reduced.

Comparing the RFs to QoIs and QiIs of the cytochrome *b* G37V and G143A single mutants, respectively, we observed that RFs were systematically lower than one (Table 1.2.). The cytochrome *b* G37V mutants were up to two-times more susceptible to QoIs than the WT, and the cytochrome *b* G143A mutants up to three times more susceptible to QiIs than the WT. These

results suggest increased susceptibility of cytochrome *b* mutants to inhibitors which act at the opposite binding site, with QiIR isolates being more sensitive to QoIs and StrR isolates more sensitive to QiIs.

Table 1.2. Susceptibility to UK-2A and azoxystrobin as measured *via in vitro* growth of *Z. tritici* isolates.

Isolate	Cytochrome <i>b</i>	UK-2A			Azoxystrobin		
		EC ₅₀ (µg/mL)	RF _{WT} ^a	RF _{StrR} ^b	EC ₅₀ (µg/mL)	RF _{WT} ^a	RF _{StrR} ^b
IPO323	WT	0.004 ± 0.002	-	3	0.043 ± 0.022	-	> 0.01
35F22	WT	0.003 ± 0.001	-	2	0.035 ± 0.012	-	> 0.01
W.F.2.44	G37V	0.80 ± 0.18	222	516	0.026 ± 0.009	0.7	> 0.01
W.F.4.14	G37V	1.0 ± 0.4	286	665	0.019 ± 0.01	0.5	> 0.01
W.F.5.10	G37V	1.6 ± 0.9	451	1050	0.022 ± 0.014	0.6	> 0.01
35F6	G143A	0.0020 ± 0.0007	0.6	-	5.1 ± 1.8	132	-
35G5	G143A	0.0014 ± 0.0006	0.4	-	3.5 ± 2.3	92	-
37-15	G143A	0.0018 ± 0.0004	0.5	-	6.5 ± 2.1	168	-
37-16	G143A	0.0009 ± 0.0002	0.3	-	4.1 ± 1.5	106	-
716.3F.2.2	G37V+G143A	0.5 ± 0.1	140	327	3.0 ± 0.2	78	0.6
716.3F.2.3	G37V+G143A	0.75 ± 0.09	209	486	3.6 ± 0.4	93	0.7
716.3F.2.4	G37V+G143A	1.28 ± 0.08	356	828	3.2 ± 0.1	83	0.7
716.3F.3.1	G37V+G143A	0.8 ± 0.05	223	519	5.3 ± 0.3	138	1
716.3F.3.2	G37V+G143A	0.28 ± 0.09	79	183	2.0 ± 0.5	53	0.4
716.3F.4.1	G37V+G143A	0.46 ± 0.07	127	296	1.7 ± 0.5	45	0.4
716.3F.4.2	G37V+G143A	0.81 ± 0.09	226	526	2.2 ± 0.2	58	0.5
716.3F.4.3	G37V+G143A	0.66 ± 0.09	185	430	0.7 ± 0.2	19	0.1
716.3F.4.4	G37V+G143A	0.73 ± 0.39	203	473	2.9 ± 0.7	76	0.6
716.3F.4.5	G37V+G143A	0.94 ± 0.33	261	608	2.6 ± 0.3	66	0.5

^aRF_{WT} is the ratio between the EC₅₀ of the resistant isolate and the average of EC₅₀ values from the WT isolates. Each EC₅₀ value is the average of at least three independent assays.

^bRF_{StrR} is the ratio between the EC₅₀ of the resistant isolate and the average of EC₅₀ values from the cytochrome *b* G143A isolates. Each EC₅₀ value is the average of at least three independent assays.

Consequences of the cytochrome *b* G37V change on complex III (or cytochrome *bc*₁ complex) activity and fungal growth rate. To evaluate the impact of cytochrome *b* G37V on enzyme activity, we first measured the activity of complex III without fungicide inhibition (Fig. 1.4.). Activity was assessed spectrophotometrically by monitoring cytochrome *c* reduction. It was normalized by the independent enzyme activity of complex IV, as complex III concentrations might vary among the different mitochondrial preparations. The cytochrome

b G37V change reduced complex III activity in the mutant strains; for IPO323-derived mutants activity was reduced by approximately 50%, and for mutants derived from the strobilurin-resistant parent 37-16 activity was reduced by about 30%. The cytochrome *b* G143A change also appeared to cause a slight (20 %) decrease in complex III activity (Fig. 1.4.) based on comparison of activity for IPO323 and 37-16, however the difference is within the range of variability for this type of experiment.

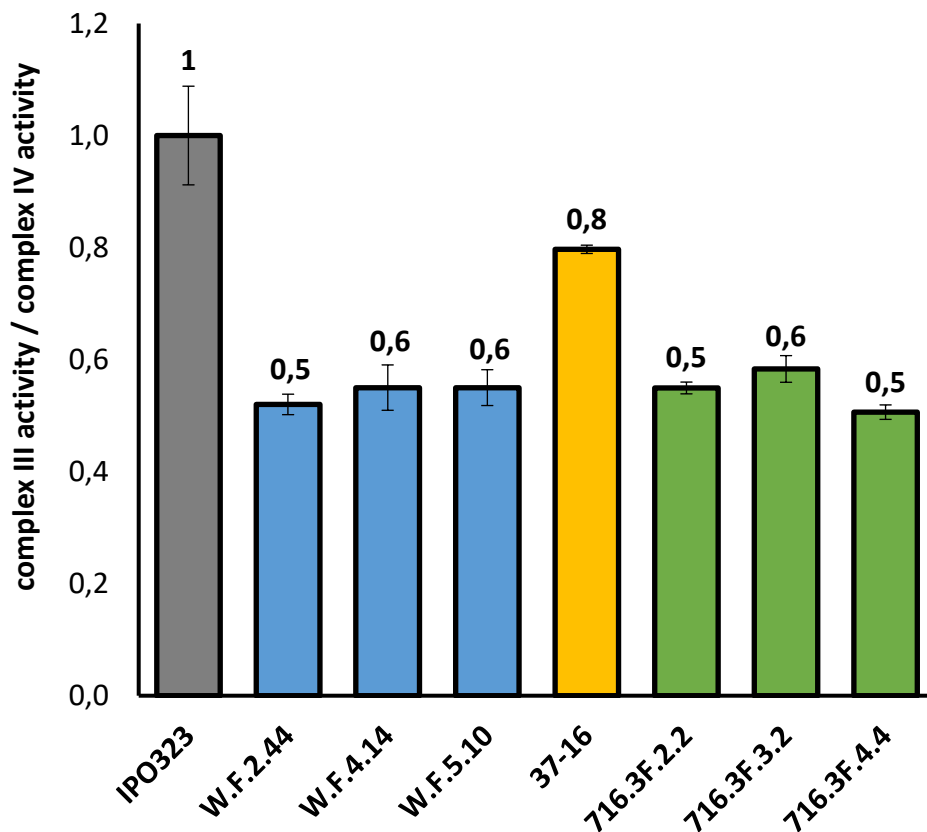


Figure 1.4. Complex III activity of different *Z. tritici* isolates. For each mitochondrial sample, complex III activity was measured as the rate of cytochrome *c* reduction, and normalized by complex IV activity. Each assay was repeated at least three times, and the values averaged. Values were normalized to a constant ratio of 1 for IPO323.

Although complex III activity was impaired by the cytochrome *b* G37V change, the growth rate in microtiter plates of fenpicoxamid-resistant mutants was similar relative to their ancestral strains when measured over 60 hours (Fig. 1.5.).

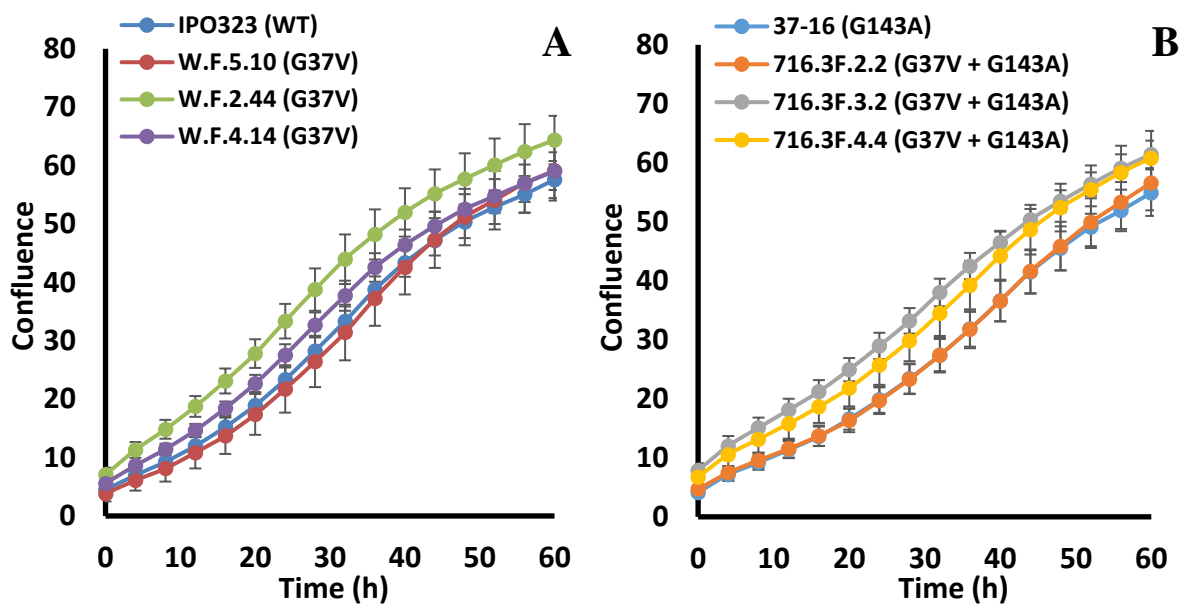


Figure 1.5. Growth of cytochrome *b* G37V mutants compared to their ancestral strains. A. Growth of cytochrome *b* G37V single mutants compared to IPO323. **B.** Growth of cytochrome *b* G37V+G143A double mutants compared to the cytochrome *b* G143A strain 37-16. The maximum growth rates estimated after using a logistic regression model were not significantly different between IPO323 and the single mutants. For the double mutants, the maximum growth rate of 716.3F.3.2 was not significantly different from that of 37-16, and the other two double mutants showed a very slight apparent increase in growth rate. However, growth rates of the 3 mutants were not statistically different from each other, and clearly any differences relative to 37-16 were small.

To confirm that the G37V modification of cytochrome *b* conferred resistance by reducing enzyme sensitivity to UK-2A, I_{50} values were determined using crude mitochondrial extracts from the parent and mutant strains (Table 1.3.). RFs based on I_{50} values for UK-2A ranged from 81 to 171 for the cytochrome *b* G37V mutants, relative to their ancestral IPO323 strain. In contrast, RFs for cytochrome *b* G37V+G143A mutants ranged from 9 to 15 when compared to IPO323 and from 16 to 26 when compared to their cytochrome *b* G143A parent strain 37-16. Therefore, double mutant enzymes were about eleven times more sensitive to UK-2A than the enzymes of cytochrome *b* G37V single mutants. Complex III of the cytochrome *b* G143A parent strain 37-16 was also 40% more susceptible to UK-2A than the enzyme from IPO323.

Regarding sensitivity to azoxystrobin, complex III from the cytochrome *b* G37V single mutants was 40-50% more susceptible than the enzyme from their parent strain. Enzyme RFs for strains with the cytochrome *b* G143A change could not be precisely determined because the I_{50} values were above the azoxystrobin solubility limit in the aqueous assay buffer. Therefore, the precise

impact of the additional cytochrome *b* G37V change on sensitivity to azoxystrobin could not be assessed in the cytochrome *b* G143A background.

Table 1.3. Impact of substitutions on complex III sensitivity to UK-2A and azoxystrobin.

Isolate	Cytochrome <i>b</i>	UK-2A			Azoxystrobin	
		I ₅₀ (μM)	RF ^a	RF ^b	I ₅₀ (μM)	RF ^a
IPO323	WT	0.012 ± 0.004	-	1.7	0.113 ± 0.003	-
W.F.2.44	G37V	2.1 ± 0.5	171	290	0.090 ± 0.004	0.5
W.F.4.14	G37V	0.968 ± 0.005	81	136	0.10 ± 0.03	0.6
W.F.5.10	G37V	1.8 ± 0.1	151	256	0.10 ± 0.01	0.6
37-16	G143A	0.0071 ± 0.0004	0.6	-	> 15	> 100
716.3F.2.2	G37V+G143A	0.18 ± 0.01	15	26	> 15	> 100
716.3F.3.2	G37V+G143A	0.114 ± 0.001	9	16	> 15	> 100
716.3F.4.4	G37V+G143A	0.140 ± 0.001	12	20	> 15	> 100

^aRF is the ratio between the I₅₀ value of the resistant mutant and the I₅₀ value of the WT IPO323 strain.

^bRF is the ratio between the I₅₀ value of the resistant mutant and the I₅₀ value of the cytochrome *b* G143A parent strain 37-16.

Effect of the cytochrome *b* G37V change on binding of UK-2A at the cytochrome *b* Q_i site. A *Z. tritici* homology model was built based on the crystal structure of the *S. cerevisiae* cytochrome *bc*₁ complex. Docking of UK-2A in the *Z. tritici* Q_i site showed the importance of cytochrome *b* residue D230 (D229 in *S. cerevisiae*, Fig. 1.6.) to stabilize the binding pose, as well as the proximity of G37 to the exocyclic methyl group and the ester tail on the bislactone ring of UK-2A. Figure 6 shows how the substitution of G37 by a valine, with its apolar isopropyl side chain, approximately halves the distance between UK-2A and residue 37.

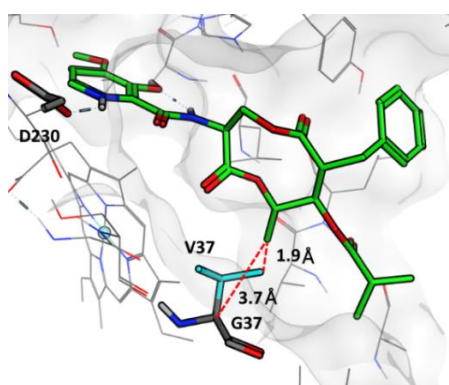


Figure 1.6. Binding of UK-2A at the Q_i site of *Z. tritici* cytochrome *b*. Consequences of the cytochrome *b* G37V substitution are shown by the reduction of the distance (red dotted lines) between the amino acid in position 37 and the UK-2A exocyclic methyl group.

DISCUSSION.

Fenpicoxamid is a new fungicide, developed to control *Z. tritici*, the causal agent of wheat STB. It inhibits the cytochrome *bc*₁ complex by binding to the Q_i site, which represents a new mode of action to control *Z. tritici*. Predicting adaptation to antifungal compounds in fungal populations represents a challenge for evolutionary biology, with practical implications in agriculture and medicine for the management of pathogens. The resistance of *Z. tritici* to fenpicoxamid constitutes a relevant model to study these questions, as outlined in the introduction.

We used directed evolution to select *Z. tritici* isolates resistant to fenpicoxamid, as successfully used for other modes of action (Hawkins and Fraaije, 2016). Evolved isolates displaying a stable phenotype all harbored the cytochrome *b* G37V change in the Q_i site of cytochrome *b*. This change led to fenpicoxamid resistance at both the whole cell and enzyme levels. However, the cytochrome *b* G37V change reduced the activity of complex III by 50%, despite no measurable impact on *in vitro* growth rate. This change also increased the susceptibility of resistant isolates to Q_o site inhibitors. Conversely, the G143A change at the Q_o site of cytochrome *b* increased sensitivity towards fenpicoxamid. In addition, RFs towards QoIs and QiIs in the cytochrome *b* G37V+G143A double mutants were on average lower than for single mutants. These findings may be related in some way to the smaller proportion of cytochrome *b* G37V isolates selected in cytochrome *b* G143A backgrounds, as compared to the WT isolate. However, a larger set of directed evolution data from experiments using multiple ancestral strains with WT, G143A-cytochrome *b* and MDR backgrounds would be required to test rigorously the hypothesis that cytochrome *b* G37V mutants may be selected less readily in a cytochrome *b* G143A background, and further explore the potential link to observed negative cross-resistance between QoIs and QiIs. Altogether, directed evolution suggests that target alteration due to the cytochrome *b* G37V change is a likely resistance mechanism to emerge in *Z. tritici* populations under fenpicoxamid selection pressure.

Directed evolution as a tool to predict resistance selection. Among different evolutionary pathways which could allow adaptation to fenpicoxamid in *Z. tritici*, the most viable should be determined by the relative fitness of selected mutants, *i.e.* their capacity to outcompete other strains in the selecting environment. In our experiments, directed evolution was used to mimic and accelerate the processes of mutation and natural selection in naive ancestral strains.

Resistant isolates were selected as early as the second cycle of the experiment, confirming the utility of this approach. Our protocol did not use a mutagenic agent to make directed evolution as realistic as possible in simulating natural selection. Natural selection depends on random genetic change, and even the most beneficial mutations can be lost due to genetic drift, especially in small populations subjected to environmental factors which restrict multiplication and spread. A similar situation can be considered to occur in experimental evolution experiments due to the small number of cells transferred in each cycle. This may explain why not all lines derived from the same ancestral isolate led to resistance. We also noticed variations according to the ancestral isolate, possibly due to epistasis and/or insufficient fitness. For example, stable resistance was never selected in MDR ancestors, and found less often in cytochrome *b* G143A strains. In the latter case, this may reflect a stronger fitness penalty for isolates displaying alterations in both the Q_i and Q_o sites of cytochrome *b*. The ability of epistatic interactions between mutations in OXPHOS complexes to increase fitness penalties has also been described in *B. cinerea* field isolates which display target site based resistance to both QoIs and SDHIs (Veloukas *et al.*, 2014). Most striking in our study was the repeated selection of the cytochrome *b* G37V change, rather than other changes in cytochrome *b*, in multiple independent lines and different genetic backgrounds, suggesting an optimal trade-off between resistance and fitness penalty for this substitution. In our experiments, additional mutations may have been selected in some isolates as reflected in the variability of RFs observed for both the enzyme activity (81 to 171 and 9 to 15 for cytochrome *b* G37V and G37V+G143A, respectively) and *in vitro* growth (222 to 451 and 79 to 356). Cytochrome *b* G37V was selected much more readily in lines exposed to the MIC as selection pressure, as compared to lines exposed to 25MIC. This is consistent with evidence from multiple organisms that sub-lethal doses enable mutations to emerge more readily (Amaradasa and Everhart, 2016; Andersson and Hughes, 2014; Busi and Powles, 2009; Rix and Cutler, 2018). Also, this highlights the importance of fungicide concentration in the experimental evolution approach.

Target site resistance as the most probable resistance mechanism. We proposed four potential resistance mechanisms which might affect susceptibility to fenpicoxamid in *Z. tritici*. Reduced conversion of fenpicoxamid to UK-2A could be envisaged as a potential resistance mechanisms. A default in metabolic activation is a known resistance mechanism for the human antifungal drug 5-flucytosine (Vermees *et al.*, 2000), and is associated with natural insensitivity of some *Botrytis cinerea* strains to cymoxanil (Tellier *et al.*, 2009). However, the scarcity of

marketed active ingredients requiring activation, together with poor exploration, may explain why this mechanism is barely described. Resistance due to a default in activation was not detected in our experiments, as shown by the positive cross-resistance between fenpicoxamid and UK-2A in isolated mutants. By analogy to other masked ester molecules, the activation mechanism for fenpicoxamid is expected to involve carboxylesterase enzymes (Tian *et al.*, 2012). Carboxylesterase(s) mediating the hydrolysis of fenpicoxamid into UK-2A have not been characterized. However, the ubiquitous occurrence of these enzymes and the fact that activation also operates *in planta* (Owen *et al.*, 2017), suggests that this resistance mechanism is not the most likely to affect fenpicoxamid susceptibility.

A second potential resistance mechanism involves activation of the AOX-mediated alternative respiratory pathway. Some weakly resistant isolates derived from MDR ancestral strains displayed a slightly reduced growth on fenpicoxamid in the droplet test in the presence of the AOX inhibitor propyl gallate, suggesting that in some strains AOX overexpression could lead to a slight decrease in susceptibility to QiIs, especially if combined with MDR. Sequencing of the *CYTb* gene in these strains did not reveal any mutation, and expression levels were not explored to validate AOX involvement. AOX expression should affect QiI fungicides as well as QoIs. In this respect, it is of interest that some QoI-resistant isolates from Ireland displayed lower RFs in the presence of SHAM, suggesting that AOX expression levels may vary among individuals and could play a role in resistance to strobilurin fungicides (Kildea *et al.*, 2019), although this effect is clearly minor compared to the impact of the cytochrome *b* G143A change. In addition, reliance on AOX is associated with lower energy production which may be insufficient to satisfy a high energy requirement during early stages of infection in *Z. tritici* (Miguez *et al.*, 2004). Altogether, these findings suggest that AOX overexpression is unlikely to be a major resistance mechanism for fenpicoxamid in *Z. tritici* field populations.

Enhanced efflux, or multidrug resistance, is a general mechanism associated with positive cross-resistance between DMIs, QoIs and SDHIs (Leroux and Walker, 2011). *In vitro* sensitivity to fenpicoxamid was reduced slightly in MDR field isolates (Table 1.1.), and RFs for fenpicoxamid were similar to those for QoIs and SDHIs (RFs 2-15) (Leroux and Walker, 2011; Omrane *et al.*, 2015, 2017). In our experiments, MDR was not detected in evolved mutants. A possible explanation is that the selection pressure was restricted to fenpicoxamid since MDR is selected more readily in field populations sprayed with mixtures of fungicides with different modes of action (Garnault *et al.*, unpublished). The frequency of MDR has increased each year in French populations since its first detection in 2008, and is generalized in

some locations (Garnault *et al.*, 2019). The effect of enhanced efflux alone on fenpicoxamid sensitivity is likely to be small. Nevertheless, it may insidiously contribute to increase RFs when combined with other resistance mechanisms, as it is largely installed in field populations and because sexual reproduction occurs multiple times per season in *Z. tritici* (Zhan *et al.*, 2003).

Surprisingly, directed evolution also led to the selection of unexpected mechanisms associated with fenpicoxamid resistance. A high number of isolates, initially characterized as resistant, rapidly lost their resistance to fenpicoxamid when subcultured without fungicide. This phenomenon has already been described for QoI-resistant *Z. tritici* field isolates (Miguez *et al.*, 2004). Miguez and colleagues isolated 46 QoI-resistant isolates growing on high azoxystrobin concentrations. However, only six isolates were still able to grow on azoxystrobin-amended medium after being subcultured on fungicide-free medium. This transient resistant state might correspond to “tolerance”, a phenomenon regularly observed in human pathogenic yeasts (reviewed in Berman and Krysan, 2020). Tolerance refers to a state in which susceptible isolates become able to grow in the presence of high antifungal concentrations, due to the modification of several metabolic pathways. Although it is a well-studied phenomenon in human pathogenic fungi, tolerance has been largely ignored in resistance studies concerning agricultural fungicides. A second hypothesis could be the existence of an unstable heteroplasmic state for mitochondrial mutations in *Z. tritici*. Heteroplasmy is defined as a cellular state where at least two different mitochondrial populations (or mitotypes) coexist in the same cell, and may be transient (Mendoza *et al.*, 2020). In the case of lowered enzyme activity in resistant mitochondria, the parental mitotype would be preferentially selected, and fungal cells would rapidly return to homoplasmy in the absence of selection pressure. Whatever the underlying mechanism, these observations should prompt further investigation of transient resistant states induced by agricultural fungicides. It could be argued that these metabolic states may allow isolates to survive longer when exposed to QoI fungicides, therefore increasing the risk of selecting resistance mutations. On the other hand, if stable resistance requires further selection to achieve a homoplasmic state, the development of resistance may be delayed.

Alteration of the target site is the most common resistance mechanism in fungi (Lucas *et al.*, 2015), and is therefore likely to be of greatest importance for fenpicoxamid resistance. Indeed, the G37V change in cytochrome *b* was repeatedly selected in independent lines and genetic backgrounds in our directed evolution studies and was associated with high RFs. In addition, mutations at this locus have conferred resistance to diverse QoIs in multiple organisms, as

reviewed in Mounkoro *et al.*, 2019. As examples, the cytochrome *b* G37A and G37V changes have been reported in lines of the human parasite *P. falciparum* resistant to the macrocyclic Q_is ML238 and BRD6923, and to antimycin A. The cytochrome *b* G37C and G37V changes were associated with antimycin A resistance in the model organism *S. cerevisiae*, cytochrome *b* G37D and G37S resulted in ilicicolin H resistance, and the cytochrome *b* G37C change conferred resistance to UK-2A. Moreover, the cytochrome *b* G37V change was also selected in *Z. tritici*, after repeated exposure to high concentrations of antimycin A (Fehr *et al.*, 2016). Parallel evolution is frequent in fungicide resistance (Hawkins *et al.*, 2019). A review of literature suggests that lab mutations associated with high RFs, and those found in multiple species, are more likely to be reported in the field (Hawkins and Fraaije, 2016). Altogether, our findings make the selection of the cytochrome *b* G37V change a realistic and most probable evolutionary scenario for *Z. tritici* populations exposed to fenpicoxamid selection pressure in the field, and suggest this could occur in both QoI-sensitive and resistant populations. This conclusion is also supported by the lack of an obvious fitness penalty on *in vitro* growth as measured in cytochrome *b* G37V single and G37V+G143A double mutants. Nevertheless, it will be important to assess fitness *in planta* during the infection cycle.

The cytochrome *b* G37V change influences complex III activity. We have demonstrated that the cytochrome *b* G37V change is responsible for at least a 100-fold decrease in enzyme sensitivity to fenpicoxamid in single mutants, and a 20-fold decrease in double mutants. According to docking calculations, the molecular distance in the resistant cytochrome *b* between valine 37 and fenpicoxamid is less than 2 Å. This distance is barely longer than a C-C covalent bond (1.54 Å), suggesting that steric hindrance is likely. The mutation was also associated with a decrease in complex III activity, likely due to ubiquinone binding impairment, or to a negative impact of the mutation on electron transfer. Indeed, some Q_i site changes have been shown to impact negatively the electron transfer between the Q_o and Q_i sites and/or between the Q_o site and the Rieske iron-sulphur protein, hence reducing complex III activity (Cooley *et al.*, 2006). A similar penalty has been described for the S34L substitution in *S. cerevisiae*, a mutation that triggers ametoctradin resistance in *P. viticola* (Mounkoro *et al.*, 2019). The S34L change confers high resistance levels, but also a 2-fold decrease in enzyme activity in yeast. In *Z. tritici*, the 2-fold decrease in enzyme activity triggered by the cytochrome *b* G37V change had no detectable impact on *in vitro* growth, suggesting that complex III activity is not limiting in these conditions. A similar observation was made for SDH laboratory mutants which displayed decreased enzyme activity of up to 90% yet showed no visible growth defect

in vitro (Scalliet *et al.*, 2012). Some of the same mutations in these laboratory mutants were also identified in field isolates (Rehfus *et al.*, 2018).

RFs for UK-2A against cytochrome *b* G37V strains were somewhat variable. Such variations may be explained by the presence of additional mutations with minor effects, which were likely acquired during experimental evolution. Differences in resistance levels to QiIs may also be due to heteroplasmy. This phenomenon has been reported for QoI resistance in various species like *Botrytis cinerea* (Hashimoto *et al.*, 2015), *Venturia inaequalis* (Villani and Cox, 2014), or *Podosphaera xanthii* (Vielba-Fernández *et al.*, 2018). The existence of a stable heteroplasmic state in *Z. tritici* has never been described but cannot be excluded.

We have also observed an interaction between the G37V and G143A changes, in the Q_i and Q_o binding sites of cytochrome *b*, respectively. The selection of cytochrome *b* G37V in parental strains containing the cytochrome *b* G143A change occurred less frequently than in the WT IPO323 strain, suggesting that the cytochrome *b* G143A change could have reduced cytochrome *b* G37V selection to some extent. Moreover, we highlighted the negative cross-resistance between QoIs and QiIs in single mutants resistant to one mode of action. An increased sensitivity to UK-2A was also apparent at the enzyme level in strains containing the cytochrome *b* G143A change (Table 1.3.). The increased sensitivity may be a consequence of reduced enzyme efficiency associated with the cytochrome *b* G37V and G143A changes. Alternatively, a mutation at one site may increase inhibitor binding at the opposite site. The slightly lower RFs to both QoIs and QiIs in cytochrome *b* G37V+G143A double mutants also point to some form of interaction between the Q_o and Q_i sites.

Consequences for fenpicoxamid use in the field. To our knowledge, this is the first study using directed evolution to predict resistance to QiIs. This confirms the interest of this approach to assess adaptation. Although laboratory mutant isolation studies cannot predict with certainty what will happen in the field, our results highlight the cytochrome *b* G37V change as a quite likely resistance mechanism since it was the only change detected and is a common locus for QiI resistance mutations in other organisms. The thorough characterization of cytochrome *b* G37V mutants showed that complex III activity was impaired in these isolates. Although their *in vitro* growth rate was similar to that of their ancestral strains, pathogenicity or fitness in the field could be affected, especially since some stages of the infection cycle require high energy production (Wood and Hollomon, 2003). In the case of QiI fungicides used to control oomycete diseases, the S34L change in the Q_i site of *P. viticola* causes resistance to ametoctradin. In a *S. cerevisiae* strain transformed with the *P. viticola* cytochrome *b* allele, this change produced a

similar decrease (2-fold) in enzyme activity (Moukoro *et al.*, 2019) to that observed for the cytochrome *b* G37V change in *Z. tritici*. The cytochrome *b* S34L change remains at low frequency in French *P. viticola* populations, suggesting a fitness cost under field conditions. In contrast, the cytochrome *b* E203-VE-V204 insertion in the Q_i site of the same oomycete, associated with resistance to cyazofamid and amisulbrom, are thriving in French vineyards, suggesting a lower, or no fitness cost (Fontaine *et al.*, 2019). Therefore, further risk assessment of the cytochrome *b* G37V mutants requires *in planta* studies.

The prediction of resistance in advance of its emergence in the field can enable sustainable management of a new mode of action. Molecular tools can be developed to allow early detection of anticipated mutants in real time (R4P-Network, 2016). This approach could allow adjustments in usage of fenpicoxamid within a fine spatio-temporal scale. Detailed knowledge of the risk of resistance may also allow the smart and informed deployment of anti-resistance strategies, including mixtures, alternation or mosaic.

EXPERIMENTAL PROCEDURES.

Isolates and media.

Isolates used in this study were either collected in the field, or generated in the laboratory by experimental evolution or sexual reproduction between field isolates. Their genotypic variations in *MFS1* and cytochrome *b* are listed in Table 1.4.

Table 1.4. Origins and phenotypes/genotypes of *Z. tritici* isolates used in this study.

Isolate	Cytochrome <i>b</i>	<i>MFS1</i>	Origin	Reference
IPO323	WT	WT	Reference isolate	Goodwin <i>et al.</i> , 2011
14-H-K3	G143A	WT	Field isolate	Lab collection
09-ASA-3a-PZ	G143A	MDR ^{Type I}	Field isolate	Leroux and Walker, 2011
09-CB1	G143A	MDR ^{Type I}	Field isolate	Leroux and Walker, 2011
11183	WT	MDR ^{Type I}	Progeny isolate, lab cross	Lab collection
35C1	G143A	WT	Progeny isolate, lab cross	Lab collection
35C3	G143A	MDR ^{Type I}	Progeny isolate, lab cross	Lab collection
35C4	G143A	MDR ^{Type I}	Progeny isolate, lab cross	Lab collection
35D16bis	G143A	WT	Progeny isolate, lab cross	Lab collection
35F6	G143A	WT	Progeny isolate, lab cross	Lab collection
35F22	WT	WT	Progeny isolate, lab cross	Lab collection
35G5	G143A	WT	Progeny isolate, lab cross	Lab collection
37-15	G143A	WT	Progeny isolate, lab cross	Lab collection

Isolate	Cytochrome <i>b</i>	<i>MFSI</i>	Origin	Reference
37-16	G143A	WT	Progeny isolate, lab cross	Lab collection
37-19	G143A	WT	Progeny isolate, lab cross	Lab collection
37-22	G143A	WT	Progeny isolate, lab cross	Lab collection
37-41	WT	MDR ^{Type I}	Progeny isolate, lab cross	Lab collection
37-59	G143A	MDR ^{Type I}	Progeny isolate, lab cross	Lab collection
W.F.2.44	G37V	WT	Experimental evolution	This study
W.F.4.14	G37V	WT	Experimental evolution	This study
W.F.5.10	G37V	WT	Experimental evolution	This study
716.3F.2.2	G37V+G143A	WT	Experimental evolution	This study
716.3F.2.3	G37V+G143A	WT	Experimental evolution	This study
716.3F.2.4	G37V+G143A	WT	Experimental evolution	This study
716.3F.3.1	G37V+G143A	WT	Experimental evolution	This study
716.3F.3.2	G37V+G143A	WT	Experimental evolution	This study
716.3F.4.1	G37V+G143A	WT	Experimental evolution	This study
716.3F.4.2	G37V+G143A	WT	Experimental evolution	This study
716.3F.4.3	G37V+G143A	WT	Experimental evolution	This study
716.3F.4.4	G37V+G143A	WT	Experimental evolution	This study
716.3F.4.5	G37V+G143A	WT	Experimental evolution	This study

All isolates were stored in 25% glycerol at -80°C, and grown in liquid or on solid YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar if needed). Apical germ-tube length measurement establishing dose-response curves for tolinaftate sensitivity assays were performed on PG solid medium (10 g/L glucose, 2 g/L K₂HPO₄, 2 g/L KH₂PO₄ and 12.5 g/L agar).

Fungicide sensitivity assays.

Germ-tube elongation assay. *Z. tritici* isolates were grown at 18°C on YPD agar plates for 72h under light. Spore suspensions in sterile water were prepared from 3-day old cultures, and adjusted to 2.10⁵ spores/mL. 300 µL of spore suspensions were plated on PG medium containing increasing tolinaftate concentrations in ethanol, or control plates without fungicide containing 0.5 µg/mL final ethanol concentration. Plates were incubated for 48h in the dark at 17°C. After incubation, the length of apical germ-tubes from 10 spores was measured under the microscope using a micrometer (magnification x200). Tests were replicated independently three times.

Fungitoxicity assay in microtiter plates. *Z. tritici* isolates were grown at 18°C on YPD agar plates for 72h under light. Cells were then suspended in liquid YPD at 10⁵ spores/mL. Wells

were filled with 1 μL of a concentrated fungicide solution in DMSO at 200 times the desired final concentration and 100 μL of YPD, then inoculated with 100 μL of the spore suspension. Final fungicide concentrations ranged between 0.0001 and 10 $\mu\text{g}/\text{mL}$ for UK-2A and azoxystrobin, using a three-fold dilution series. Plates were incubated at 18°C for 72h. Growth was assessed in a NepheloStar plate reader, and EC_{50} values were determined by non-linear regression (curve-fit) using GraphPad Prism (GraphPad Software, La Jolla, CA, United States). EC_{50} values were the average of three technical replicates per assay and each assay was repeated at least twice.

Selection and characterization of fenpicoxamid-resistant *Z. tritici* isolates.

Experimental evolution. Ancestral isolates were either susceptible to QoIs and QiIs (IPO323), resistant to QoIs and susceptible to QiIs (35C1, 35D16bis, 35G5, 37-16, 37-19 and 37-22), MDR isolates susceptible to QoIs and QiIs (11183 and 37-41) or MDR isolates resistant to QoIs and susceptible to QiIs (35C4 and 37-59). Each experiment encompassed 3 or 4 independent lines for each ancestral isolate. 10^7 spores were incubated at 17°C in 25 mL of liquid YPD containing 100 $\mu\text{g}/\text{mL}$ propyl gallate and fenpicoxamid with shaking at 150 rpm in the dark for 7 days. Fenpicoxamid concentrations used were the MIC or 25MIC for each isolate, as established in preliminary experiments using the same experimental conditions. New selection cycles were initiated by transferring 0.5% of cells from the previous cycle to fresh medium. A concentration of 10^7 cells at the start of each cycle was achieved by adding cells from an untreated control cell line. At the end of each cycle, 150 μL of each population were plated on fenpicoxamid-amended YPD at the selecting concentration, to detect resistant isolates. Experiments were stopped after eight selection cycles. Isolates were subcultured twice on fenpicoxamid-amended YPD before storage.

Characterization of fenpicoxamid-resistant isolates. Resistant isolates were grown in 25 mL of liquid YPD at 17°C with shaking at 150 rpm, in the dark for 3 days. OD at 560 nm was measured for each culture to standardize spore concentration. Spore suspensions were plated using three 10-fold serial dilutions on YPD agar with selecting compounds: 2 $\mu\text{g}/\text{mL}$ fenpicoxamid, 0.5 $\mu\text{g}/\text{mL}$ UK-2A, 2 $\mu\text{g}/\text{mL}$ tolnaftate, 2.5 $\mu\text{g}/\text{mL}$ azoxystrobin, 100 $\mu\text{g}/\text{mL}$ propyl gallate, 100 $\mu\text{g}/\text{mL}$ SHAM, and mixtures of fenpicoxamid and propyl gallate, or fenpicoxamid and SHAM, at the same concentrations. Plates were incubated at 17°C in the dark for 7 days before scoring. Scores ranged from 0 to 5 depending on growth intensity at each concentration.

Molecular characterization of resistant isolates. After total DNA extraction, *CYTb* PCR was performed using the primers Zt_Cytb_2_F (5'CCTGACTGGTATCATATTGTGT3') and Zt_Cytb_1_R (5'TATATTACTAGGTTATTTTCGTG3'). PCR conditions were an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 20 s, 56°C for 20 s, 72°C for 2 min, and a final extension of 72°C for 5 min, giving a 1572 kb amplicon. Sequencing reactions with the same primers were performed by Eurofins genomics (Ebersberg, Germany).

Impact of target site mutations on complex III.

Mitochondria extraction. *Z. tritici* mitochondria were prepared according to a protocol adapted from Lemaire and Dujardin, 2008 and Scalliet *et al.*, 2012. Briefly, 10⁷ spores were incubated in 1 L of liquid YPD, at 17°C, in the dark with shaking at 150 rpm for 3 days. After centrifugation, the pellet was rinsed with sterile water, and immersed in liquid nitrogen before grinding. The resulting powder was resuspended in buffer A (0.7 M sorbitol, 50 mM Tris HCl pH 7.5, 0.2 mM EDTA pH 7.5). Mitochondria were prepared by differential centrifugation, and stored in buffer B (buffer A with 0.5% BSA) at -80°C.

Complex III activity. Decylubiquinol-cytochrome *c* reductase activities were determined at room temperature by measuring the reduction of cytochrome *c* (final concentration of 20 µM) at 550 nm *versus* 540 nm over a 2-min time-course. Mitochondria (final concentration to have a constant initial rate between 0.4 and 0.5) were added in 1 mL of 10 mM potassium phosphate pH 7, 0.01% (w/v) lauryl-maltoside and 1 mM KCN to inhibit complex IV activity. The reaction was initiated by adding decylubiquinol (final concentration of 40 µM) and the initial cytochrome *c* reduction rates were recorded. The measurements were performed in the presence of increasing concentration of inhibitors. The mid-point inhibition concentrations (I₅₀) were estimated from the inhibitor titration plots. Each measurement was repeated at least twice and independent values were averaged.

Complex IV activity. The activity of complex IV was determined at 25°C by measuring oxygen consumption with an oxygen electrode. Complex IV activity was used to normalize complex III activity in the absence of a reliable method for quantifying cytochrome *bc*₁ complex in *Z. tritici* mitochondrial extracts. Mitochondria were added to 1 mL of 50 mM potassium phosphate pH 7, 10 mM ascorbate and 50 µM N,N,N',N'-tetramethyl-p-phenylenediamine at pH 7. The reaction was initiated by the addition of 50 µM cytochrome *c*. The initial oxygen consumption rates were measured at least 3 times and independent values were averaged.

In vitro kinetic growth assays. *Z. tritici* spores were suspended in liquid YPD at 10^5 spores/mL. 200 μ L of cell suspensions were placed in wells of 96-well microtiter plates and incubated at 18°C for 2h to allow spores to settle on the well surface. Plates were then incubated in a Cytation 3 instrument for 60h. Wells were imaged every 4 hours and confluence was calculated after Z-stacking (4 heights). Curves were modeled using the self-starting logistic model (sslogis) from the R software (The R Foundation for Statistical Computing), by the equation “Growth(Time) = Asym/(1 + exp((xmid – Time)/scal))”. “Asym” a numeric parameter representing the asymptote, “xmid” a numeric parameter representing the x value at the inflection point of the curve and “scal” is a numeric scale parameter on the input axis, here corresponding to the maximum growth rate. Maximum growth rates as Δ confluence/hour were determined. Statistical comparisons between strains were performed using the R package “emmeans”. Values of maximum growth rates and strain comparisons p-values are given in the following tables.

Table 1.5. Maximum growth rates and statistical groups.

Strain	Scal	SD	Group
IPO323	12,3255	0,0988	a
W.F.5.10	12,3451	0,0979	a
W.F.2.44	12,5582	0,0951	a
W.F.4.14	12,3048	0,0974	a
37-16	15,214	0,147	a
716.3F.2.2	16,122	0,152	b
716.3F.3.2	15,616	0,141	ab
716.3F.4.4	15,776	0,143	b

Table 1.6. Strains’ maximum growth rate comparisons and associated p-value.

Contrast	Estimate	SD	t.ratio	p-value
IPO323 - W.F.5.10	-0.0196	0.139	-0.141	0.9990
IPO323 - W.F.2.44	-0.2327	0.137	-1.697	0.3255
IPO323 - W.F.4.14	0.0207	0.139	0.149	0.9988
W.F.5.10 - W.F.2.44	-0.2131	0.137	-1.561	0.4013
W.F.5.10 - W.F.4.14	0.0402	0.138	0.291	0.9914
W.F.2.44 - W.F.4.14	0.2533	0.136	1.861	0.2455
37-16 - 716.3F.2.2	-0.909	0.211	-4.299	0.0001
37-16 - 716.3F.3.2	-0.402	0.203	-1.977	0.1973
37-16 - 716.3F.4.4	-0.562	0.204	-2.747	0.0309
716.3F.2.2 - 716.3F.3.2	0.507	0.208	2.440	0.0702
716.3F.2.2 - 716.3F.4.4	0.347	0.209	1.663	0.3440
716.3F.3.2 - 716.3F.4.4	-0.160	0.201	-0.796	0.8563

Molecular docking of UK-2A at the Z. tritici Q_i site. The *Z. tritici* homology model was built based on a template of the *S. cerevisiae* cytochrome *bc*₁ complex (PDB: 1EZV) crystal structure (Hunte *et al.*, 2000), which shares a sequence identity of 60% with the *Z. tritici* protein. The model was refined with the Amber14:EHT force field. UK-2A was then docked into the Q_i site of the model using induced fit docking. A total of 30 UK-2A binding poses were generated, from which the most likely binding pose was selected based on the docking energy score and knowledge of target site mutation effects in yeast (Young *et al.*, 2018). All calculations were performed with the MOE software (Chemical Computing Group Inc., Montreal, QC, Canada).

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Chapter 2.

Evaluation of the risk of practical resistance for fenpicoxamid.

1. INTRODUCTION.

In the previous chapter, we have demonstrated through biochemical characterization that the cytochrome *b* Q_i site change G37V is responsible for fenpicoxamid resistance, but also for a decrease in complex III enzyme activity in *Zymoseptoria tritici*. This two-fold decrease had no noticeable impact on *in vitro* growth, showing that complex III activity was not limiting in these conditions. However, the situation in the field might be different. Indeed, *in vitro*, conditions are optimal for fungal growth, whereas the field environment is always fluctuating. Moreover, it has been shown that early stages of plant infection require strong energy production (Wood and Hollomon, 2003), implying that a reduced complex III activity could be deleterious. Such a situation has been described in the oomycete *Plasmopara viticola*, in which the Q_i site change S34L triggers resistance to ametoctradin. Indeed, in a laboratory mutant of *S. cerevisiae* transformed with the *P. viticola* *CYTb* allele, the insertion of the S34L change triggered a 2-fold decrease in complex III activity (Mounkoro *et al.*, 2019). As *P. viticola* resistant isolates bearing the S34L change remain at low frequencies in French populations, it may suggest the existence of a fitness cost expressed under field conditions (Fontaine *et al.*, 2019). On the contrary, examples of fitness cost measured *in vitro* or in the greenhouse but not in the field have been documented in different species (reviewed in Hawkins and Fraaije, 2018). For example, in *Z. tritici*, the fact that cytochrome *b* G143A isolates displaying reduced pathogenicity in greenhouse assays (Hagerty and Mundt, 2016) are generalized in *Z. tritici* European populations (Garnault *et al.*, 2019; Kildea *et al.*, 2019) seems to exclude such fitness penalty linked to the cytochrome *b* G143A change. A similar situation was observed in *Botrytis cinerea* (Veloukas *et al.*, 2014). In this latter fungus, mutations in the *sdhB* gene encoding the subunit B of the succinate dehydrogenase, which conferred resistance to the SDHI boscalid, decreased complex II activity in the laboratory and *in vitro* fitness (Lalève *et al.*, 2014a, 2014b). However, these mutations were found regularly in field surveys, highlighting again a discrepancy between the fitness cost observed *in vitro* and its repercussion under field conditions, especially concerning respiratory inhibitors. Such discrepancies could be explained by the limitations of fitness assessment *in vitro*, the difficulty in estimating the absolute fitness of an isolate, or the existence of compensatory mechanisms in field strains (Introduction, 5.4.). In this context, assessing the risk associated with resistance under conditions as close as possible to those of the field is a challenge of primary importance. Thus, the main question remains: what is the risk for fenpicoxamid efficacy related to the selection of resistance in the field?

One way to evaluate this risk is to study the cytochrome *b* G37V isolates *in planta*, by measuring their fitness on one hand, and evaluating their fenpicoxamid resistance levels on the other hand. Indeed, the overall field resistance risk is characterized by the balance between the fitness of the resistant strains *in planta*, which is mitigated by the cost associated with this resistance, and the level of fungicide resistance with regard to the labeled doses, as represented in Table 2.1.

Table 2.1. Risk of field resistance defined by the balance between the absolute fitness and the level of resistance.

Risk of field resistance			Efficacy loss <i>in planta</i> at labeled dose		
			Low A	Moderate B	High C
Fitness <i>in planta</i> (absolute or in competition)	Reduced compared to WT	1	Low	Moderate	Moderate
	Equal to WT	2	Moderate	Moderate	High
	Improved compared to WT	3	Moderate	High	High

For example, the *P. viticola* isolates bearing the S34L change, display a high level of resistance to ametoctradin in the field but seem to have reduced fitness (Fontaine *et al.*, 2019). Therefore they would be classified in C1, meaning they pose a moderate risk of field resistance to ametoctradin. In the same pathogen, isolates displaying the Q_i site insertion E203-VE-V204, show high levels of resistance towards cyazofamid and amisulbrom and seem to be at least as fit as the WT isolates, as shown by their increasing frequency in populations (Fontaine *et al.*, 2019). Therefore these isolates would be classified into C2 or C3, representing a high level of resistance risk towards these two Q_iIs. In *Z. tritici*, the cytochrome *b* G143A isolates would be classified in C2 regarding strobilurin resistance (Fraaije *et al.*, 2005c; Garnault *et al.*, 2019). Isolates bearing the C-H152R change in the subunit C of the SDH would be classified in C1 for SDHI resistance (Dooley *et al.*, 2016; Scalliet *et al.*, 2012). MDR isolates in A2-A3 for QoI, QiI, SDHI and DMI resistance (Garnault *et al.*, 2019; Leroux and Walker, 2011; Omrane *et al.*, 2015, 2017).

Absolute fitness of a strain is defined as its ability to cause disease year to year and to reproduce, while in competition with other genotypes (Oliver and Hewitt, 2014). Therefore, assessing fitness remains complicated. First, because it is characterized by many life traits (like the latency period, the amount and size of pycnidia, the quantity of pycnidiospores...) that can vary widely. Moreover, any of these traits can be more or less compensated depending on the genotype, making it difficult to compare isolates. As mentioned above, assessing fitness *in vitro* regularly generates discrepancies with fitness *in planta*, while assessing fitness in the plant does not take into account abiotic stresses and some stages of the fungal life cycle such as overwintering or sexual reproduction. Ideally, fitness should be assessed in the field, where the environment is variable, where the host genotypes can vary, where phytopathogenic fungi are competing with various other microorganisms, and where all stages of the fungal life cycle can be challenged (Walker *et al.*, 2017). However, even if it were technically possible to perform such assays in the field, the genetic diversity of field populations would make difficult to attribute the results to one particular genotype. In spite of these issues, assessing fitness remains a prerequisite to evaluate the resistance risk for a given fungicide *in planta*.

The objective of this still ongoing work is to assess the risk represented by the selection of fenpicoxamid resistance in the field. To achieve this goal, we decided to use the cytochrome *b* G37V isolates together with their respective ancestral strains, therefore limiting genetic variability by using near-isogenic strains. This should allow us to relate observed differences to the cytochrome *b* G37V change. In order to assess the risk as accurately as possible, we designed three experiments: two to assess the different aspects of *in planta* fitness, and the third one to assess the impact of resistance on fenpicoxamid efficacy *in planta*. First, we evaluated the pathogenicity of the cytochrome *b* G37V isolates to verify their ability to perform a complete infectious cycle on wheat, in greenhouses, from spore germination to pycnidia production. Pathogenicity is an important feature, as a complete or partial loss of virulence would inform the level of threat posed by the resistant isolates in the field. Second, we carried out a competition assay between the cytochrome *b* G37V mutants and their respective ancestral strains, to assay the ability of the cytochrome *b* G37V isolates to propagate in plant tissues while competing with other genotypes. Finally, we used dose-response curves *in planta* to quantify EC_{50s} and RFs of cytochrome *b* G37V isolates. These results are essential to evaluate the impact of the mutation on fungicide field efficacy, in relation to registered doses. It is commonly accepted that RFs measured *in vitro* in optimal controlled conditions may not necessarily reflect RFs *in planta*. However, a recent study showed that there was actually a

good correlation between both values for QoI and DMI resistance in *Z. tritici* (Blake *et al.*, 2018). Regardless, when associated to high frequencies in field populations, high RFs *in vitro* are usually responsible for field efficacy losses because MIC values of resistant strains *in planta* are usually higher than the recommended doses (Walker A.-S., personal communication).

We intend to assess pathogenicity and competition between IPO323 and the 3 derived cytochrome *b* G37V isolates, and between 37-16 and the three double mutants (Table 2.2).

All experiments conducted in this chapter are being performed at the Corteva research center in Indianapolis, USA, since I have initiated this work during my stay in February 2020. The postdoctoral researcher Michelle Souza Oliveira is performing all *in planta* assays. As designing and running such experiments on plant material is time-consuming, not to mention the closure of laboratories due to the lock down in 2020, the results presented here are only preliminary. The final results are expected for Fall 2021 and will be published in a separate article, or combined with the results from chapter 3.

2. EXPERIMENTAL PROCEDURES.

2.1. Fungal material.

Isolates used in this study were generated in the laboratory by experimental evolution from laboratory strains as described in chapter 1. Their genotypic variations in *CYTb* are listed in Table 2.2.

Table 2.2. Origins and phenotypes/genotypes of *Z. tritici* isolates used in this study.

Isolate	Cytochrome <i>b</i>	Origin	Ancestral strain	Reference
IPO323	WT	Reference isolate	-	Goodwin <i>et al.</i> , 2011
37-16	G143A	Progeny isolate, lab cross	-	Lab collection
W.F.2.44	G37V	Experimental evolution	IPO323	This study, chapter 1
W.F.4.14	G37V	Experimental evolution	IPO323	This study, chapter 1
W.F.5.10	G37V	Experimental evolution	IPO323	This study, chapter 1
716.3F.2.2	G37V+G143A	Experimental evolution	37-16	This study, chapter 1
716.3F.3.2	G37V+G143A	Experimental evolution	37-16	This study, chapter 1
716.3F.4.4	G37V+G143A	Experimental evolution	37-16	This study, chapter 1

2.2. Assessment of *in planta* fitness of the cytochrome *b* G37V *Z. tritici* isolates.

Pathogenicity assay (Fig. 2.1.).

All *Z. tritici* strains listed in table 2.2. were grown on yeast malt agar medium (YMA) (10 g/L dextrose, 5 g/L peptone, 3 g/L malt extract, 3 g/L yeast extract and 20 g/L agar) plates at 18°C under light for four days. Spore suspensions at 10⁶ spore/mL were prepared for each isolate in sterile water and inoculated with a round paintbrush (size 14) over a 7.5 cm length. The inoculation was conducted on 3-week old wheat seedlings of the cultivar Lancer, highly susceptible to most *Z. tritici* isolates and especially to the IPO-323 reference isolate. In particular, we used the first completely unrolled leaf for inoculation, excluding cotyledonary leaves. The inoculation was repeated on twenty-five wheat leaves for each isolate. Inoculated plants were placed in a dew chamber at 20°C, 100% humidity for 72 h, of which 24 h in the dark followed by 48 h with a 16 h photoperiod. Plants were then moved to a greenhouse kept at 18°C, 70% humidity and with a 16 h photoperiod. Disease severity was assessed as the percentage of leaf area covered by symptoms (chlorotic and necrotic) from 14 to 28 days post-infection (dpi). Experiment was repeated twice.

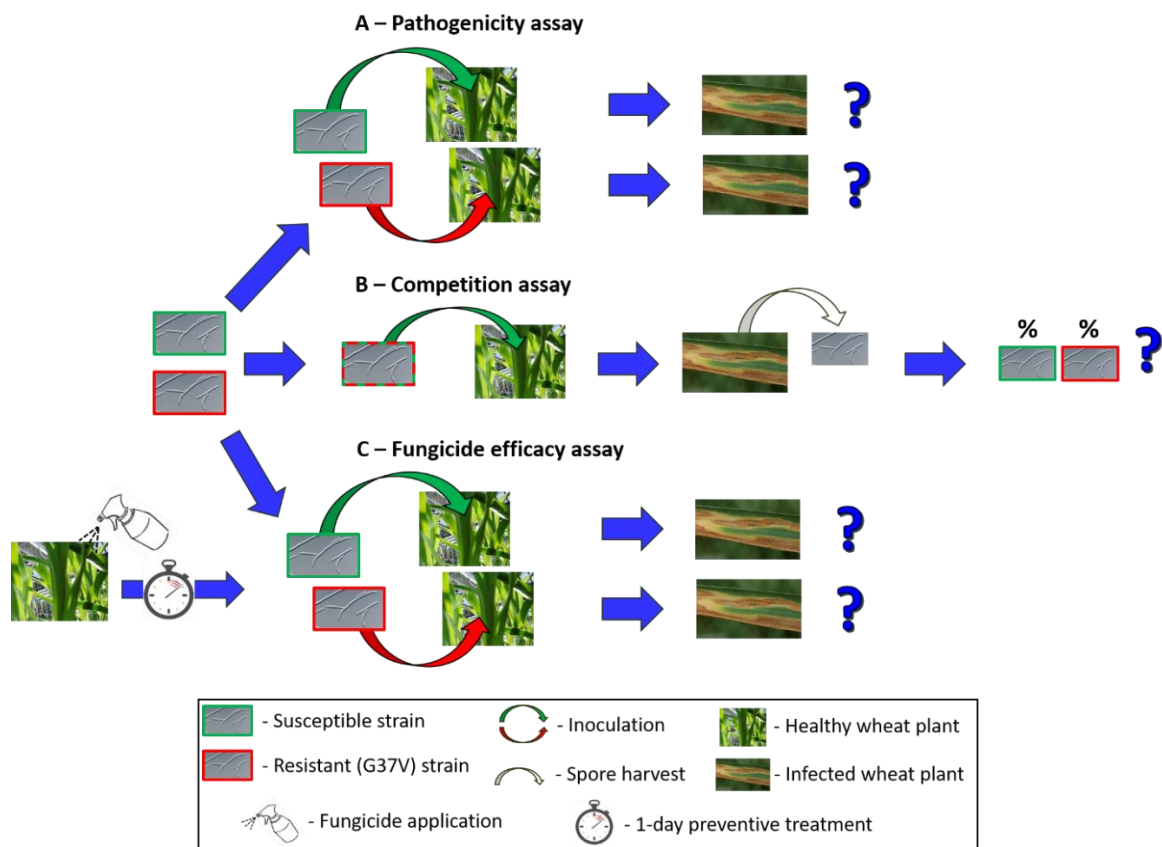


Figure 2.1. Experiments set up to assess the fitness *in planta* of cytochrome *b* G37V mutant isolates.

Competition assay (Fig. 2.1.).

Zymoseptoria tritici strains 37-16 and 716.3F.4.4 were grown on YMA plates at 18°C under continuous light for four days. Individual spore suspensions were prepared in sterile distilled water for each strain and used to make mixed spore suspensions at 10^6 spore/mL, with a 1:1 ratio of each isolate. The wheat cultivar used was Lancer. Three-week old wheat plants were inoculated with spore mixtures with an air assisted paint gun at 15 psi, and incubated as described previously. Spores from the same strain modality were harvested at 21 dpi (*i.e.* when pycnidia were visible) by dipping together all symptomatic leaves in 200 mL sterile water for 40 minutes. The concentration of this spore suspension was counted under microscope with the aid of a hemocytometer and adjusted to 10^6 spores/mL. It was then used to inoculate new seedlings, as described previously, and start a new infection cycle. Each experiment lasted for three infection cycles.

Three milliliters of the remaining spore suspensions were transferred for DNA extraction into three lysing matrix tubes A and centrifuged at 18,000 g for 5 min. Supernatant was discarded and 400 μ L of DNAzol were added before grinding spore pellets in a Fast-Prep 24 device (Mp biomedical, USA) at 4.0 m/s for 40 s. Tubes were incubated at room temperature (RT) for 5 min. Four hundred microliters of chloroform were added, and tubes were shaken by hand before incubation at room temperature for 5 min. Tubes were centrifuged at 18,000 g for 10 min, and the aqueous phase was collected. Three hundred microliters of 100% ethanol were added, before mixing by carefully inverting the tubes and incubating again at room temperature for 5 min. Tubes were then centrifuged at 5,000 g for 5 minutes. Pellets were rinsed with 300 μ L of a DNAzol/ethanol (4:3) mix before centrifugation at 5000 g for 5 min. Pellets were rinsed again with 300 μ L of 75% ethanol before centrifugation at 5000 g for 5 min. Supernatants were discarded and tubes were air-dried for 5 min. Pellets were resuspended in 50 μ L of ultra-pure water. The quality of the DNA extracted was evaluated using a NanoDrop 2000 (Thermo Fisher Scientific) and the proportion of mutants and wild-type DNA was assessed by digital droplet PCR (ddPCR) (not shown). ddPCR allows an absolute quantification of the nucleic acids independent of the PCR efficiency. Its principle is to distribute the DNA in several thousand droplets (10.000 to 20.000) producing as many independent PCR reactions. The detection of the PCR reaction is done at the end, based on fluorescence detection. The results are analyzed by counting the number of positive droplets in relation to their total number, and then calculating a number of copies/ μ L of DNA detected. The ddPCR method for cytochrome *b* G37V quantification was developed in 2020 by Corteva teams from Johnston, USA.

Competition between a cytochrome *b* G37V single mutant and a G37V+G143A double mutant requires quantification of the cytochrome *b* G143A allele instead of the G37V allele.

2.3. Assessment of the loss of fungicide efficacy *in planta*.

In planta fungicide efficacy assay (Fig. 2.1.).

The plants were sprayed with Inatreq™ (commercial formulation of fenpicoxamid) at increasing concentrations (Table 2.3.), using four pots of five plants per treatment. Treatment was applied using a Teejet Twinjet 8002E nozzle, at 32 psi, with a 150 L/ha application volume and at a 50 cm application height. Plants were dried for 24h, before inoculation. *Zymoseptoria tritici* strains IPO323 (WT), 37-16 (cytochrome *b* G143A), and derived mutants, were grown on YMA plates under light for four days. 10⁶ spore/mL spore suspensions were prepared in distilled sterile water. The 3-weeks old wheat plants used for inoculation were from the cultivar Lancer. Plants were inoculated and incubated as described previously. Disease severity in each inoculated plant was assessed at 20 dpi, as the percentage of the plant covered by disease. EC₅₀ is the fungicide concentration that halves the percentage of the plant covered by disease compared to the untreated control. EC₅₀ values are the average of the four independent replicates. Resistance factor (RF) is the ratio between the EC₅₀ of the resistance strain and the EC₅₀ of its parental strain (Table 2.2.).

Table 2.3. Inatreq™ concentrations used in the efficacy assay *in planta*.

Inatreq™ rate (g/ha)	Strains	
-	0	All
Field / 100	0.75	IPO323, 37-16
Field / 30	2.5	IPO323, 37-16
Field / 10	7.5	IPO323, 37-16
Field / 3	25	IPO323, 37-16
Field ^a	75	All cytochrome <i>b</i> G37V strains
Max label	100	All cytochrome <i>b</i> G37V strains
Max label x 3	300	All cytochrome <i>b</i> G37V strains

^aLabel dose for a use against STB on wheat. The label dose for a use in mixture with prothioconazole is 50 g/ha.

3. RESULTS.

3.1. Pathogenicity of cytochrome *b* G37V isolates.

Pathogenicity was assessed for two series of ancestral strains (IPO323, susceptible to all fungicides and 37-16, resistant to azoxystrobin, due to the cytochrome *b* G143A change) and

their derived cytochrome *b* G37V mutants (four mutants per original background) as produced by experimental evolution (chapter 1). All strains originated from Bioger and were sent to Indianapolis. In Bioger, IPO323 is regularly used for pathogenicity assays and produces good levels of disease on susceptible cultivars. However, during *in planta* assays in Indianapolis on cultivar Lancer considered as susceptible, the level of disease obtained with the isolates of IPO323 background was too low to provide consistent results (Fig. 2.2.A). On the contrary, disease levels obtained for strains of the 37-16 background provided consistent results. Pathogenicity was assessed in two independent experiments (Fig. 2.2.B and 2.2.C). In both experiments, disease levels triggered by 37-16 and the double mutants did not seem different (Fig. 2.2.D), even if the variability between the two experiments was high. Altogether, these results suggest that cytochrome *b* G143A and cytochrome *b* G143A+G37V strains may have similar pathogenicity on this susceptible wheat cultivar.

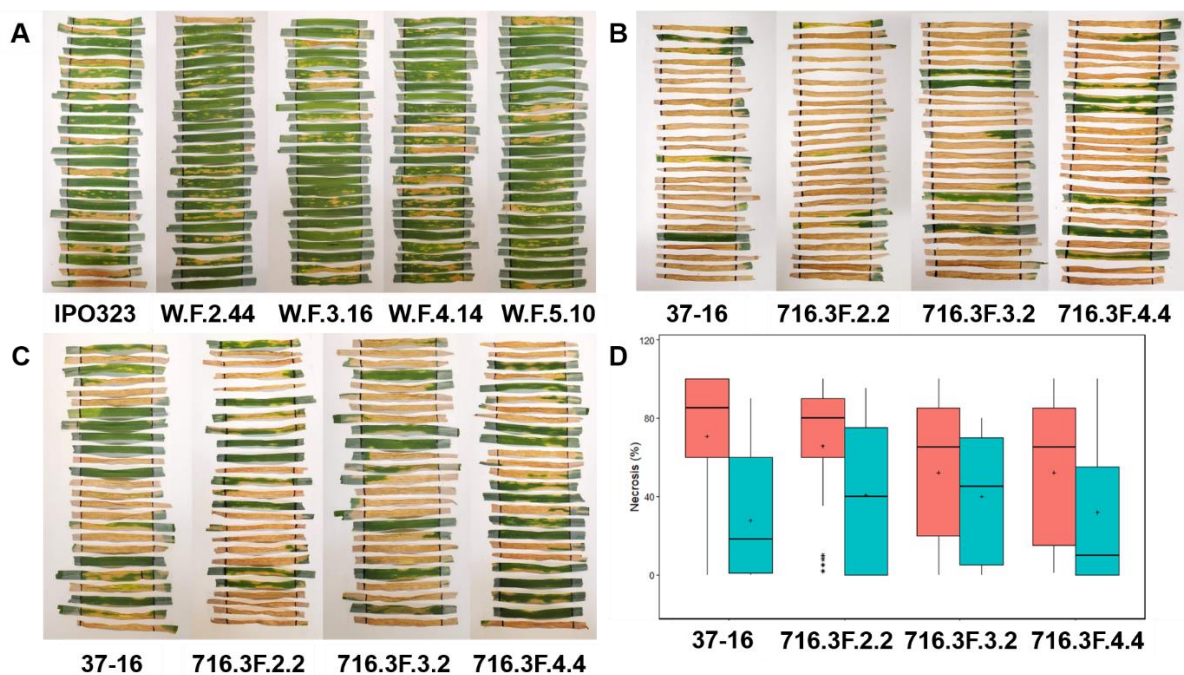


Figure 2.2. Pathogenicity assays of *Z. tritici* cytochrome *b* G37V mutants and ancestral strains. A. Level of disease obtained on 25 leaves with IPO323 and derived cytochrome *b* G37V mutants. **B and C.** Level of disease obtained on 25 leaves with the cytochrome *b* G143A strain 37-16, and derived cytochrome *b* G37V mutants in two independent experiments. **D.** Box plot representing disease severity from the two independent experiments conducted with 37-16 and derived cytochrome *b* G37V mutants. Pink-red boxes represent the first experiment (B), and the blue-green boxes represent the second experiment (C).

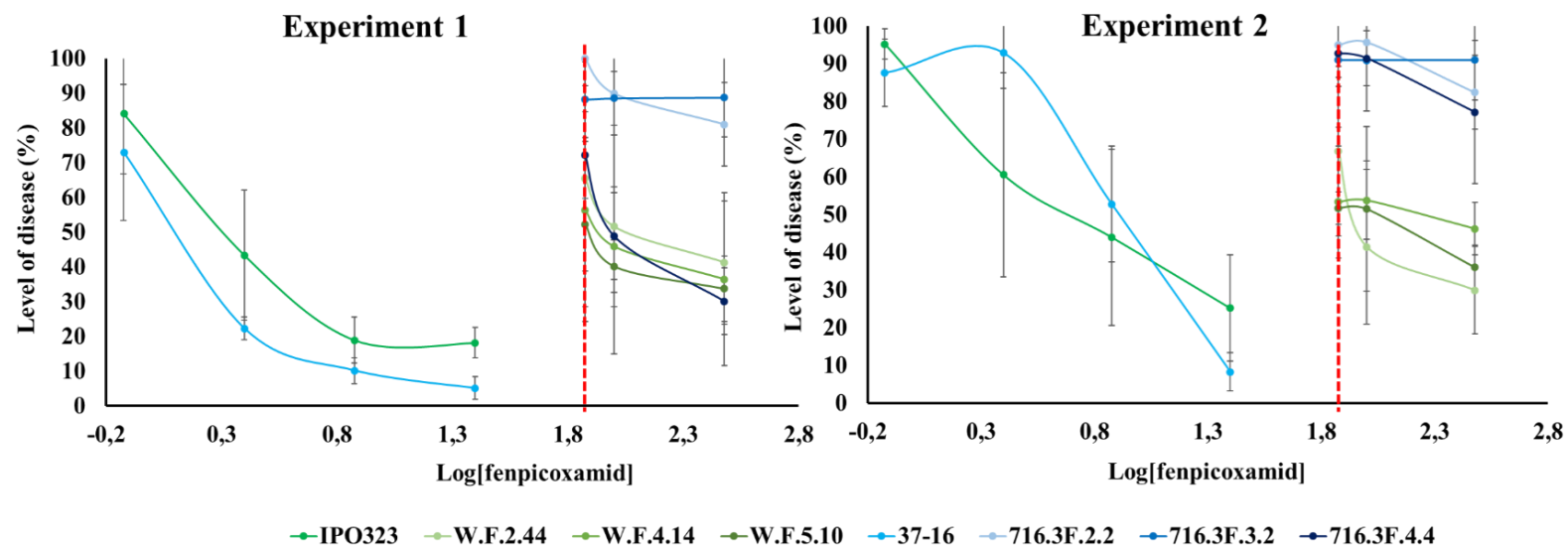
3.2. Competitiveness of cytochrome *b* G37V isolates.

A preliminary experiment was performed using a 1:1 mixture of spores from strain 37-16 (cytochrome *b* G143A) and the double mutant 716.3F.4.4 (cytochrome *b* G143A+G37V) that was inoculated on wheat plants. Both genotypes were detected after a complete infectious cycle. Cytochrome *b* G37V quantification was not performed after this preliminary experiment. Further experiments are still ongoing.

3.3. Fenpicoxamid efficacy assay.

Two preliminary experiments were conducted with the two ancestral strains IPO323 and 37-16 (of cytochrome *b* G143A background), and their derived single (W.F.2.44, W.F.4.14, W.F.5.10) and double (716.3F.2.2, 716.3F.3.2, 716.3F.4.4) mutants resistant to fenpicoxamid, respectively. Results are shown in Figure 2.3.

The amount of disease scored *in planta* for a given various isolates is generally variable within and between experiments, but ranking of the isolates was consistent between the two experiments (except for the isolate 716.3F.4.4 which induced much more disease in the second experiment compared to the first one). The ancestral susceptible strains were both controlled (maximum 30% disease in the two experiments) at one third of the label dose (75 g/ha). IPO323 seemed as susceptible to fenpicoxamid as the cytochrome *b* G143A isolate *in planta*. RFs of the cytochrome *b* G37V single mutants ranged between 18 and 58, with EC_{50s} superior to the label dose, except for W.F.5.10 in the first experiment (Figure 2.3.). The control of single mutants provided by fenpicoxamid at the label dose was below 50% in all cases. The RFs of double mutants were higher than that of the single mutants (*e.g.* by ≈ 2.5 to > 7.5 times in the first experiment), with EC_{50s} greater than the highest dose assayed (300 g/ha, *i.e.* 4 times the label dose). Efficacy of fenpicoxamid at the label dose on the double mutants was systematically lower than 20%. The only exception was the 716.3F.4.4 isolate in the first experiment, displaying an EC₅₀ of 133 g/ha and controlled at 28% by fenpicoxamid at the label dose. EC_{50s} of cytochrome *b* G37V mutants imply that MIC might be above the maximal dose, triggering a loss of efficacy in the field, should the frequency of the mutants in the population reach a sufficient level. Indeed, the loss of efficacy observed in these experiments corresponds to the maximal efficacy loss, in the case where the population is composed only of resistant isolates.



Isolate	Cytochrome <i>b</i>	EC ₅₀ (g/ha) (Exp. 1)	RF (Exp. 1)	Efficacy at label dose (%) (Exp. 1)	EC ₅₀ (g/ha) (Exp. 2)	RF (Exp. 2)	Efficacy at label dose (%) (Exp. 2)
IPO323	WT	3	-	-	6	-	-
W.F.2.44	G37V	158	58	34	110	18	33
W.F.4.14	G37V	98	36	44	98	16	47
W.F.5.10	G37V	65	24	48	162	27	48
37-16	G143A	1	-	-	7	-	-
716.3F.2.2	G37V+G143A	> 300	> 300	0	> 300	> 40	5
716.3F.3.2	G37V+G143A	> 300	> 300	12	> 300	> 40	9
716.3F.4.4	G37V+G143A	133	102	28	> 300	> 40	7

Figure 2.3. Fenpicoxamid efficacy *in planta*. Top: Level of disease triggered by the two ancestral strains IPO323 and 37-16, and derived cytochrome *b* G37V mutants, relative to the untreated control, in two independent experiments. The red dotted line indicates the label dose for fenpicoxamid solo formulation on wheat (75 g/ha). Bottom: Table showing the EC₅₀s, RFs (calculated regarding the corresponding ancestral strain) and fenpicoxamid efficacy at the label dose in both experiments.

4. DISCUSSION.

4.1. Assessing the impact of the cytochrome *b* G37V change on fitness.

Neither pathogenicity nor competitiveness could be assessed in IPO323 derivative cytochrome *b* G37V mutants. These levels of virulence displayed by IPO323 and its derived single cytochrome *b* G37V mutants were unexpected and potentially due to the experimental conditions or the wheat cultivar. At INRAE Bioger, IPO323 is regularly used for pathogenicity assays, and it is able to cause disease on whole plants of susceptible cultivars. Modifications of the assay conditions have also been considered, like using a growth chamber or a dew chamber in which conditions can be more strictly controlled than in a greenhouse, like increasing the inoculum size, performing the assay in Bioger or using another wheat cultivar. At first, the Lancer cultivar used for this assay seemed one of the most susceptible to IPO323. Recently, the use of inoculum with higher spore concentrations seems to trigger higher disease levels.

Regarding the cytochrome *b* G37V+G143A double mutants, which are likely to be selected in populations from Western Europe, preliminary results showed that the presence of cytochrome *b* G37V does not affect the ability of isolates to cause disease, and to produce pycnidia and pycnidiospores. Moreover, in spite of the high variability between the two experiments, which are frequent in *in planta* assays, there were no significant differences in pathogenicity between the double mutants and their ancestral cytochrome *b* G143A strain. Regarding the competitiveness of the double mutants, preliminary experiments allowed the detection of both types of spores (cytochrome *b* G143A and cytochrome *b* G37V+G143A) after one infection cycle, meaning that both isolates are able to reproduce when infecting a plant simultaneously. This could mean that the 2-fold decrease in complex III activity triggered by cytochrome *b* G37V observed *in vitro*, does not affect *in planta* fitness, at least in the cytochrome *b* G143A background, which dominates in contemporary *Z. tritici* populations of Europe. However, assays need to be repeated, including other double mutants, to draw more solid conclusions. Results from ongoing experiments with quantification of the two strains following symptom development will help understanding the relative competitiveness of the strains.

Impact of the cytochrome *b* G37V change in the single mutants might be as minor as in the double mutants on *in planta* and *in natura* fitness, but needs to be verified. Indeed, assessing fitness and competitiveness of single mutants is also important as strains without the cytochrome *b* G143A change are still present in European populations, at various frequencies, and *in vitro* selection of the cytochrome *b* G37V change in these isolates appeared easier in our

conditions. Therefore, since the strobilurin selection pressure is now low on *Z. tritici* populations due to the reduced usage of this mode of action, single cytochrome *b* G37V mutants may emerge more rapidly in the field.

Fitness is one of the parameters that influences selection of resistant strains in the field (Table 2.1.). The fitter the resistant isolates are, the more rapidly they may be selected. Given the above preliminary results, we conclude that fitness *in planta* of cytochrome *b* G37V strains is not likely to highly delay or prevent their selection in the field. However, further assays are needed to confirm these first observations, especially with more isolates, including the cytochrome *b* G37V single mutants.

4.2. Cytochrome *b* G37V may trigger detectable field resistance.

RFs to fenpicoxamid of cytochrome *b* G37V strains measured *in vitro* were high, and above 100 for the double mutants. Similar RFs were associated to the cytochrome *b* G143A change determining high field resistance in Europe (Leroux *et al.*, 2006). A recent study showed that high RFs in the laboratory were well correlated to a decrease field efficacy of DMIs and QoIs on *Z. tritici* (Blake *et al.*, 2018). *In planta*, EC₅₀s of fenpicoxamid on cytochrome *b* G37V single and double mutants were almost systematically equal to the maximal registered field dose, suggesting that the cytochrome *b* G37V change is likely to affect fenpicoxamid field efficacy, should the frequency of these mutants in the population reach a certain level. Indeed, the control of cytochrome *b* G37V isolates provided by fenpicoxamid at the label dose was always lower than 50% and even decreased severely to less than 10% on double mutants in the second experiment. Even though, these observations were made on six different mutant isolates in two independent experiments, more repetitions are needed to validate these preliminary observations.

Ideally, other fungicides should be assayed too. A QoI would be a good reference, especially regarding the loss of efficacy measured historically. Representatives of SDHIs and DMIs would be also valuable candidates to achieve consistency with the results from chapter 3, and because they are the most widely used fungicide classes against *Z. tritici*. Introduction of other phenotypes in the assay would also be informative, especially MDR strains, again to compare efficacy loss.

To conclude, given that cytochrome *b* G37V strains, especially in a cytochrome *b* G143A genetic background, do not show a severe fitness penalty *in planta*, and that their resistance levels to fenpicoxamid are high, one can conclude that the cytochrome *b* G37V change

represents a threat for fenpicoxamid efficacy, should such mutants be selected in the field (Table 2.4.).

Table 2.4. Estimate of the risk of field resistance to fenpicoxamid represented by the cytochrome *b* G37V change. Results based on preliminary assessment of their fitness and levels of resistance.

Risk of field resistance			Efficacy loss <i>in planta</i> at labeled dose		
			Low A	Moderate B	High C
Fitness <i>in planta</i> (absolute or in competition)	Reduced compared to WT	1	Low	Moderate	Moderate
	Equal to WT	2	Moderate	Moderate	High
	Improved compared to WT	3	Moderate	High	High

Aside from the experiments that need to be repeated, susceptibility of cytochrome *b* G37V isolates to other modes of action will also be assessed *in planta*. Indeed, the cytochrome *b* G37V change may trigger some shifts in sensitivity to other modes of action, as it has been observed *in vitro* for QoI susceptibility (see chapter 1). Moreover, as the risk of resistance triggered by the cytochrome *b* G37V change appears to be high, and fitness may not delay selection of these isolates, identifying good anti-resistance strategies is therefore essential. This encompasses the identification of the modes of action to be associated preferentially to QiIs, and of the relevant components to be best combined to enhance sustainability. In the next chapter, we chose to evaluate fungicide-based anti-resistance strategies *via* an *in vitro* approach of experimental evolution.

Chapter 3.

Managing resistance to fenpicoxamid in *Zymoseptoria tritici*.

1. INTRODUCTION.

1.1. Resistance dynamics in populations and its drivers.

Resistance is an adaptive evolutionary process of fungi in response to fungicide treatments. It results in the progressive increase of advantageous alleles in populations. Resistance dynamics is usually represented by a sigmoid curve, defining the emergence, the selection and the stabilization phases (Fig. 3.1.; van den Bosch and Gilligan, 2008; Mikaberidze *et al.*, 2017; zur Wiesch *et al.*, 2011).

The emergence phase relies on mutation (either *de novo* or present in standing variation; see introduction 5.2.) and then selection, to allow advantageous alleles to arise in populations. Resistance alleles are usually at very low frequency and this phase is characterized by the time resistance takes to be first detected in populations. The duration of the emergence phase (TE; Fig. 3.1.) is highly variable, depending on pathogens, fungicides and cropping systems (crop rotation, use of tolerant cultivars...) (Grimmer *et al.*, 2015). It depends on both the mutation rate of the pathogen, estimated around 10^{-10} changes per site per cell division in fungi (see introduction 5.2.), but increased by fungicide exposure (Amaradasa and Everhart, 2016), and compensatory mechanisms, as not all mutations emerge due to their fitness and genetic drift. For example, in *Z. tritici* French populations, resistance emerged after 6 years of field use for QoIs and SDHIs, and after 10 years for the first DMIs (Garnault *et al.*, 2019).

The selection phase frames the increase of resistance frequency in populations (Fig. 3.1.). Field resistance can be observed beyond a certain threshold of resistance frequency. In the meantime, depending on dispersion traits of the pathogen and on selection pressure because of product usage, resistance distributes spatially. The selection phase relies mostly on selection as an evolutionary force. The rate of resistance increase is determined by the selection coefficient, which is the difference in fitness between the resistant and susceptible isolates due to the application of the corresponding fungicide (van den Bosch *et al.*, 2014):

$$S = r_R - r_S \quad \text{Equation 1}$$

Where r_R and r_S are the average rate of increase “*per capita*”, for the resistant and susceptible isolates, respectively. The rate of resistance then results from a trade-off between the relative fitness of resistant *vs.* susceptible strains. As examples, proxies of the selection coefficient were modelled from large-scale *Z. tritici* empirical data in France between 1980 and 2017. The highest rates were estimated for the StrR (almost twice as rapid as the rest of the population)

and TriHR strains (about 1.5 times more rapid), followed by TriMR (until 2011) and MDR strains (Garnault *et al.*, 2019).

During the stabilization phase, the frequency of resistant isolates in populations reaches a plateau (TG; Fig. 3.1.). It is generally stabilized at high resistance frequency but the height of this plateau depends on the balance between selection and fitness. When the selection pressure is removed, the frequency of resistant strains may decrease if there is a fitness cost associated to the resistance allele, but it is rarely observed in the field. Globally, very long time periods without fungicide application are necessary to recover resistance frequencies low enough for the fungicide to be efficient again (Walker *et al.*, 2013).

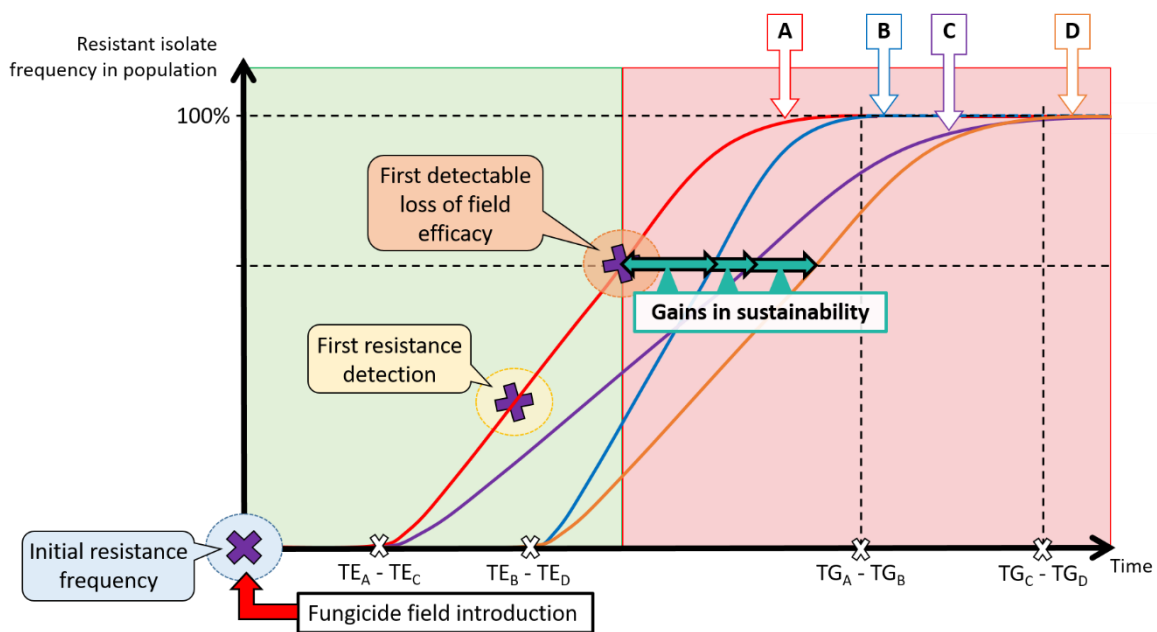


Figure 3.1. Dynamics of resistance in populations and interest of resistance management. Time of emergence (TE) and time of generalization (TG) of a resistant allele in a population. **A.** Evolution of allele frequency without management strategy. **B, C and D.** Evolution of allele frequency with fungicide-based management strategies. Strategy **B** delays the time of emergence, strategy **C** delays the time of generalization and strategy **D** delays both.

1.2. Delaying resistance evolution.

The mutation rate can hardly be mitigated in natural conditions but several options are possible to decrease the selection coefficient. Given equation 1, the selection coefficient is decreased when (Fig. 3.2.; van den Bosch *et al.*, 2014):

- Rationale 1: the increase of both susceptible and resistant strains is reduced (r_S and r_R respectively);
- Rationale 2: the increase of the resistant strain (r_R) is reduced, relatively to the susceptible strain (r_S). This is the case, for example, in situations with effective resistance cost;
- Rationale 3: the time span over which selection takes place (exposure time) is reduced:

$$sT = (r_R - r_S)T \quad \text{Equation 2}$$

Where T is the exposure time of the pathogen population to the fungicide. In practice, this leads to a voluntary reduction of fungicide use, to the benefit of other control measures.

Anti-resistance strategies (*i.e.* the smart deployment of fungicide modes of action in time, space and dose) aim at minimizing the selection coefficient *via* one or several of the options detailed above. Strategies are all the more effective as the initial resistance frequency is low, *i.e.* that the resistance rate is low and the strategy is implemented early, because genetic drift is more efficient at losing low-frequency alleles, especially if populations are kept at small size. Consequently, early identification of the best fungicide-based strategies to delay resistance selection represents a primary challenge (Rex consortium, 2013).

To conclude, reliable anti-resistance strategies decrease the selection coefficient while maximizing the heterogeneity of the selection pressure, and then delay field resistance (reviewed in Rex consortium, 2013).

1.3. Anti-resistance strategies.

Selection heterogeneity can be achieved by modulating the use of fungicides in time or space, or by modulating the dose (Fig. 3.2.). Combined fungicides should be efficient on the targeted pathogen, display different modes of action and should not exhibit positive cross-resistance, regardless of the resistance mechanism.

Among strategies, fungicide alternation refers to the use of fungicides staggered in time, with different possible time steps (a season, a year or several years). If an individual survives to the application of the first fungicide, then the second fungicide, displaying a different mode of action efficient on the pathogen, will kill its progeny, resulting in an intergenerational killing. Such a temporal heterogeneity can reveal useful as it could leave the time for a putative resistance cost or fitness penalty to express (Rex consortium, 2013).

A second strategy is the mosaic, which consists in using different fungicides in different locations, resulting in a spatial heterogeneity whose grain should be adapted to the migration traits of the pathogen. Using this strategy, a resistant individual and its progeny that migrates from one location to another are killed by the second fungicide. Like for alternation, mosaic exerts an intergenerational killing, on migrant generations. Mosaic is for example used to manage Bt resistance in insect pests (Zhao *et al.*, 2010) and is clearly an untapped option for other *taxa* such as fungi. Mosaic can be used along with alternation to increase selection heterogeneity over space and time (Rex consortium, 2013).

Mixture designates the simultaneous use of two (or more) fungicides. Practically, fungicides can be combined as pre-mixtures (fungicides formulated together) or as extemporaneous tank-mixtures (fungicides formulated separately). Mixture relies on the process of redundant killing of the same genotype (the fungus is killed “two” times, differently) (Hobbelen *et al.*, 2011). Both active ingredients are applied onto the same generation, thus performing intragenerational killing. This strategy requires that both partners are efficient on the pathogen, that they are used at their efficient dose, and that the resistance to one of the two partners has not been selected previously in the field (Genet *et al.*, 2006; Mavroeidi and Shaw, 2006).

Finally, dose modulation aims at limiting the fungicide doses used in a field, which may have consequences on resistance management. Contrary to high doses that tend to select only highly resistant isolates, dose modulation allows the survival of some susceptible isolates and isolates with intermediate resistance levels. The survival and reproduction of a part of the susceptible population limits the spread of resistant individuals (reviewed in van den Bosch *et al.*, 2011). However, this strategy may favor quantitative resistance as described for DMI resistance in *Z. tritici* (see introduction, 5.8.3.; van den Bosch *et al.*, 2011).

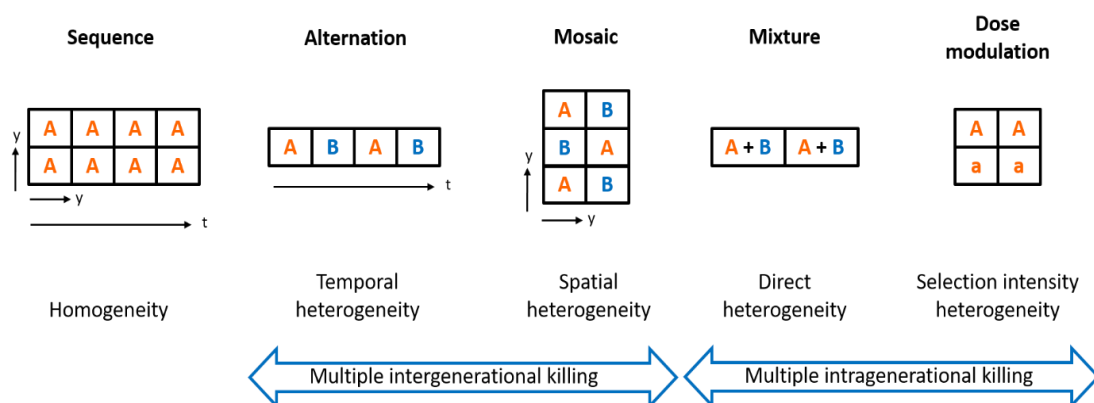


Figure 3.2. Representation of fungicide-based anti-resistance strategies (Walker A.-S., unpublished). A and B are two fungicides that do not display cross-resistance.

1.4. Comparing anti-resistance strategies.

At least three approaches have been employed so far to compare strategies: theoretical mathematical modeling, statistical modeling of empirical data and experimental evolution. Each approach comes with its own advantages and limitations when comparing the various experimental components (Table 3.1.).

Modeling has been widely used to try determining what the best anti-resistance strategy would be. *In silico* mathematical models (van den Bosch *et al.*, 2014; Hobbelen *et al.*, 2011, 2013) are purely theoretical and may be eventually validated by confrontation to empirical data. Mathematical models constitute the most artificial approach, but allows a simplified and precise assessment of the influence of each parameter. Simplicity may sometimes be also a limitation, as too simplified models provide less accurate representation of the reality. Moreover, our lack of knowledge in some aspects of fungicide resistance can harden the development of very accurate models (Birkegård *et al.*, 2018).

Table 3.1. Strengths and weaknesses of available approaches to compare fungicide-base anti-resistance strategies.

	Statistical inference of empirical data	Experimental evolution	Mathematical modelling
Time needed to achieve strategy comparisons	+	++	+++
Need for field empirical data	+++	-	-
Realism /field conditions	+++	++	+
Diversity of influencing factors	+++	++	+
Pathogen biology made explicit	+++	++	-
Genetics of resistance made explicit	+++	+++	+
Informs on the outcome of the evolution	++ (monitoring)	+++	-
Number of compared strategies	+	++	+++
Factors influencing evolution are easy to characterize	+	+++	+++

Statistical inferring models could also help to evaluate anti-resistance strategies (Grimmer *et al.*, 2014, 2015). These models are much more realistic, considering the heterogeneity related to the host, the environment and the general fitness of selected resistant isolates. However, they may display some confounding effects, as for example the higher selection pressure exerted by

fungicide mixtures, due to synergy between fungicide partners. Moreover, these models require generally high calculating capacities, which limits the number of possible comparisons. Statistical models are based on large datasets, and cannot be used to predict situation to new modes of action prior to their use in the field. In addition, in such pre-existing datasets, information about the diversity of selected resistance mechanisms and precise fitness assessment of resistant isolates is almost never found, except when monitoring is performed.

Experimental evolution is clearly an untapped approach when comparing strategies, especially for fungi (Fisher and Lang, 2016). Indeed, in spite of its high potential, experimental evolution has rarely been applied to compare anti-resistance strategies (Lagator *et al.*, 2013; Zhao *et al.*, 2010). Yet, it represents a compromise between the two other approaches, a compromise between the size of the experimental design and the reality, and then may contribute to shed light on the debate about the respective interest on strategies. The advantage in using experimental evolution is that fungicide exposure can be precisely assessed, while comparing multiple selection regimes in controlled laboratory or greenhouse conditions, and while dissecting its components in a non-confounding manner. It allows making thorough characterization of the selected resistant isolates, if needed. Moreover, experimental evolution *in vitro* presents the advantage of generating results rapidly due to much shorter generation time, thus considering evolution on many generations (*e.g.* corresponding to large time-scales in the field). Among limitations is the fact that spatial heterogeneity (mosaic) cannot be assessed and that the host plant as selective parameter is not considered neither. Experimental evolution *in planta* integrates the host plant parameter, making it more “field-realistic” than experimental evolution *in vitro*. It possesses the same advantages as experimental evolution in the laboratory, except for the generation time that is much longer in the plant (between 9 to 30 days, see introduction 3.6.), and the fact that it is much more resource consuming. In both the laboratory and the greenhouse, experimental evolution does not allow taking into account the impact of sexual reproduction.

To conclude, in our study we chose experimental evolution for being a compromise between other approaches. Moreover, this method being the fastest, it was more suitable for a short-term project aiming at assessing the risk of resistance selection to a new active ingredient.

1.5. Identifying the best anti-resistance strategy for fenpicoxamid facing *Z. tritici*.

In chapter 1, using directed evolution *in vitro*, we showed that target site resistance through the selection of the cytochrome *b* change G37V, was likely to be selected in *Z. tritici* as resistance mechanism towards fenpicoxamid. In chapter 2, we hypothesized that the risk of practical resistance due to this mechanism was high, because of lack of fitness penalty and the high potential for fenpicoxamid efficacy loss in field conditions. In this chapter, our objective was to identify which parameters are the most important to consider in order to make anti-resistance strategies efficient at delaying resistance to fenpicoxamid, over the various phases of resistance dynamics (delay resistance emergence and/or resistance selection), and prior to the launch of this fungicide on the market. In particular, we wanted to analyze which strategy components are important for resistance management for this specific example of resistance. To this end, we used experimental evolution as a way to find out how strategies would mitigate resistance dynamics. For strategy drivers, we compared the number of active ingredients used for selection, their intrinsic risk of resistance, and the timing of fungicide application. For practical reasons, we limited our experiment to a comparison between sequence (straight application), alternation and mixture, as they are the most commonly used in the field, and the most commonly compared in modeling analyses (Hobbelen *et al.*, 2013).

I conducted all experiments of this chapter at Bioger. Agathe Ballu kindly provided statistical analyses to explore primarily the data.

2. EXPERIMENTAL PROCEDURES.

2.1. Isolates of *Z. tritici* and ancestral populations.

Isolates used in this study were described in chapters 1 and 2. Their genotypic variations in *CYTb* are listed in Table 3.2.

Table 3.2. Origin and characteristics of *Z. tritici* isolates used in this study.

Isolate	Phenotype	Origin	Ancestral strain	Reference
IPO-323	TriS, CarS, QoIS, QiIS	Reference isolate	-	Goodwin <i>et al.</i> , 2011
37-16	TriS, CarS, QoIR, QiIS	Progeny isolate (lab cross)	IPO-323 and G143A field strain	Lab collection
716.3F.2.2	TriS, CarS, QoIR, QiIR	Experimental evolution	37-16	This study, chapter 1

These isolates were used to found ancestral populations in experimental evolution: either “pure population of 37-16, or a mixed population encompassing of 99% of 37.161% and 1% of the derived 716.3F.2.2 isolate.

2.2. Fungicides and determination of selection doses.

Selection was achieved using fenpicoxamid (FPX), and azoxystrobin (AZ), benzovindiflupyr (BEN) or prothioconazole-desthio (PRO), the active form of prothioconazole, as possible partners. This choice was driven by the fact that SDHIs and DMIs are the main two fungicide classes currently used in the field to control *Z. tritici*. In spite of the G143A change in ancestral strains, azoxystrobin, representing QoIs, presented an interest in our study as this family of fungicides targets the same protein as QiIs (cytochrome *b*) but displays a different binding site. This choice also had an educational interest, while enabling to study strategies where one of the partners is already concerned by resistance.

Dose response-curves were established prior to the choice of selection doses. Ranges of concentrations were 0.005 to 0.65 $\mu\text{g/mL}$ for fenpicoxamid using 3-fold increase; 0.0001 to 0.0018 $\mu\text{g/mL}$ for prothioconazole-desthio using 2.2 and 1.5-fold increase; 0.005 to 1 $\mu\text{g/mL}$ for benzovindiflupyr using 3 and 3.75-fold increase; and mixtures of fenpicoxamid with azoxystrobin, prothioconazole-desthio or benzovindiflupyr. Isolates 37-16 and IPO-323 were incubated in 25 mL of YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) at 17°C and 150 rpm in the dark for 7 days. 10^7 spores were used to inoculate 25 mL of fresh YPD, before the addition of 125 μL of a 200-fold concentrated fungicide solution in a 1:1 ethanol/acetone mixture, for the previous ranges of concentrations. Amended spore suspensions were incubated at 17°C and 150 rpm in the dark for 7 days. The optical density at 405 nm ($\text{OD}_{405\text{nm}}$) was determined at day 7 using a SpectraMax M2 spectrophotometer (Molecular Devices, San Jose, CA, USA). Minimal inhibitory concentrations (MICs) were determined using the GaphPad Prism program (GaphPad Software). Each MIC value is the average of two independent assays using three technical replicates each. MICs calculated from dose-response curves were adjusted experimentally as correct selection dose in preliminary experiment before starting experimental evolution. Briefly, strains were grown as described previously with each fungicide MIC, and several surrounding concentrations, for 7 days. After 7 days, relative growth compared to the untreated control was determined, and the selection concentrations for experimental evolution were adjusted to the concentration providing the best control of growth. MIC was not achieved for azoxystrobin in cytochrome *b* G143A isolates, and was fixed at 6 $\mu\text{g/mL}$ considered its solubility limit in aqueous media.

2.3. Synergy calculation.

EC₅₀s and MICs for mixtures of fenpicoxamid with each possible partner were established as described above for isolates 37-16 and IPO-323. Concentration ranges for dose-response curves were established while using percentages of the MICs obtained for single compounds. Concentrations ranged between: 1 and 80% for the FPX/AZ mixture (*i.e.* 0.008 to 0.64 µg/mL for FPX and 0.06 to 4.8 µg/mL for AZ); 5 and 60% for the FPX/PRO mixture (*i.e.* 0.04 to 0.48 µg/mL for FPX and 0.00004 to 0.00048 µg/mL for PRO); 2 and 10% for the FPX/BEN mixture (*i.e.* 0.016 to 0.08 µg/mL for FPX and 0.01 to 0.05 µg/mL for AZ). The ratio was the same for each partner, meaning, for example, that 20% of the fenpicoxamid MIC was mixed with 20% of the azoxystrobin MIC.

Synergy between fungicides used in mixtures were calculated using the Wadley formula (Wadley, 1945). It describes the theoretical response of fungicides (EC₅₀^{theo}) of a two-component mixture as:

$$EC_{50}^{theo} = (a + b) / (a/EC_{50}A + b/EC_{50}B) \quad \text{Equation 3}$$

Where A and B represent the two tested compounds (A = fenpicoxamid and B = partner, in our study), and a and b are their ratio in the mixture, respectively. The level of interaction is calculated as:

$$R = (EC_{50}^{theo}) / (EC_{50}^{obs}) \quad \text{Equation 4}$$

In which EC₅₀^{obs} is the observed EC₅₀ value of the specific mixture. By definition, additive interaction is considered if R = 1, synergism if R > 1 and antagonism if R < 1. Because of the biological variations of the tested systems, synergism is considered significant if R ≥ 1.5 and antagonism if R ≤ 0.5 (Gisi *et al.*, 1985; Stergiopoulos and Dewaard, 2002).

2.4. Experimental evolution and data analysis.

Each experiment encompassed three independent lines for each selection regime. Experimental procedure was the same as in chapter 1. Ancestral strains used were either 37-16 (cytochrome *b* G143A) or a mixed population composed of 99% 37-16 and 1% (minimal detectable frequency) of 716.3F.2.2 (cytochrome *b* G37V+G143A). Evolution of the pure strain allows comparing the effect of selection regimes on both the emergence and selection phases. The cytochrome *b* G143A ancestral strain, 37-16, was preferentially used to be closer to the field

situation where the cytochrome *b* G143A change is almost generalized (Garnault *et al.*, 2019; Kildea *et al.*, 2019). The introduction of the double mutant allows skipping the emergence phase of the resistance that can be unequal between the evolved lines, even those subjected to the same selection regime. It allowed comparing the impact of selection regimes on the selection phase without being dependent on the emergence phase.

Briefly, 10^7 spores (either 100% 37-16, or 99% 37-16 + 1% 716.3F.2.2) were incubated in 25 mL of liquid YPD at 17°C shaken at 150 rpm, in the dark for 7 days, with selecting fungicide at their established selection dose, or selecting mixture of two fungicides at half their selection dose each. New selection cycles were inoculated using a 2% transfer rate from the previous cycle. When needed, immigration from the untreated control line or reconstituted population enabled to complement serial transfer to reach 10^7 spores in populations starting the next cycle. The experiment lasted for 8 cycles. At the end of each cycle, OD_{405nm} was determined using a SpectraMax M2 spectrophotometer.

OD_{405nm} values were used to calculate the normalized Malthusian growth. Malthusian growth corresponds to:

$$\text{Malthusian growth} = \ln(Cd^{end}/Cd^{start}) \quad \text{Equation 5}$$

Where Cd^{end} and Cd^{start} are the population sizes (in number of spores) at the end and at the beginning of the considered cycle respectively. Malthusian growth of the treated line is normalized by that of the corresponding untreated control line. Mean values of the three lines were plotted using R software (The R Foundation for Statistical Computing). Areas under the curves (AUCs) were determined using the package “emmeans” from the R software. For the statistical comparisons of the means, p.values were adjusted using the Tukey method for multiple analyses.

2.5. Selection regimes.

Selection was performed at a similar selection dose (*i.e.* either the selection concentration for solo compounds, or half these concentrations for each compound in mixtures), meaning that all lines received the same total fungicide dose at the end of the eight cycles.

Table 3.3. Selection regimes used in experimental evolution and parameters influencing the different phases of resistance selection assessed by each regime.

Strategy	Fungicide 1	Fungicide 2	Dose of selection	Assessed parameters influencing the phases of resistance selection		
				Active ingredients	Intrinsic risk to select resistance ¹	Temporal heterogeneity
Sequence	FPX (QiI)	-	SC ²	1	High	none
	PRO (DMI)	-	SC	1	Medium	none
	BEN (SDHI)	-	SC	1	Medium	none
Alt	FPX (QiI)	AZ (QoI)	SC/SC	2	High/high	intergenerational
	FPX (QiI)	PRO (DMI)	SC/SC	2	High/medium	intergenerational
	FPX (QiI)	BEN (SDHI)	SC/SC	2	High/medium	intergenerational
Mixt	FPX (QiI)	AZ (QoI)	0.5SC+0.5SC	2	High/high	intragenerational
	FPX (QiI)	PRO (DMI)	0.5SC+0.5SC	2	High/medium	intragenerational
	FPX (QiI)	BEN (SDHI)	0.5SC+0.5SC	2	High/medium	intragenerational

¹Intrinsic risk of resistance as predicted by chapter 2 (fenpicoxamid) or by FRAC (other fungicides).

²SC is the selection concentration.

The first parameter assessed was the number of active ingredients in the strategy, meaning that we compared efficacy of mixture or alternation, as opposed to straight selection (Table 3.3.). The second parameter was the intrinsic risk of the partners in the strategy, thus comparing strategies using two high risk fungicides, or one high risk fungicide and one medium risk fungicide. The last parameter was the temporal heterogeneity, which meant comparing mixture (multiple intragenerational killings) to alternation (multiple intergenerational killings). We aimed at identifying which parameter had the greatest influence on emergence and/or resistance selection.

2.6. Characterization of evolved lines.

At the end of experimental evolution (cycle 8), OD_{405nm} was measured for each line to standardize spore concentration to the lowest. Spore suspensions were plated using three 10-fold serial dilutions, on YPD agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar), amended with discriminatory doses of various fungicides, established in chapter 1. They were: 2 µg/mL fenpicoxamid; 1.5 µg/mL azoxystrobin; 0.01 µg/mL prothioconazole-desthio; 1.5 µg/mL benzovindiflupyr; 2 µg/mL tolnaftate; 0.1 µg/mL carbendazim; 100 µg/mL propyl gallate; and mixture of fenpicoxamid and propyl gallate at the same concentrations.

Plates were incubated at 17°C in the dark for 7 days before scoring. Scores ranged from 0 to 5 depending on growth intensity at each spore concentration.

2.7. Quantification of resistance frequency in evolved lines.

After each cycle, depending on the spore concentration, 200 or 400 µL of each line was sampled for DNA extraction. The relative amount of the cytochrome *b* G37V allele will be quantified by ddPCR (see chapter 2) by Corteva Johnston, USA, in the forthcoming weeks.

3. RESULTS.

3.1. Susceptibility to fungicides used alone or in mixtures in experimental evolution.

In order to determine fungicide selection concentrations to be used in experimental evolution, we performed dose-response curves with the cytochrome *b* G143A strain 37-16, in the conditions of the experimental evolution, and with IPO323, for comparison. We determined the EC₅₀s and the MICs for all single fungicides in our conditions, except for azoxystrobin for which it could not be achieved due to the presence of the G143A change. This change induces high resistance, which would imply using concentrations greater than azoxystrobin solubility (Table 3.4.). For consistency, we decided to use the MICs of the 37-16 isolate for all lines, including for the mixed population lines.

MICs were adjusted after complementary assay to the final selection concentrations used in experimental evolution, established at 0.5 µg/mL for fenpicoxamid (89% growth control), 0.002 µg/mL for prothioconazole-desthio (81% growth control), and 0.5 µg/mL for benzovindiflupyr (87% growth control). Control of fungal growth was not fully achieved, in our conditions, suggesting that we may have finally used sub-lethal concentrations (EC₈₀-EC₉₀), but was rather similar among fungi. Likely, the residual growth observed is an artifact due to OD measurement, as even dead cells may trigger some absorbance, which is not visible in control lines because of the high spore density. This hypothesis is supported by the fact that even higher doses displayed similar control.

Table 3.4. Susceptibility of the WT strain IPO323 and the G143A strain 37-16 to different fungicides and to fenpicoxamid-based mixtures, and synergy coefficients of mixtures.

		IPO323				37-16			
		Single	Mixture with			Single	Mixture with		
			AZ	PRO	BEN		AZ	PRO	BEN
EC₅₀ ($\mu\text{g/mL}$)	FPX	0.126 \pm 0.06	0.022 \pm 0.004	0.038 \pm 0.003	0.009 \pm 0.001	0.06 \pm 0.01	0.0060 \pm 0.0006	0.0048 \pm 0.0006	0.0018 \pm 0.0006
	AZ	0.218 \pm 0.04	0.039 \pm 0.007			6 ^a	0.60 \pm 0.06		
	PRO	0.00055 \pm 0.0001		0.00017 \pm 0.00001		0.00043 \pm 0.00005		0.000034 \pm 0.000004	
	BEN	0.386 \pm 0.09			0.027 \pm 0.004	0.07 \pm 0.01			0.0021 \pm 0.0007
	Synergy coeff. (Wadley)		2.8	1.7	7.1		5.0	6.3	16.7
MIC ($\mu\text{g/mL}$)	FPX	0.87 \pm 0.08	0.24 \pm 0.04	0.37 \pm 0.02	0.096 \pm 0.009	0.7 \pm 0.1	0.154 \pm 0.007	0.133 \pm 0.007	0.035 \pm 0.007
	AZ	0.6 \pm 0.2	0.17 \pm 0.03			6 ^a	1.32 \pm 0.06		
	PRO	0.00084 \pm 0.00003		0.00036 \pm 0.00002		0.0012 \pm 0.0001		0.00023 \pm 0.00001	
	BEN	1.33 \pm 0.07			0.15 \pm 0.01	0.56 \pm 0.04			0.028 \pm 0.006
	Synergy coeff. (Wadley)		1.8	1.2	4.5		2.3	2.6	10.0

^aAI = active ingredient.

^bEC₅₀ and MIC of azoxystrobin for the 37-16 strain were fixed at 6 $\mu\text{g/mL}$, its solubility limit in aqueous media.

^cSynergy coefficients are calculated according to the Wadley formula (see “Experimental procedures” for details).

^dExample of percentage of the MIC of each partner used to achieve the MIC of the mixture. Here 28% of the MIC of FPX and AZ gave 100% of growth control in mixture. Therefore, the final concentration of FPX in the mixture was 28% of 0.87 $\mu\text{g/mL}$, equal to 0.24 $\mu\text{g/mL}$.

We then performed dose-response curves for mixtures of fenpicoxamid with three possible partners, displaying different intrinsic resistance risks (Table 3.4.). The MIC of the fungicide mixtures for the 37-16 strain was achieved by using 22% of the MICs of fenpicoxamid and azoxystrobin, 19% of the MICs of fenpicoxamid and prothioconazole-desthio, and 5% of the MICs of fenpicoxamid and benzovindiflupyr, suggesting high synergy between these fungicides, including azoxystrobin, despite resistance in the tested isolates. For IPO3232, MICs were achieved with 28, 43 and 11% of the MICs of the partners, respectively.

Synergy is defined by the fact that the activity of two (or more) drugs used in a mixture is greater than the sum of the individual activities of each partner (Sen *et al.*, 2019). The determination of the synergy coefficients according to the Wadley method based on MIC values, confirmed synergy of fenpicoxamid with all tested modes of action (1.7-16.7 based on EC₅₀s and 1.2-10 based on MICs). However, the synergy between fenpicoxamid and

prothioconazole-desthio was not significant against IPO323, based on the empirical level of 1.5. The mixture fenpicoxamid + benzovindiflupyr displayed the highest synergy against both IPO323 and 37-16 (7.1-16.7 based on EC_{50} s and 4.5-10 based on MICs). Synergies between fenpicoxamid and prothioconazole-desthio or azoxystrobin were similar against both strains, but the synergy between fenpicoxamid and azoxystrobin was slightly higher against IPO323 compared to that between fenpicoxamid and prothioconazole-desthio (synergy coefficient of 1.8 versus 1.2), as opposed to synergies against 37-16 (synergy coefficient of 2.3 versus 2.6). In spite of the G143A change, fenpicoxamid and azoxystrobin also acted synergistically against 37-16. Considering all mixtures, synergy was always between twice and three times higher against 37-16 than against IPO323.

For the experimental evolution, selection doses in mixture lines were finally chosen as a mixture containing 50% of the MIC of each fungicide as determined in solo dose-response curves, instead of the MIC of the mixture. Thus, each line (*i.e.* sequence, alternation, mixture) was exposed to the same total fungicide dose (same equivalent MICs) at the end of the experiment.

3.2. Anti-resistance strategies in comparison to straight selection.

Experimental evolution was performed using 37-16 (cytochrome *b* G143A) as an ancestral strain, or a population of 99% 37-16 + 1% 716.3F.2.2 (cytochrome *b* G37V+G143A). Considering fenpicoxamid resistance due to the cytochrome *b* G37V change, this design allowed comparing the efficacy of nine strategies either on the emergence and selection phases or on the selection phase only, respectively. At the end of each cycle, the 60 lines were analyzed for their relative Malthusian growth (see experimental procedures for calculation) (Fig. 3.3.). Areas under the curves (AUCs) were calculated in order to compare the different lines more easily (Fig. 3.4.). Resistance was considered as generalized when growth of the treated line reached 90% of the untreated control growth.

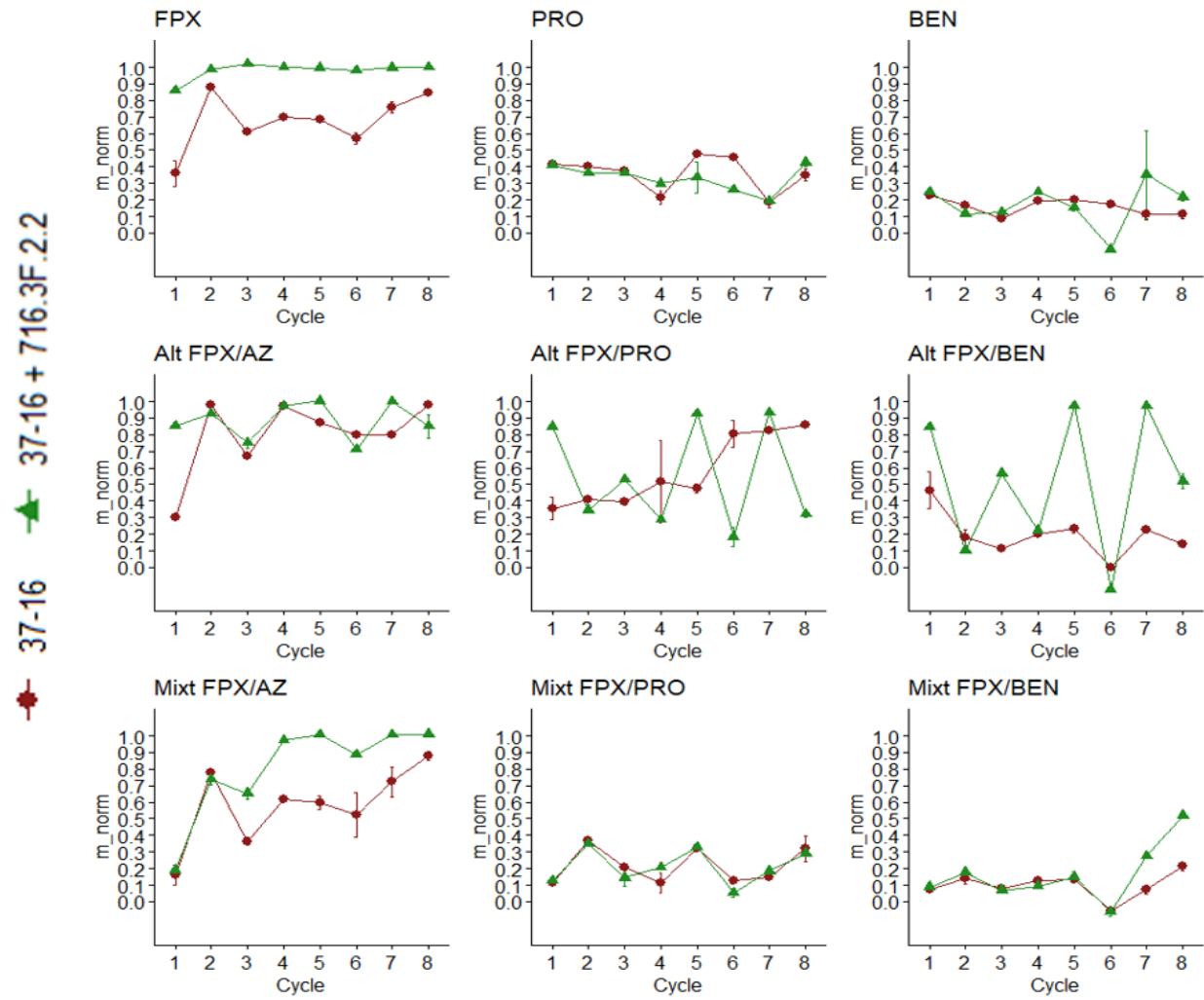


Figure 3.3. Evolution of relative growth during experimental evolution for the strain 37-16 and the population 37-16 (99%) + 716.3F.2.2 (1%). M_{norm} : relative Malthusian growth of the treated line compared to the untreated control line; for alternations, fenpicoxamid was always applied first (uneven cycle numbers).

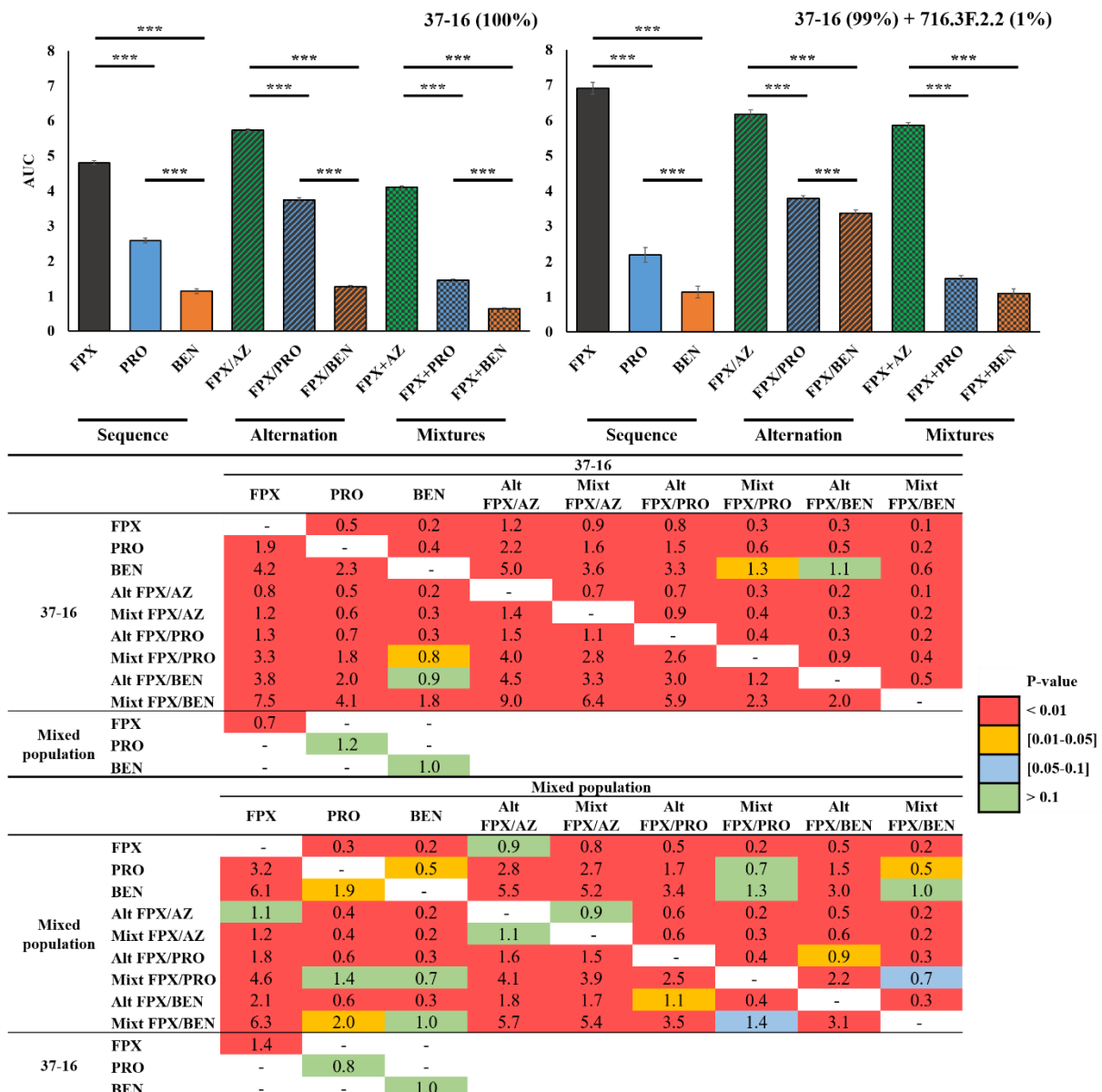


Figure 3.4. Efficacy of the different selection regimes in experimental evolution. Top: Representation of AUCs obtained with the different selection regimes, by starting population (left: 37-16, right: mixed population). AUC is used as an estimate of the efficiency of selection regimes. ***: indicates the statistically significant differences ($\alpha = 0.05$) between fungicide treatments for each starting population. Bottom: ratios of the AUCs for each pair of selection regimes (the AUC of the selection regime on top of the column is the numerator in the ratio), with significant differences (red and orange). Comparisons were made for all regimes, independently for each starting population (top table: 37-16, bottom table: mixed population), as well as comparisons of straight selections between populations.

Single fungicides (fenpicoxamid, prothioconazole-desthio and benzovindiflupyr) displayed different activities of growth control at the previously determined selection concentrations. AUCs of fenpicoxamid-treated lines was 1.4 times higher when the cytochrome *b* G37V change

was introduced at 1% in the initial population, compared to when it was not. Generalization was achieved after 2 cycles in evolving mixed population lines. This is consistent with a rapid selection of the cytochrome *b* G37V change in concerned populations. Resistance to fenpicoxamid was quickly selected in lines evolving the pure cytochrome *b* G143A isolate, and fluctuated between 60% and 90% of the control growth from the 2nd cycle up to the end of the experiment. AUCs in fenpicoxamid-treated lines were also significantly higher (2-3 times and 4-6 times), compared to the straight use of prothioconazole-desthio and benzovindiflupyr, respectively. At the chosen selection doses, fungal growth was about twice as high with prothioconazole-desthio as with benzovindiflupyr (Fig. 3.3. and 3.4.).

Adding a fungicide to fenpicoxamid in alternation or mixture strategies decreased AUCs by a factor 1.2 to 7.5. The sole exception was for the alternation between fenpicoxamid and azoxystrobin in 37-16 lines, where the total growth was significantly 1.2 times greater compared to the one achieved with the straight use of fenpicoxamid. Noteworthy, the reduction in AUC was not significant in alternation of fenpicoxamid and azoxystrobin, compared to the straight sequence of fenpicoxamid on mixed populations, contrary to mixtures. Strategies including benzovindiflupyr or prothioconazole-desthio systematically reduced AUCs (1.3 to 7.5 times for 37-16, and 1.8 to 6.3 for the mixed population) in comparison to straight fenpicoxamid sequences, regardless of the ancestral population, confirming the interest of adding one of these partners to this high-risk fungicide.

3.3. Respective interest of fungicide partners to delay resistance to fenpicoxamid.

As mentioned previously, adding azoxystrobin to fenpicoxamid, either in mixture or alternation, had poor interest since AUCs were either similar to the straight use of fenpicoxamid or even increased fungal growth (alternation on 37-16). AUC was significantly reduced in one situation (mixture on 37-16) but this decrease was the significantly smallest ever noticed among other strategies (factor 1.2) (Fig. 3.4.). These results were expected, as the cytochrome *b* G143A change causing high resistance was introduced in the ancestral population. However, they confirm that convenient partners should not be concerned by resistance on their own, *i.e.* that they should be efficient *per se* on local populations.

By contrast, alternations and mixtures of fenpicoxamid with prothioconazole-desthio and benzovindiflupyr always significantly reduced total fungal growth (1.3-7.5 times). Nevertheless, AUC reduction was more efficient with benzovindiflupyr (2-7.5 times) than with prothioconazole-desthio (1.3-4.6), corroborating the greater efficacy of benzovindiflupyr at the

used dose already mentioned in straight lines. Prothioconazole and benzovindiflupyr had similar protective effects in only one situation, *i.e.* when used as mixtures with fenpicoxamid on the mixed ancestral population. However, in both cases, mixture outcompeted alternation, performing twice and three times better against 37-16 and the mixed population, respectively (Fig. 3.4.).

3.4. Interest of temporal heterogeneity to delay resistance to fenpicoxamid.

As mentioned in the introduction, alternation and mixture do not exert the same kind of heterogeneity in populations. In our experiment, mixtures always provided significantly better control of growth than alternations (AUC reduction by a factor 1.2-7.5 *vs.* 0.8-3.8). The only configuration where it was not observed was the combination of fenpicoxamid and azoxystrobin against the mixed population, in which alternation and mixture provided similar control. In particular, mixtures including benzovindiflupyr or prothioconazole-desthio performed between 2 and 3.1 times better than alternations, when comparing AUCs. The difference in control obtained by mixing fenpicoxamid and prothioconazole-desthio, rather than alternating them, was of the same order against the pure strain or the mixed population. In both cases, mixing provided 2.5 and 2.6 times better control than alternating respectively. Regarding the control of the mixed population, mixture and alternation of fenpicoxamid and benzovindiflupyr were statistically performing like the association of fenpicoxamid and prothioconazole-desthio (Fig. 3.4.).

3.5. Impact of strategies on the phases of resistance dynamics.

Evolution of fenpicoxamid resistance under various scenarios of selection was observed for a fenpicoxamid susceptible ancestral strain (37-16), and on an ancestral population including the double cytochrome *b* mutant G37V+G143A. In this experimental design, we hypothesized that a greater variety of resistance mechanisms (including the cytochrome *b* G37V change) might emerge and be selected in 37-16-derived lines, whereas the cytochrome *b* G37V change should be almost exclusively selected in the artificial population-derived lines. As the cytochrome *b* G37V change displays high resistance and low fitness penalty, we expect strategies to be defaulted more easily when starting with the mixed population (“worst case scenario”). By contrast, we expect strategies to be more efficient when applied on a fenpicoxamid-naïve strain, as we learnt from chapter 1 that additional resistance mechanisms of weaker interest and/or tolerance might be selected by fenpicoxamid, and might be as well in the field (“best case

scenario”). The overall population structure, depending on the evolutionary forces at work, might indeed mitigate the efficacy of strategies.

AUCs of the lines from the mixed population were overall larger (about 1.25 times) than those of the lines from the pure strain were, with the exceptions of lines treated by prothioconazole-desthio and benzovindiflupyr alone, which was expected, as both the pure strain and the population were susceptible to these fungicides. In particular, the efficacy of benzovindiflupyr-based strategies was less important, relatively to prothioconazole-desthio-based strategies, in lines derived from the artificial population. These findings suggest the fast selection of the cytochrome *b* G37V change as a main resistance mechanism in mixed-population-derived lines and confirm the greatest risk of resistance of this mechanism, compared to those that might have been selected in lines derived from the pure susceptible strain. Growth peaks in alternation lines for the cycles where fenpicoxamid was applied also exemplify this (Fig. 3). Such peaks were not observed for the pure strain-derived lines, which suggest that weaker mechanisms were selected. The correlations between AUC and the frequency of the cytochrome *b* G37V allele assessed by ddPCR in the forthcoming weeks should verify this hypothesis.

The phenotypic diversity of evolved lines was assessed using a droplet test on selection media at the end of the last cycle of experimental evolution (data not shown). However, the conditions of the assay were not sufficient to show the whole diversity of selected resistance mechanisms. It would have required further optimization that could not be made due to lack of time. Moreover, some lines displayed too low surviving spore concentration so no growth was observed, even on the control fungicide-free medium. For the majority of the lines, the resistant spore concentration was not high enough, thus preventing us from capturing the diversity. Consequently, from observed results, diversity appeared low but was likely underestimated. Droplet tests should be repeated after technical optimization.

4. DISCUSSION.

In the previous chapters, we demonstrated that the risk of field resistance for fenpicoxamid was high, given the high resistance observed *in vitro* and *in planta* and the low fitness penalty associated to the cytochrome *b* G37V change, most likely to be selected. Consequently, we aimed at identifying the fenpicoxamid-based anti-resistance strategies that would increase the most the heterogeneity of the selection pressure in this specific context, thus delaying resistance selection and increasing fenpicoxamid overall sustainability. From an operational point of view, the early identification and implementation of appropriate strategies should allow avoiding

repeating mistakes from the past, like, for example the ones made with the unrestricted use of strobilurins (Bartlett *et al.*, 2002). Using experimental evolution, we tried to decipher which and how components of the strategies (*i.e.* number of active ingredients, their intrinsic risk to select resistance and the temporal heterogeneity of the selection pressure over fungal generations) would influence the different phases of the resistance dynamics (*i.e.* emergence or selection). We conducted the experiment using a constant dose of fungicides, close to the MIC, over 8 cycles. We also tried to determine if the strategies would affect the phenotypic diversity of the resistance.

4.1. The optimal strategy results from the smart combination of its components.

Our experiment suggests that the intrinsic risk of resistance attributed to each fungicide might rank first to guarantee the efficacy of a strategy. Confirming previous observations, straight fenpicoxamid was not able to control the frequency increase of the cytochrome *b* G37V mutant in the mixed population, which was expected from our previous work. In comparison to the other fungicides tested, resistance was selected faster in fenpicoxamid treated lines, in our conditions. This hypothesis is corroborated by the results of chapter 1, where resistance was selected as soon as the second cycle in some lines. This demonstrates that fenpicoxamid exhibits a higher risk to select resistance than prothioconazole-desthio or benzovindiflupyr. In addition, prothioconazole-desthio selected resistance significantly faster than benzovindiflupyr, implying that at this concentration and in our experimental conditions, prothioconazole-desthio may provide lower protection to fenpicoxamid. This stresses the importance of the choice of partners, and of their respective efficacy (that is decreased by the corresponding resistance) when designing a strategy. Indeed, strategies including two partners (*e.g.* fenpicoxamid and azoxystrobin) sometimes performed worse than straight applications, confirming that the intrinsic risk of resistance of a fungicide outcompetes other components when determining sustainability. The contrasted behaviors of benzovindiflupyr and prothioconazole-desthio also modulate the ranking of resistance risk provided by the FRAC, with SDHIs considered at medium to high risk, and DMIs at medium risk. This discrepancy might rely on the accuracy by which selection concentrations were established, as the dose-response curves are sometimes leptokurtic and then difficult to model. Moderate control of fungal growth ($\approx 40\%$ compared to the control, despite acceptable control $\approx 80\%$ in preliminary validation assays) even at the first cycle suggests that the selection concentration of prothioconazole-desthio might have been underestimated. From a practical point of view, this partial control might mimic to the one of

DMIs in field populations from Western Europe, where DMI efficacy is partly compromised because of resistance (Jørgensen *et al.*, 2021).

The temporal heterogeneity of the selection pressure ranked second, in terms of importance for reducing resistance evolution. Redundant intragenerational killing provided by mixtures seemed more efficient than straight selection (1.3 times better if including strategies with azoxystrobin, 2.7 times otherwise) or that intergenerational killing provided by alternations (1.6 times better if including strategies with azoxystrobin, 2.6 times otherwise). However, this component might be more sensitive to population structure (*i.e.* to the type of resistance mechanisms to be selected and their proportions), as established from its efficacy on pure *versus* mixed ancestral population. In our example, mixture might reduce the selection coefficient because redundant killing decreases the multiplication rate both of the susceptible and the resistant strains (rationale 1; see introduction). The efficacy of alternation is based on rationale 2 and assumes a reduction in increase of resistant strains, comparatively to susceptible ones. This is allowed in cycles in which the selection fungicide is not used, and if it entails a fitness cost. Here, we concluded in chapter 2 that cytochrome *b* G37V mutants exhibited fitness similar to the WT strains. This may explain the lower interest of alternation compared to mixture in our specific case. Moreover, our experimental design may suffer from a bias due to a confounding factor. Indeed, to ensure comparison between strategies (8 selection doses of each fungicide over the course of the experiment), mixtures were constituted by 0.5 the selection dose of each fungicide, and not a dose equivalent to the MIC efficacy for single fungicides. Consequently, mixtures applied relatively higher selection dose than other regimes, because of strong synergy observed between tested active ingredients. This may introduce a bias when comparing alternation and mixture (Amaradasa and Everhart, 2016), and an experiment at equivalent efficacy (*i.e.* using the MIC of the mixture) would be necessary to draw more solid conclusions about the superiority of mixtures over alternations. The same bias is observed in modeling studies that often designate mixture as the most efficient strategy (Hobbelen *et al.*, 2013), mostly because mixed fungicide partners are also used at their maximal dose in the field (Rex consortium, 2013). Therefore, comparisons with other strategies should always be taken with caution, especially knowing that the use of mixtures favors the selection of multiple resistance or of generalist resistance mechanisms, such as MDR (reviewed in Jansen *et al.*, 2013; PhD Maxime Garnault, unpublished). In addition, some recent experimental evolution studies from our laboratory showed that alternation might outcompete mixture, thus reviving the debate (van den Bosch *et al.*, 2014; Rex consortium, 2013; Ballu *et al.*, unpublished).

The third component influencing resistance dynamics was the number of active ingredients to be used in the strategy. This parameter seemed to affect resistance selection to the same extent as temporal heterogeneity. Indeed, when associated with a partner of lower risk of resistance (*e.g.* prothioconazole-desthio or benzovindiflupyr), fenpicoxamid resistance was systematically delayed (AUCs on average 1.5 times lower) compared to straight selection, with little influence of the resistance mechanism selected. Otherwise, association with a partner of high resistance risk has a neutral or detrimental impact on resistance evolution. This highlights again the major importance of AIs mode of action and suggest that solo use can be an option, in some situations, especially in the European context of reducing pesticide use.

4.2. Synergy modulates the efficient dose of mixtures and then govern their sustainability.

Dose-response curves established for mixtures clearly demonstrate synergy between fenpicoxamid and other fungicides, *i.e.* their activity used in a mixture is greater than the sum of the individual activities of each partner (Sen *et al.*, 2019). From a practical point of view, this means that to reach the MIC of the mixture, the concentration of each partner in the mixture should be lower than 50% of its own MIC. Then, synergy assessment suggests that this phenomenon modulates the efficient dose of mixtures, and so may govern sustainability since dose reduction may accelerate the selection of quantitative resistance (van den Bosch *et al.*, 2011; Busi and Powles, 2009). This could bring some insights about the way we generally design fungicide association use in the field, by preferring associations that maximize the synergy coefficient against most of the field isolates. Therefore, the acclaimed interest of mixture in literature and by stakeholders might be rather due to the relatively higher doses of the partners, due to their synergy, rather than to the asserted redundant killing effect. Indeed, commercial mixtures often contain fungicides each at a high dose (*i.e.* close to the dose used in a solo formulation), implying that they may be beyond the point where synergy is the highest. Consequently, optimizing mixtures would mean optimizing the synergy between partners.

It appeared from our calculation that synergy was higher when combining two respiratory inhibitors (except if the isolate is resistant to one of them) than that observed when combining fenpicoxamid with prothioconazole-desthio, which targets sterol biosynthesis. In addition, synergy between inhibitors of two independent complexes of the mitochondrial respiratory chain (*i.e.* QiIs and SDHIs) was higher than that of the association QiI/QoI that both target the same protein. We also noticed differences in synergy depending on the tested strain. We may also assume that resistance to one or to both partners should reduce synergy. Finally, the strong

synergy measured here between fenpicoxamid and benzovindiflupyr or prothioconazole-desthio might not be as strong with current European field strains already resistant to these fungicide partners. Synergy is then to be established for each pair of fungicides, and for each genotype. These findings jeopardize the prediction of synergy, and then of the efficacy of a given mixture under field conditions, as the efficient fungicide dose reaching the fungus might be highly dependent on the environmental conditions, and as populations encompass various genotypes, in variable proportions. Synergy coefficient might evolve over time through populations change. Therefore, the robustness of unisite fungicide mixtures seems difficult to anticipate in all situations and is part of a gamble, not knowing how populations will evolve and how fast.

This then sets the discussion about using high-risk fungicides at their maximal dose rate (as authorized by its toxicity and ecotoxicity) but less frequently, instead of a mixture of poor synergy (*e.g.* because of evolving resistance), that might select generalist strains hardly controlled and expose the partner unnecessarily. This strategy, to be tested, for example, in further experimental evolution, exploits rationale 3, that suggests decreasing exposure time, as well as rationale 1, because of the high dose.

4.3. Recommendations for tailor-made anti-resistance strategies for fenpicoxamid.

Given that fenpicoxamid has a high intrinsic risk to select resistance (based on the results of chapters 1, 2 and 3), it needs to be protected by fungicides with a lower intrinsic risk and highly efficient, while improving the impact of the other parameters of the strategies. To this point, benzovindiflupyr appears as the best fungicide partner for fenpicoxamid among those tested here. Indeed, strategies including benzovindiflupyr were the most efficient in all situations, corroborating that benzovindiflupyr selected the least resistance among all fungicides used alone. Moreover, benzovindiflupyr presented the highest synergy coefficient with fenpicoxamid. However, other partners (*e.g.* other DMIs or SDHIs or metyltetraprole) may display similar interest and should be tested in a more exhaustive manner. In addition, in the field, anti-STB strategies need to be integrated among strategies aiming at controlling other foliar diseases such as rusts, against which strobilurins present a high efficacy, and benzovindiflupyr is one of the most efficient SDHI.

To conclude, the best strategy emerging from our study is the use of a mixture of fenpicoxamid and benzovindiflupyr at the efficient dose of both partners. This strategy allowed good control of fenpicoxamid resistance emergence as well as resistance selection when the cytochrome *b*

G37V change was introduced in the population, implying significantly greater durability associated to this pair. However, one limitation in the field would be the yearly increasing frequency of SDHI-resistant isolates in populations. We assume here that the component settings combined in this strategy are the more favorable to fenpicoxamid sustainability, and should be searched when designing other options. Indeed, our observations highlight the importance of building strategies adapted to each new active ingredient, to each pathogen and to resistance status in field populations. In other words, it would be difficult, or even irresponsible, to recommend a universal strategy that may fit all situations. This study provides some insights about the way to build the best anti-resistance strategies to maintain fenpicoxamid sustainability, while analyzing the relative efficacy of some of their components. Our main findings are the following:

- Fenpicoxamid appears as a high-risk fungicide, considering the characteristics of the resistant mutants most likely to be selected in populations (chapters 1 and 2), and growth control in lines treated by fenpicoxamid alone (Fig. 3.3. and 3.4.).
- Its most adapted partners need to display a few characteristics. First, they must not share positive cross-resistance, linked to the target or not. They should be at lower risk of resistance and exhibit a high degree of synergism, ideally with the maximum synergy coefficient obtained for the doses used in the field. They need to be highly efficient on their own at the dose used. These criteria should be adapted to local field genotypes for each active ingredient.
- The interest of mixture over alternation could be explained by the lack of significant resistance cost associated to fenpicoxamid resistance that could express during untreated sequences, contributing to the decrease in resistance frequency. It can also be due to the higher global selection pressure exerted by mixtures, because of high synergy measured on fenpicoxamid-sensitive strains. However, synergy might evolve over time with the generalization of SDHI resistance.
- Due to its high intrinsic risk to select resistance, the deliberate limitation of fenpicoxamid general use in the field, *e.g.* less than one application per year (considering that French recommendations now include single-spray strategies), but at the highest possible dose, to the benefit of other control measures, might also help preserving its efficacy in a long-term view. These recommendations have already been implemented for SDHIs in France and contributed to delay this resistance, by contrast to some other European countries.

GENERAL DISCUSSION

The control of target pathogens (bacteria, viruses, and fungi) depends on our capacity to manage their adaptation (*i.e.* resistance selection) by developing new drugs while ensuring their sustainable management (*i.e.* maintaining a heterogeneous selection pressure). This arms race is the result of a process similar to the co-evolution described for host-pathogens interactions, where the introduction of a new drug creates a change in the environment of the target organism, which, on its turn, adapts by developing resistance, thus reducing drug efficacy, requiring a “new drug”, and so on (Knobler *et al.*, 2003). Therefore, predicting adaptation to drugs as early as possible has become a primary issue, to anticipate the implementation of efficient management strategies. Resistance selection and evolution have been thoroughly studied, in order to identify their main drivers, and determine how one can influence them to delay resistance selection (reviewed by Hawkins, 2019). The purpose of this study was to provide a conceptual framework for the prediction of resistance selection to new drugs, prior to their use in practice, by exploiting the case of a new agricultural fungicide, namely fenpicoxamid. This framework aims to improve the sustainable management of new drugs, regardless of the field of use or targeted organism.

Fenpicoxamid displays a new mode of action to control *Zymoseptoria tritici*, the causal agent of *Septoria tritici* blotch (STB), the most important foliar disease of wheat. Fenpicoxamid is a picolinamide QiI, which inhibits the mitochondrial respiration by binding to the cytochrome *b* Qi site. Consequently, no previous data were available on the interaction between picolinamides and *Z. tritici*, making this study entirely predictive. The first question to be addressed was the identification of the main resistance mechanism (or mechanisms) to be developed by *Z. tritici* under fenpicoxamid exposure. Evaluating the risk of selecting resistance in practice was the second objective, using a thorough characterization of the resistant isolates. The final part of the project aimed at understanding how different strategies might delay fenpicoxamid resistance emergence and selection and their basis.

Using directed evolution, we were able to demonstrate that target site resistance was the most likely resistance mechanism to be selected by fenpicoxamid, as it is true for most cases of fungicide resistance in phytopathogenic fungi. We identified the cytochrome *b* Qi site change G37V as the most probable substitution to trigger fenpicoxamid resistance. Isolates bearing this change displayed high resistance factors (RFs) to fenpicoxamid at both the enzyme and cellular levels. The cytochrome *b* G37V change also halved the enzyme activity but without visible impact on *in vitro* growth or pathogenicity in the absence of inhibitor. RFs *in planta* were lower but fenpicoxamid efficacy was reduced by more than 50% against the resistant population.

These RFs are likely to cause a decrease in fenpicoxamid efficacy in the field if resistant isolates were to be present at sufficiently high frequencies in populations. The frequency threshold before practical efficacy loss is yet to be determined. We have identified that the sustainability of fenpicoxamid-based strategies depended mainly on the combination with different modes of action (heterogeneity of the selection pressure), in particular with a partner fungicide not affected by cross-resistance, preferably with a lower intrinsic risk of resistance, and highly synergistic with fenpicoxamid. Such strategies should be the most efficient in delaying the emergence and selection of fenpicoxamid resistance, whether it is triggered by the cytochrome *b* G37V change or by any other resistance mechanism.

1. Identification of the fungal keys to success.

In the arms race between fungal pathogens and fungicide development, characterizing the strengths and weaknesses of the fungal enemy is important in considering effective control strategies. The identification of resistance mechanisms is important as not all mechanisms affect fungicide efficacy to the same extent, and the corresponding control measures would not be the same. The fitness of resistant isolates should give insights into the likelihood of selecting these isolates in practical conditions. Finally, designing the best anti-resistance strategies is crucial, because the earlier they are implemented in the field, the more resistance selection is delayed.

1.2. Predicting resistance emergence and resistance mechanisms.

Assessing evolutionary responses to a selection pressure is not so straightforward. Indeed, simulating evolution in a natural environment carries several challenges. In order to circumvent these difficulties, several approaches were developed, each of which comes with its own advantages and bias.

1.2.1. Mutagenesis to increase the mutation rate.

Mutagenesis has been employed in the laboratories for more than 50 years in order to predict resistance (Hawkins and Fraaije, 2016). Indeed, mutagenic agents increase the mutation rate, therefore increasing the possibility of selecting resistant mutants. However, even if this method is more exhaustive, genotypes are not selected according to their fitness. Its predictive value may then remain limited. Indeed, literature abounds with examples of mutations selected in the laboratory but never found *in natura* (Hawkins and Fraaije, 2016; Rehfus *et al.*, 2018). This approach displays other limitations, as listed in chapter 1, but proved accurate in some cases (Azam *et al.*, 2003; Denholm *et al.*, 1998; Rawal *et al.*, 2013; Scalliet *et al.*, 2012).

To avoid additional mutations that could be triggered by random mutagenesis, the evolution in molecular biology techniques allows performing site-directed mutagenesis, thus introducing mutations in the target gene (Lalève *et al.*, 2014b). However, that approach cannot be applied without *a priori* knowledge of the resistance mechanism, and is mostly used to perform functional validation of mutations already identified, sometimes in other species displaying orthologous genes, where resistance to similar chemistries or modes of action has been demonstrated. Consequently, it is more difficult to assess resistance to a new mode of action, or a new chemical group with this approach. Moreover, molecular biology techniques are not suitable for mitochondrial genes, in most species.

1.2.2. Protein structure to identify the impact of mutations.

The increasing knowledge in modeling protein conformation and calculating its stability, as well as in deciphering the energy calculation that governs protein-protein interactions or ligand binding, allowed the development of new approaches to assess drug resistance. Some studies have used genomic sequences to predict whether a mutation is likely to trigger resistance, by estimating stability of mutated protein, and affinity for its inhibitor(s) and natural substrate(s) (for review Cao *et al.*, 2005; Frey *et al.*, 2010; Phelan *et al.*, 2016). It is therefore possible to evaluate unlimited possible mutations, hence performing structure-based virtual screening, using tools adapted to any ligand-protein interaction (Wu *et al.*, 2020). Such approaches have been used to predict resistance mutations in viruses (HIV-1 and hepatitis C virus), bacteria (*Mycobacterium tuberculosis*, *Neisseria meningitides*, *Helicobacter pylori*...), parasites like *Plasmodium falciparum*, and cancer cells (reviewed in Cao *et al.*, 2005).

1.2.3. Machine learning.

Progress in molecular biology and decreasing costs of sequencing triggered the emergence of big genomic datasets, used to implement computer programs and develop machine-learning approaches that predict antimicrobial resistance. Using the sequenced genome of new isolates, machine learning is able to predict their resistance status towards various antibiotics, simply by extrapolation from implemented data associating genotypes with resistance status, an approach similar to GWAS (Genome-Wide Association Study). It proved particularly accurate in predicting antibiotic resistance in human pathogenic bacteria (reviewed in Pesesky *et al.*, 2016), as in *Escherichia coli* where resistance is regularly linked to genes located in dispensable parts of the genome (Her and Wu, 2018), with huge variations among isolates. Combined with transcriptomic data, this approach allowed early identification of *Pseudomonas aeruginosa*

isolates' resistance profiles, using loci outside from the targeted genes (Khaledi *et al.*, 2020). Limitations of this approach would be the need for a large set of empirical data for prediction. Therefore, it cannot be applied to new chemistries or new modes of action, where there is a lack of data. Moreover, resistant isolates need to be detected and sequenced first, meaning that the predictive value of this approach is limited.

1.3. Predicting resistance selection.

1.3.1. Mathematical models (theory driven).

The characteristics and relevance of mathematical and statistical models to predict resistance dynamics were discussed in chapter 3. Mathematical models predict the evolution of resistance (in microbes and cancer cells) knowing that resistance has emerged, but without any assumptions regarding the putative mechanisms and their implications, and often without validation from empirical data (for review Birkegård *et al.*, 2018; Lavi *et al.*, 2012). They have been used to predict the efficacy of fungicide-based strategies (van den Bosch *et al.*, 2011; Hobbelen *et al.*, 2013), and their impact on the different phases of resistance selection (Hobbelen *et al.*, 2014). However, mathematical models are simplified descriptions of the reality, removed from the pathogen's biology (reviewed in Birkegård *et al.*, 2018; Lavi *et al.*, 2012). Consequently, their conclusions can hardly be extrapolated to other situations, especially regarding a new mode of action (Rex consortium, 2013).

1.3.2. Statistical models (data driven).

Statistical models using empirical data model and learn from past evolution to predict future evolution of resistance. They have been successfully used to describe quantitatively resistance evolution in French populations of *Z. tritici* (Garnault *et al.*, 2019) and identify its determinants, or to evaluate the risk of resistance selection (Grimmer *et al.*, 2014). In the medical field, these models have been used intensively to predict evolution of drug resistance, especially in cancer cells (reviewed in Sun and Hu, 2018). Statistical modeling is a powerful tool to predict resistance selection and evolution. Moreover, it has benefited from the considerable and ongoing progress of computer technology, as well as from the ever-increasing accumulation of empirical data. Among limitations of modeling is the decrease in its accuracy when extrapolating to new situations (*i.e.* new drugs or new target organism for example), which is not even always possible.

1.4. Evolution in the laboratory.

One may consider that mutagenesis (random or directed) followed by mutant selection on drug-amended medium is somewhat similar to directed evolution. In my opinion, directed evolution is different, at least considering the way we have used it in our study. Here, directed evolution allowed mimicking natural selection, *i.e.* natural evolution of genotypes under drug selection pressure, without the use of mutagenic agents (reviewed in Kawecki *et al.*, 2012). Therefore, in directed evolution, adaptive landscapes are more similar to those observed *in natura* (in our study this applies to the pycnidiospore/blastospore stage, see introduction 3.4.), with the least fit mutants eliminated over the generations, or rescued by compensatory mechanisms. Indeed, the selection of compensatory mechanisms in the field is one of the reasons put forward to explain the discrepancies between mutant fitness observed in the laboratory and in the field (Lalève *et al.*, 2014a; Mikaberidze and McDonald, 2015). Even if there is a gap between experimental evolution and field conditions, making prediction more difficult, the predictive value of experimental evolution remains superior to that of mutagenesis in my opinion. Indeed, resistance is selected only by fungicide selection pressure, and resistant isolates can be thoroughly characterized, thus giving insight on the likelihood of their selection in nature. Experimental evolution is a good compromise to assess drug resistance selection for a drug, in particular prior to its use in practice, with the advantage of giving insights on both resistance selection and its outcome.

1.5. Fitness is an important driver of resistance selection.

In the absence of any anti-resistance strategy, the selection of the resistant isolates in the field relies on several factors, the main one being the fitness of these isolates. The main issues regarding the definition and the measurement of fitness were detailed in the introduction and in chapter 2. The difficulty in assessing fitness outside of the field situation relies on the many traits that cannot be measured in the laboratory or the greenhouse. Regarding the situation of *Z. tritici*, the inability to assess traits related to sexual reproduction or overwintering periods is critical, as they are direct components of its absolute fitness (Suffert *et al.*, 2015). Moreover, fitness also encompasses the ability to grow and reproduce under fluctuating conditions, while conditions in the laboratory or the greenhouse are always stable and controlled (Jallet *et al.*, 2020). Nevertheless, thorough characterization of resistant isolates at different levels (enzyme, whole cell, *in vitro* and in the host plant) may partially compensate for the lack of measured traits by assessing the relative fitness of resistant isolates compared to susceptible ones. Moreover, comparing strains with high genotypic similarity, even isogenic if possible increases

the significance of the study. Such assessment of relative fitness under laboratory and greenhouse conditions therefore gives a reliable estimate of the risk of selecting these resistant isolates under field conditions.

1.6. Assessing the efficacy of anti-resistance strategies.

Trying to predict the effectiveness of anti-resistance strategies without testing them in the field is also a challenge. Like assessing fitness of resistant isolates, assuming to evaluate strategy efficacy in the laboratory may seem unrealistic, in conditions so different from the field environment. However, several tools are available to help filling this gap as best as possible. As seen previously, experimental evolution allows mimicking the different phases of natural resistance selection, under miniaturized laboratory conditions. Consequently, it becomes possible to change different experimental parameters to simulate fungicide-based strategies. Performing such assays simply means evaluating the pathogen's adaptation to different growth conditions, as it has been done in many studies (reviewed in Kawecki *et al.*, 2012). Interestingly, in spite of this obvious opportunity, experimental evolution has rarely been used in fungi to evaluate the efficacy of anti-resistance strategies, highlighting the under-utilization of experimental evolution in fungi (Fisher and Lang, 2016). In contrast, this type of experiment has been widely used to evaluate strategies to delay antibiotic resistance in human pathogenic bacteria (reviewed in Jansen *et al.*, 2013). This discrepancy may be explained by the overall longer generation time of fungi compared to bacteria, or simply by our scientific culture. Concerning fungi, theoretical mathematical models have been widely used to assess the efficacy of different strategies on the different phases of fungicide resistance evolution (van den Berg *et al.*, 2013; van den Bosch *et al.*, 2014; Hobbelen *et al.*, 2013, 2014). Models allow understanding how the different components of a strategy affect the different phases of resistance selection on their own, or in combination. The advantage of models is that they can simulate in-field conditions, within certain limitations as mentioned above.

1.7. Advantages and limitations of our study.

Predicting the risk of selecting fenpicoxamid resistance in *Z. tritici* field populations presented a number of challenges. We used experimental evolution based on its similarities to natural selection by fungicide exposure in the field. Moreover, experimental evolution is particularly adapted to phytopathogenic fungi, as fungicide resistance is more prone to emerge from *de novo* mutations (Hawkins *et al.*, 2019). However, as shown in chapter 1, resistance selection by directed evolution was highly variable, suggesting that we might have failed to select stable

resistant mutants in the first place. Nevertheless, it was possible to evaluate the likelihood of selected resistance mechanisms by comparing results to existing data, and by assessing the fitness of resistant isolates. These results should make the prediction more reliable. In our case, target site resistance was selected, which is known to be the most common resistance mechanism in fungi (Lucas *et al.*, 2015). The selection of the cytochrome *b* G37V change was also significant, because in other species codon 37 had already been associated with resistance to UK-2A and antimycin A, a compound structurally related to fenpicoxamid. In addition, cytochrome *b* G37V isolates did not display reduced pathogenicity *in planta*, meaning that the likelihood of cytochrome *b* G37V selection in field isolates under fenpicoxamid selection pressure is high. Therefore, experimental evolution appeared as a powerful method to assess the risk of resistance selection, which was the main goal of this project.

Nevertheless, our study also had some limitations. The first one was the randomness of resistance selection as explained in chapter 1. Indeed, given that outcomes of selection were highly variable in the first place, any conclusions related to the implementation of anti-resistance strategies, assayed under the same conditions, should be taken with caution. This variability may be explained by the lack of exhaustiveness in sampling growing colonies, for technical reasons. In fact, this bias should be taken into account when using such approach to study the risk of resistance selection to other compounds, especially the newly developed ones. The observed variability may also come from fungicide dose fluctuations. Indeed, when dispatching concentrated fungicide solutions of highly active compounds, small volume variations may trigger some significant variations in fungicide concentration. Moreover, fungicide concentrations may vary over time in cultures, due to metabolism that would depend on cell concentration (hence fungal biomass). These observations revive the dose rate debate (van den Bosch *et al.*, 2011), which is not specific to experimental evolution. The fungicide dose has been shown to influence the outcome of selection (Gutiérrez-Alonso *et al.*, 2017), and the success of anti-resistance strategies (van den Berg *et al.*, 2013), in various and sometimes contradictory ways. One possibility to circumvent this issue would be using bigger volumes of less concentrated solutions, but this may involve problems regarding higher solvent concentrations. Another possibility would be automation to allow more accurate pipetting, as probably used in the industry.

The second main limitation of our study would be the assessment of *in vitro* evolution of a pathogen that usually evolves in a fluctuating field environment, a limitation shared by many approaches when it comes to resistance evolution studies. Indeed, working with a single

genotype, there is no variability in fungicide susceptibility in the starting population. On the contrary, in the field, the very diverse genotypes present introduce a variability in fungicide susceptibility among populations, therefore challenging the resistant isolates to various extent depending on the population composition. Moreover, other competing microorganisms, weather conditions or host plant genotypes, all challenge resistant isolates too (Walker *et al.*, 2017), even more so in a world where climate conditions are evolving due to global warming. For example, in recent years, levels of STB in French wheat fields were low due to particular dry springs (Arvalis, 2019), which may affect resistance dynamics. Only a few attempts to perform experimental evolution in the field have been made (for review Kawecki *et al.*, 2012; Zhan and McDonald, 2013), due to obvious logistical, legal or ethical issues. The impossibility of preventing the dissemination of resistant isolates remains the major issue, especially when studying resistance to a new fungicide prior to its field use. Consequently, it is not possible to fill completely the gap between field and laboratory conditions, especially in the case of our study. However, our choice to assess as many traits of the resistant isolates as possible and to compare our findings to existing data was a reliable manner to evaluate the likelihood of selecting fenpicoxamid resistance in the field.

Experimental evolution also revealed the existence of a transient resistant state in *Z. tritici*. We suspect this could either be due to an unstable heteroplasmic state or to a tolerance phenomenon (see chapter 1, Results and discussion). Tolerance, often described for human pathogenic yeasts or bacteria, was never reported for a phytopathogenic fungus to our knowledge. Tolerance is based on the activation and repression of several metabolic pathways (reviewed in Berman and Krysan, 2020), while the fungal cell remains in a quiescent stage (Bojsen *et al.*, 2017). Tolerance results in slow growth under fungicide exposure, and is not heritable, contrary to resistance. We can hypothesize that reduced growth is deleterious in the field due to the host's seasonality, compared to human pathogens evolving in a stable environment. This may explain why this phenomenon has never been explored and reported in fungal field populations. However, tolerance has been shown to enable resistance selection towards human antifungals (Arastehfar *et al.*, 2020); hence, it may represent a threat for agricultural fungicide sustainability as well. So far, tolerance has been reported in several yeast species like *Candida* species or *Cryptococcus neoformans* but never in filamentous fungi, including human pathogens such as *Aspergillus fumigatus*. Consequently, the phenomenon we observed in *Z. tritici* would have required further assessment to decipher the underlying mechanisms, and assess whether or not it is likely to appear in the field. Nevertheless, highlighting the existence of such transient

resistant phenotypes may be important when implementing anti-resistance strategies against phytopathogenic fungi.

2. The specific situation of mitochondrial respiratory inhibitors.

As key organelles, mitochondria play an essential role in the eukaryotic cell life cycle by participating in many metabolic processes, including virulence or drug resistance and tolerance in fungi. Indeed, regarding human pathogenic yeasts, mitochondria have been found to contribute to this drug tolerance phenomenon, through their role in lipid homeostasis (Shingu-Vazquez and Traven, 2011), and in activating the overexpression of membrane transporters, highlighting again the role of mitochondria as central cellular organelles. Due to their role, mitochondria have been prime targets for antifungals used in agriculture, as shown by the development of strobilurins, SDHs and QiIs. Dealing with mitochondrial target proteins, especially the ones encoded in the mitochondrial genome (*i.e.* cytochrome *b* as opposed to the SDH encoded in the nucleus), is a situation somewhat different from what is encountered for other modes of action. Indeed, mitochondria constitute a world apart in the cell, with specific features.

2.1. The mitochondrial matrix, a highly mutagenic environment.

Due to their enzyme activity, OXPHOS protein complexes produce large amount of ROS like superoxide, singlet oxygen or peroxides, especially through complexes III and I (Murphy, 2009). Moreover, ROS production has been proven to self-activate when reaching a certain level (Zorov *et al.*, 2014). Under physiological mitochondrial functioning, excess of ROS is scavenged by several enzyme classes such as glutathione peroxidases, glutaredoxins, peroxiredoxins, superoxide dismutases, thioredoxins and glutathione-S-transferases, which constitute the ROS defense system (Starkov, 2008). However, when mitochondrial function is impaired, ROS accumulation may overtake scavenging capacities of the defense system, triggering a highly oxidative environment. Consequently, ROS overproduction triggers genome instability, which was shown to be involved in cellular ageing or in the development of human diseases such as cancers (Yang *et al.*, 2016), neurodegenerative diseases (Weissman *et al.*, 2007) or depression (Czarny *et al.*, 2018).

The environment of the mitochondrial matrix implies that the mitochondrial DNA is exposed to more extensive and persistent oxidative damage than the nuclear DNA (Stein and Sia, 2017). In this context, DNA mutation rate is likely to be higher in the mitochondria than in the nucleus, which is observed in mammals (Saccone *et al.*, 2000). However, *Z. tritici* displays an extremely

conserved mitochondrial genome while its nuclear genome presents an extraordinary plasticity (Torriani *et al.*, 2008), which may seem paradoxical. If the explanation for this paradox is to be found inside the cell, it seems unlikely to result from a lower mutation rate in the mitochondrion. It could be explained by the presence of several copies of the mitochondrial chromosome, which may favor preserving non mutated copies (Stein and Sia, 2017), and/or by the presence of specific mitochondrial DNA repair mechanisms (Stein and Sia, 2017) that may be more efficient compared to those of the nucleus. These mechanisms associated with systematic elimination of deleterious mutations, which are more likely to appear in the mitochondrial genome that contain only essential genes (Christie *et al.*, 2015), may explain the paradox of *Z. tritici* mitochondria.

Given that the emergence phase of resistance relies mainly on the mutation rate of the considered pathogenic species, it can be hypothesized that the mitochondrial environment being more mutagenic, it may accelerate the emergence of resistance when due to a mitochondrial gene mutation. Moreover, mitochondrial respiratory inhibitors are known to increase ROS production due to the impairment of the electron transfer (Schwarzländer *et al.*, 2009). In addition, it has been shown that even fungicides with non-mitochondrial targets, such as multisites or DMIs, are responsible for mitochondrial stress in several organisms like mouse, zebra fish, insects or human neuronal cells (Domico *et al.*, 2006; Petricca *et al.*, 2019; Sanchez *et al.*, 2020; Syromyatnikov *et al.*, 2020). It cannot be excluded that such situation also happens in fungi, implying that fungicide treatments, especially the use of mixtures, may increase the mutation rate in fungal mitochondria, hence accelerating the emergence of resistance to respiratory inhibitors (QoIs and QiIs). We can hypothesize that the exposure to fungicide selection pressure has consequences for the balance between DNA mutation rate and maintenance, which may favor emergence of mutations in such situations.

2.2. Consequences for resistance selection.

The history of resistance selection in *Z. tritici* seems to corroborate these assumptions. Indeed, among all unisite modes of action, resistance to QoIs is the one that emerged the most rapidly (reviewed in Lucas *et al.*, 2015). In the UK, QoIs were introduced in 1997, and isolates bearing the cytochrome *b* G143A change were detected as soon as 2001. Regarding the other modes of action, it is noticeable that resistance to benzimidazoles (targeting the β -tubulin) also emerged and spread very fast. It has been reported that about 50% of the field isolates displayed resistance less than ten years after benzimidazole field introduction in the UK. However, little was published about the emergence of benzimidazole resistance in *Z. tritici* (likely because it

was the first unisite fungicide class, so resistance was not anticipated, and because *Z. tritici* was a less important pathogen at that time), making it difficult to compare with QoIs. Nevertheless, in France, QoI resistance was almost generalized in the field seven years after their introduction, meaning that it spread very quickly. As explained in the introduction, the unrestricted use of strobilurins in fields where resistant isolates were already present, increased the dissemination of the cytochrome *b* G143A mitotype by influencing parenthood during sexual reproduction (Kema *et al.*, 2018). These observations highlight the higher risk of selecting resistance to fenpicoxamid that may spread very fast without any fungicide-based strategy implemented early.

2.3. The unique case of two modes of action with the same target protein.

Fenpicoxamid opens the way to a never before observed interaction between two modes of action, and its consequences for resistance selection. Indeed, QoIs and QiIs both target the mitochondrial cytochrome *b*, but bind to different sites, each of which is a catalytic site indispensable for proper functioning of the OXPHOS complex III. This implies that the evolution of resistance to QiIs may be unique. At the outset of the project, questions about the outcome of fenpicoxamid resistance selection, in a genetic context where cytochrome *b* already contained at the Q_o site, were numerous. Early in the study, based on results of directed evolution experiments, we hypothesized that the co-occurrence of the cytochrome *b* G143A and G37V changes was very unlikely as we failed selecting the double mutants, until subsequent experiment proved us wrong. However, the question remains whether the more difficult selection of cytochrome *b* G37V in a cytochrome *b* G143A context supports our early hypothesis, and whether early failure to detect double mutants was due to insufficient sampling and the impossibility of collecting growing colonies exhaustively during experimental evolution. The forthcoming assessment of cytochrome *b* G37V frequency in all experimentally evolved lines by ddPCR should provide further insights.

Aside from the uneven selection of cytochrome *b* G37V in cytochrome *b* G143A backgrounds, some of our other results seemed to point at interactions between both the Q_o and Q_i sites, influenced by the two resistance conferring changes. Indeed, we noticed an increased susceptibility of single mutants (cytochrome *b* G37V or G143A) to inhibitors of the respective opposite binding site, and lower *in vitro* RFs of the double mutants to QiI and QoIs compared to the respective resistant single mutants. However, RFs to fenpicoxamid *in planta* were higher in the double mutants than in the single cytochrome *b* G37V mutants, indicating that there might be a discrepancy in resistance observed *in vitro* and in the host plant, emphasizing the interest

of associating both approaches for precise risk assessment. Given the low levels of disease triggered by cytochrome *b* G37V single mutants and their ancestor strain IPO323, these results still need to be confirmed. Nevertheless, *in vitro* observations were validated at both the fungal cell and the enzyme levels, suggesting interactions at the protein level. Indeed, the functional interdependency of both sites has already been described (Cooley *et al.*, 2009), as mentioned in chapter 1. It has also been demonstrated that some mutations in or near the Q_i site, as well as the binding of Q_i site inhibitors, could impair the electron transfer between both sites and between the Q_o site and the Rieske iron-sulphur protein (Cooley *et al.*, 2005; Song *et al.*, 2015). It seems likely that the cytochrome *b* Q_o site change G143A may interfere in some way with fenpicoxamid resistance selection in the field. However, making any assumption on the outcome of such influence would not be realistic with so few evidences. To understand better the interaction between both sites, in the context of our study, spectral analyses like the ones described in existing literature would be of interest. I would allow evaluating the molecular dialog between the Q_o and Q_i sites, or between the Q_o site and the Rieske iron-sulphur protein, in the presence of fenpicoxamid, and in the cytochrome *b* G37V and/or G143A context.

3. Fenpicoxamid, a new mode of action, biochemistry and risk of resistance.

Displaying a new mode of action against *Z. tritici*, fenpicoxamid is at the same stage as boscalid was ten years ago. The efficacy of the ever-expanding SDHI class has been preserved since, even though SDHI-resistant isolates have increased in frequency in European populations in the past few years (Dooley *et al.*, 2016; Garnault *et al.*, 2019; Rehfus *et al.*, 2018). The implementation of management strategies and recommendations (limited number of applications and a systematic use in mixtures) have contributed to this sustainable situation, avoiding or at least delaying scenarios similar to that of strobilurins or benzimidazoles. Nevertheless, there are some particular considerations for SDHIs. The SDH catalytic core comprises three different subunits encoded in the nucleus. Consequently, mutations in different subunits have been shown to affect to different extents the enzyme activity and susceptibility of the target pathogens to SDHIs, with incomplete cross-resistance between active ingredients depending on the mutations involved (Lalève *et al.*, 2014b; Rehfus *et al.*, 2018; Scalliet *et al.*, 2012; Veloukas *et al.*, 2012). The SDHI case suggests to me that SDHI resistance is more likely to evolve quantitatively than qualitatively, similar to what was observed for DMIs. In the case of QiIs, a similar trend seems unlikely. However, future development of new QiIs may reshuffle the deck with respect to the results of our study and this assumption.

Fenpicoxamid also displays a brand new chemistry among fungicides. Picolinamides, such as UK-2A, have been known for a long time but have never been further developed commercially, probably due to their lack of stability when exposed to UVs (Owen *et al.*, 2017; Ueki *et al.*, 1996). Fenpicoxamid's chemical structure is different to those of the two other QiIs used in agriculture, cyazofamid and amisulbrom, which display exclusively an anti-oomycete activity, or anti-parasitic QiIs like HDQ (see introduction, 4.2.4.6.2.). Therefore, no extrapolation from the situation of existing QiIs can be made to predict resistance evolution to picolinamides. One exception would be AOX-based resistance that can putatively affect any inhibitor of the complexes III or IV. However, regarding AOX mediated resistance, the situation does not depend on the inhibitor, but on the considered pathogen as explained in the introduction (5.6.2.2.).

Another specific feature of fenpicoxamid is that it is a required metabolic activation. Profungicides represented only 17% of all agricultural fungicides in 2017, roughly as many proinsecticides (20%), but far behind proherbicides (37%) (Casida, 2017). This situation remains higher than that from the medical field, where prodrugs represented only about 12% of all registered drugs in 2018 (Rautio *et al.*, 2018). In spite of their relative low presence on the market, prodrugs are designed to display advantages such as better physicochemical properties (like for fenpicoxamid), better pharmacokinetic properties, the possibility to be targeted or specific, or a decrease in toxicity for example (Casida, 2017). On the other hand, prodrugs can also be the victims of their need to be bio activated. Indeed, resistance development by a decrease or a lack of activation has been reported in some cases, albeit rarely in fungi (see introduction, 5.6.2.3.2.). Profungicides being activated by different enzyme families, the situation for fenpicoxamid regarding potential resistance development through default in activation is unlikely but remains difficult to predict (see chapter 1).

4. Summary, conclusion and perspectives.

Our study revealed that the risk to select resistance to fenpicoxamid in *Z. tritici* field populations is high. Our approach tried to mimic as realistically as possible resistance selection in the field, and estimated the likelihood of resistant isolates to be selected by comparing our findings to preexisting data, and thoroughly characterizing these isolates. Even though fenpicoxamid brings a new context for resistance selection by the many aspects detailed above, our approach presents the advantage to incorporate a majority of these characteristics. To my knowledge, it

is the first time that such risk assessment is achieved, prior to a drug use in practice. It may be because such studies, if they exist, may have never been published.

The earlier anti-resistance strategies are implemented, the greater the durability of the fungicide (Rex consortium, 2013). Consequently, being able to identify the best strategies prior to the fungicide use in the field is a great asset to prevent resistance emergence and selection. To this end, our study provides guidance to protect fenpicoxamid. As already implemented for SDHIs in France, limiting the exposure time to fenpicoxamid is essential. The fungicide should be applied one time per season maximum in the case of several anti-STB treatments per season, but fungicide rotation can also be considered over several seasons. Indeed, the use of fenpicoxamid in the field must be considered within the general framework of crop protection, and needs to be adapted to the number of treatments per season, *i.e.* adapted to each country. Spatial alternation (mosaic) is also a possibility, albeit harder to implement in practice. Fenpicoxamid should be better protected if applied in mixtures, on the sole condition that there is no cross-resistance between the compounds and they are used at doses where each is effective. This means that neither fenpicoxamid nor its partner should be affected by resistance in the field, implying a close monitoring of resistance and an adaptation of strategies at fine geographical scales. Of course, we suspect that these recommendations may be difficult to apply in the field where the economic dimension is obviously crucial for farmers, and where coordinated action of the different actors can sometimes be difficult to achieve. Nevertheless, the use of mixtures in the field must always be carefully considered and precisely implemented as the distribution of both fungicides and their persistence can vary, increasing the risk of resistance selection for the least protected partner. Therefore, any reduction in the fungicide dose, which may be tempting due to obvious economic and agroecological reasons, should be considered wisely, and adapted to each local situation.

The framework we are providing through this study can serve as a model to anticipate resistance selection against agricultural or medical compounds, as it can be transposed easily to any case of study. This work aims to contribute to improved resistance management, in a current context of high pressure on agrochemical products and medical drugs, as exemplified by the many private pharmaceutical companies that ceased their research on antimicrobials (Årdal *et al.*, 2020) due to the rapid and systematic development of resistance. In my opinion, our innovative approach brings a new dimension to resistance management. We have confirmed that efficient resistance management relies on the deployment of optimal strategies, the success of which depends on their influence on the evolutionary adaptation of target organisms. Being able to

characterize thoroughly and realistically the different aspects of this adaptation opens the way to a different management of resistance, by anticipation and no longer *a posteriori*. This should allow the earlier implementation of efficient strategies, and thus increase the durability of new active substances like fenpicoxamid.

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Titre : Estimation du risque de sélection de la résistance aux fongicides de type QiII chez *Zymoseptoria tritici*.

Mots clés : Septoriose du blé ; Mitochondrie ; Inhibiteurs respiratoires ; Résistance liée à la cible ; Cytochrome *b* ; Antifongiques.

Résumé : La résistance aux fongicides représente un défi majeur pour la protection des plantes, et soulève la question théorique de la prédiction et de la réduction de l'adaptation chez les champignons. Cette thèse illustre cette problématique en étudiant l'évolution du pathogène fongique *Zymoseptoria tritici*, responsable au champ de la septoriose du blé, et du fenpicoxamide, représentant un nouveau mode d'action anti-septoriose en cours de développement, et inhibant la respiration mitochondriale en se liant au cytochrome *b*, au niveau du site de fixation interne de l'ubiquinone (site Qi). Nous avons abordé trois questions principales : 1/ Quel(s) est/sont le(s) mécanisme(s) de résistance le(s) plus susceptibles(s) d'émerger ? ; 2/ Quel est le risque pratique de résistance au fenpicoxamide en conditions contrôlées ? ; 3/ Quelles seraient les stratégies optimales pour réduire ce risque de résistance au fenpicoxamide ?

En utilisant l'évolution dirigée, nous avons identifié la résistance de cible, et en particulier une substitution au niveau du site Qi du cytochrome *b* : G37V, comme le mécanisme de résistance le plus probable, ce qui est cohérent avec l'évolution parallèle observée chez d'autres taxons résistants aux QiIs. Les isolats G37V présentaient des facteurs de résistance élevés contre le fenpicoxamide, à la fois au niveau cellulaire et enzymatique. La substitution G37V entraînait également une réduction de l'activité enzymatique de 50%, sans toutefois avoir d'impact visible sur la croissance *in vitro*. Les premiers résultats ont également révélé l'absence d'effet de cette réduction d'activité enzymatique sur la pathogénicité, chez des mutants portant les altérations du cytochrome *b* G37V et G143A. Les tests *in planta* indiquaient par ailleurs un fort impact de la substitution G37V sur l'efficacité du fenpicoxamide. L'ensemble de ces résultats suggère un risque élevé de résistance en pratique pour le fenpicoxamide, et souligne l'importance primordiale d'implémenter des stratégies efficaces à retarder l'évolution de la résistance au champ. Par évolution expérimentale, nous avons donc évalué divers scénarios de déploiement de fongicides, incluant le fenpicoxamide, mais différant par le nombre de différentes substances actives, leurs risques inhérents de sélectionner la résistance, et la stratégie d'application (par exemple, en alternance ou en mélange). Les premiers résultats indiquaient que le développement de la résistance peut être significativement retardé par des mélanges incluant un partenaire avec un mode d'action différent et présentant une forte synergie avec le fenpicoxamide. En effet, la synergie augmente la dose relative de fongicide appliquée, et permet un contrôle suffisant des génotypes résistants. Nous discutons cependant des limites de cette stratégie.

Les résultats de cette thèse ont démontré que l'évolution expérimentale est un outil précieux chez *Z. tritici* pour établir des hypothèses quant à l'évolution des champignons pathogènes, pour ce qui concerne la résistance aux fongicides. D'un point de vue opérationnel, elle a ouvert de nouvelles perspectives pour mettre en œuvre des recommandations responsables préalablement à la mise sur le marché de nouveaux fongicides, en accord avec la demande d'une utilisation plus prudente des produits phytosanitaires d'origine synthétique.

Title: Assessing the risk of resistance selection towards QiII fungicides in *Zymoseptoria tritici*.

Keywords: *Septoria tritici* blotch; Mitochondria; Respiratory inhibitors; Target site resistance; Cytochrome *b*; Antifungals.

Summary: Resistance to fungicides represents a major challenge for plant protection, and underlies the theoretical question of predicting and mitigating adaptation. This thesis exemplified this problem while studying the evolution of the highly adaptive pathogen *Zymoseptoria tritici*, responsible in the field for wheat *Septoria tritici* blotch (STB), and fenpicoxamid, a newly developed anti-STB mode of action, inhibiting mitochondrial respiration while binding at the cytochrome *b* inner ubiquinone binding (Qi) site. We addressed three main questions: 1/ What is/are the most plausible resistance mechanism(s) to emerge? ; 2/ How high is the risk of fenpicoxamid resistance under controlled conditions? ; 3/ What would be the optimal strategies to reduce the risk of fenpicoxamid resistance development?

Using directed evolution, we identified target site resistance, and in particular the cytochrome *b* Qi site change G37V as the most likely resistance mechanism, consistent with parallel evolution observed in other taxa resistant to QiIs. G37V isolates displayed high resistance factors against fenpicoxamid, at the cellular and enzyme levels. The G37V change reduced enzyme activity in the absence of inhibitor by 50% although with no visible impact on *in vitro* growth. First results also revealed no effect of the reduced enzyme activity on pathogenicity of mutants bearing the cytochrome *b* G37V change, with or without the G143A Qi site change. Susceptibility assays *in planta* indicated a strong impact of the G37V substitution on fenpicoxamid efficacy. Altogether, this suggested high risk of resistance for fenpicoxamid and stressed the primary importance of strategies efficient at delaying resistance development in the field. By experimental evolution, we then evaluated various scenarios of fungicide deployment, including fenpicoxamid but differing according to the number of different active ingredients, their respective inherent risk of resistance and the application strategy (e.g. alternation vs. in mixture). First results indicated that mixtures could significantly delay the development of resistance, when including a partner with different mode of action exhibiting strong synergy with fenpicoxamid. This increased the overall fungicide dose and allowed sufficient control of resistant genotypes. However, we discuss the limitations of this strategy.

The results of this work provided evidence that experimental evolution is a valuable tool in *Z. tritici* to predict evolutionary outcomes for fungicide resistance. From the practical point of view, it offered new perspectives to implement responsible recommendations prior to fungicide release, in agreement with the demand for more cautious use of synthetic plant protection products.