Master of Science thesis (“Cand. scient. speciale”)

Status on emerging resistance to succinate dehydrogenase inhibitors in *Zymoseptoria tritici* in Denmark and Sweden

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Sammendrag

Zymoseptoria tritici forårsager svampesygdommen hvedegråplet på hvede; en af de vigtigste hvedesygdomme under danske og nordvest Europæiske vækstbetingelser. For at holde sygdommen under kontrol, og sikre høje og stabile udbytter, sprøjter landmændene med svampemidler (fungicider). I Danmark sprøjtes der normalt en til tre gange med fungicider i løbet af vækstsæsonen. De mest anvendte fungicider i både Danmark og Europa hører til tre grupper af fungicider, som er demethylase inhibitorer (DMI/azoler), quinone-outside inhibitorer (QoI/strobiluriner), og succinate dehydrogenase inhibitorer (SDHI). DMI -og SDHI-fungiciderne er de primært anvendte fungicider i bekæmpelsen af hvedegråplet, da QoI fungiciderne ikke længere er effektive, grundet resistensudvikling i *Z. tritici* populationen. Det er vigtigt at variere sit valg af fungicider, da man ved konstant brug af fungicider med den samme virkningsmetode, øger risikoen for at selektere for resistens i svampe populationen over for de anvendte fungicider.

I dette studie, blev to mutationer (C-T79N og C-N86S) i succinate dehydrogenase genet (*Sdh*) og én mutation (S524T) i *Cyp51* genet, undersøgt for deres frekvenser i *Z. tritici* populationerne i Danmark og Sverige, med *Z. tritici* isolater indsamlet fra vækstsæsonerne 2019 og 2020. De to mutationer i *Sdh* genet blev fundet i lave frekvenser, dog blev en lille vækst observeret fra 2019-20 i både Danmark og Sverige. S524T mutationen blev fundet i højere grad, både i Danmark og i Sverige, og her blev der også observeret en vækst i frekvensen af denne mutation imellem de to sæsoner.

I *in vitro* forsøg blev sensitiviteten overfor prothioconazole-desthio (DMI) og fluxapyroxad (SDHI) målt i danske og svenske isolater af *Z. tritici.* I alt blev der undersøgt 740 isolater.

De målte EC_{50} værdier som udtrykker sensitiviteten overfor disse midler, varierede mellem vækstårene fra 2016 til 2020, dog forblev sensitiviteten stabil igennem årrækken. Isolater med de specifikke identificerede mutationer havde en signifikant nedsat følsomhed overfor henholdsvis azoler og SDHI'er. Baseret på en mindre udvalgt population (30) blev der fundet krydsresistens imellem SDHI'et boscalid og fluopyram, mens dette var mindre klart for fluopyram og fluxapyroxad samt boscalid og fluxapyroxad.

Desuden blev isolaterne testet for tilstedeværelsen af promotor inserts i *MFS1*, som kan lede til en multi-fungicid resistens. En meget lille andel (1-3%) af populationen for hvert år, havde en af de tre typer af inserts.

For at vurdere effekten af forskellige fungiciders selektion for øget frekvens af de nævnte mutationer, blev bladprøver indsamlet fra fem markforsøg i Danmark og et i Sverige, hvor forskellige sprøjtestrategier blev testet. Data indikerede at behandlinger med prothioconazole i høj grad øgede frekvensen af S524T mutationen. Behandlinger med fluxapyroxad selekterede for C-N86S og C-T79N mutationerne, specielt ved fuld dosis og split behandlinger, ligesom mindre potente SDHI'er også selekterede mindre for disse mutationer. Disse resultater understøtter de danske anbefalinger om at begrænse antallet af sprøjtninger, anvende reducerede doseringer, blandingsprodukter af SDHI og azoler og kun én SDHI-behandling per sæson for at mindske resistensopbygning.

Summary

Zymoseptoria tritici, the causal agent of septoria tritici blotch (STB) on wheat, is one of the most important diseases on wheat under Danish and North-Western European growth conditions. To keep the disease under control and ensure high and stable yields, farmers often spray with fungicides. In Denmark farmers typically spray one to three times during the growing season. The most commonly used fungicides in Denmark and the rest of Europe belong to three groups of fungicides, namely demethylase inhibitors (DMIs/azoles), quinone-outside inhibitors (QoIs/strobilurins), and succinate dehydrogenase inhibitors (SDHIs). DMIs and SDHIs are the primary fungicides used in the control of STB, since QoI fungicides are no longer effective due to resistance development in the *Z. tritici* populations. It is important to diversify the use of fungicides, since constant use of fungicides with the same mode of action (MOA), increases the risk of resistance development in the fungal population towards fungicides within the respective fungicide group.

In this study, the frequency of the two mutations (C-T79N and C-N86S) in the succinate dehydrogenase gene (*Sdh*) and one mutation in the *Cyp51* gene (S524T) was investigated in Danish and Swedish *Z. tritici* populations from the growing seasons of 2019 and 2020. The two *Sdh* mutations were found in low frequencies, however, a small increase was observed from 2019 to 2020 in both Denmark and Sweden. The S524T mutation was found to a greater extent in both Denmark and Sweden and a pronounced increase in frequency was observed from 2019 to 2020.

In *in vitro* experiments, the sensitivity of *Z. tritici* isolates from Denmark and Sweden towards prothioconazole-desthio (DMI) and fluxapyroxad (SDHI) was measured. In total, 740 isolates were tested. The measured EC_{50} values, which express the sensitivity towards the fungicides, fluctuated between the growing years of 2016 to 2020. The overall sensitivity, however, remained stable throughout this time span. Isolates that harbored the specific investigated mutations showed a significantly decreased sensitivity towards DMI or SDHI fungicides, respective of the mutation. Based on a small sub population (30 isolates), cross-resistance was identified between the two SDHI fungicides boscalid and fluopyram. This cross-resistance was identified less clearly between fluopyram and fluxapyroxad and between boscalid and fluxapyroxad.

Furthermore, all isolates were screened for the presence of inserts in the promotor region of *MFS1*, which shows multidrug resistance. A very small fraction (1-3%) of the total collection carried either of the three types of inserts.

To access the effect of different fungicides' impact on selection towards an increase in the frequency of the investigated mutations, leaf samples were collected from five field trials in Denmark and one in Sweden, in which different fungicide schemes were carried out. The data indicated that treatments with prothioconazole increased the frequency of the S524T mutation to a high degree. The treatments with fluxapyroxad selected for the two SDH mutations (C-T79N and C-N86S) to a high degree. This was particularly pronounced in treatments with full dose and in split treatments. Less potent SDHI fungicides did also select for these mutations, however, to a lesser extent. These results support the Danish recommendations on fungicide use, which include limiting the number of treatments, use of adjusted doses, use of SDHI and DMI fungicides in mixture products, and to only apply one SDHI treatment per season, to delay resistance development.

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I would also like to thank Gunilla Berg, for providing leaf samples from the Swedish field trial. Without her contribution, this study would have been limited to only focus on the Danish *Z. tritici* population.

List of abbreviations

ATP	adenosine triphosphate
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie
bp	base pair
ct	cycling time
Cyp51	sterol 14α-demethylase cytochrome P450 gene
CYP51	sterol 14α-demethylase cytochrome P450 enzyme
Cyt b	cytochrome b gene
CYTB	cytochrome bc_1 complex (encoded by *Cyt b*)
DMI	demethylation inhibitor
dNTP	deoxyribonucleotide triphosphate
EC_{50}	effective concentration of 50% inhibition
FRAC	Fungicide Resistance Action Committee
Fw	forward
GS	growth stage
ha	hectare
Kb	kilobase pair
MDR	multi drug resistance
MOA	mode of action
MFS	major facilitator superfamily
NTC	no-template control
PCR	polymerase chain reaction
ppm	part per million
PDA	potato dextrose agar
QoI	quinone-outside inhibitor
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
rpm	rounds per minute
Sdh	succinate dehydrogenase gene
SDH	succinate dehydrogenase enzyme
SdhB	succinate dehydrogenase subunit B gene
SdhC	succinate dehydrogenase subunit C gene
SdhD	succinate dehydrogenase subunit D gene
SDH-B	succinate dehydrogenase subunit B
SDH-C	succinate dehydrogenase subunit C
SDH-D	succinate dehydrogenase subunit D
SDHI	succinate dehydrogenase inhibitor
STB	septoria tritici blotch

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1 Introduction

The global population has experienced exponential growth since the 1960s (Roser, 2013), made possible by the Green Revolution and the prospects that came with it. Increasing demand for stable and nutritious crops has since followed the same trend. The projected annual increase in yields for three of the major staple crops is shown in figure 1. A significant gap exists between the projected increase in yields and the projected demand for wheat and rice yields (Long et al., 2015). Several options are available to meet the growing world food consumption. A change to a vegetarian diet could prove very efficient in reducing emissions of greenhouse gasses while feeding more people, as opposed to a diet relying on meat (Tilman & Clark, 2014). This, however, poses the challenge of changing the diet of entire nations, which might prove quite difficult. In the scenario in which we continue with modern practices, the major staple crops will still play a significant role, if we are to feed the entire global population. To ensure that the increase in food demand matches the increase in yields, drastic measures must be taken. As projected, if we continue with the practices and advances that have been made over the last few decades, the increase in yields will not be sufficient in providing enough food for the global population (Ray et al., 2013). In the case of wheat, estimates of yield increase demands by 2050 are up to 70% of current yields (Driscoll et al., 2014). One of the main issues with growing crops is closing the gap between potential yields and actual yields (Anderson, 2010). Yields are often expressed as a function of the GxExM interaction (genetics, environment, and management). Improvements have been made in genetics through breeding for several decades, which took off during the Green Revolution (Hedden, 2003). Emerging technologies like the CRISPR-CAS9 bio editing technology have the potential to take breeding to new heights (Uauy et al., 2017). While genetics and management are factors that can be manipulated and utilized by farmers to ensure optimum growth, quality, and yield of grown crops. The environment, on the other hand, is less manageable, as farmers are often at the mercy of the elements in each growing season when it comes to the growth of cultivated crops. Anderson (2010) found that in field trials in Western Australia, 80% of the variability in grain yields could be accounted for as an effect of the environment only. The environment can be unpredictable, and farmers often rely on the weather forecast to determine whether to irrigate the crop when rainfall is not sufficient within the growing season. Water is essential to the yield prospects, since water limitations impact the transpiration rate of plants, which reduces the photosynthetic apparatus. That, in agricultural terms, limits the harvested yields. Apart from management practices such as irrigation, other environmental factors are difficult to manipulate. These include the most important factor, being radiation emitted from the sun, which is essential in order for photosynthesis to take place.

One of the important management aspects is the control of in-field diseases, which can have a severe impact on the yields and quality of several cultivated crops (Savary et al., 2019). The majority of agricultural practices across the globe rely on the chemical control of many of the important crop pests (Oerke, 2006). The pests in the field are weeds, fungal and bacterial pathogens, and insects (Savary et al., 2019). Each group of pests requires different management strategies, along with varying degrees of importance in the aspect of yield reduction. Still, chemical control plays a significant role for each one of them. However, the reliance on chemical control has led to the development of resistance towards each of the pesticide classes (fungicides, herbicides, and insecticides), and specifically in many important pathogens on major cultivated crops (Van den Bosch et al., 2011; Savary et al., 2019).

The fungal wheat pathogen *Zymoseptoria tritici* (*Z. tritici*) is one of the most important pathogens in many wheat-growing areas of the world. The control of the pathogen mainly relies on varietal cultivar resistance and chemical fungicides, predominantly demethylation inhibitors (DMI) and succinate dehydrogenase inhibitors (SDHI), and to some extent multi-site inhibitors such as folpet (Torriani et al., 2015). The efficacy of QoI (quinone-outside inhibitor) fungicides was previously excellent but is almost non-existing today due to resistance (Rehfus, 2018), while a sensitivity shift has been observed for the DMI fungicides towards less sensitive *Z. tritici* populations (Cools & Fraaije, 2008; Garnault et al., 2019; Heick et al., 2020). The SDHI fungicides are generally still providing effective control of several important pathogens, including *Z. tritici*, but cases of resistance have been reported in the last few years (Rehfus et al., 2016). The mechanism of resistance development works mainly through amino acid substitutions at the target site of the aforementioned fungicides, which is a result of mutations in the target genes of these fungicides (Barrès et al., 2016).

In this master project, I aim to estimate the sensitivity and prevalence of important mutations in the target site of DMI and SDHI fungicides in single isolates of *Z. tritici* from 2019 and 2020. Furthermore, the frequency of inserts in the *MFS1* promotor region of *Z. tritici* will be estimated in the same samples. Similarly, leaf samples from different field trials carried out in 2020 with STB symptoms are screened for frequency of important mutations in the *Sdh* gene (C-T79N and C-N86S), along with the S524T mutation in the *Cyp51* gene in order to reveal the impact of different control strategies on mutation selection.

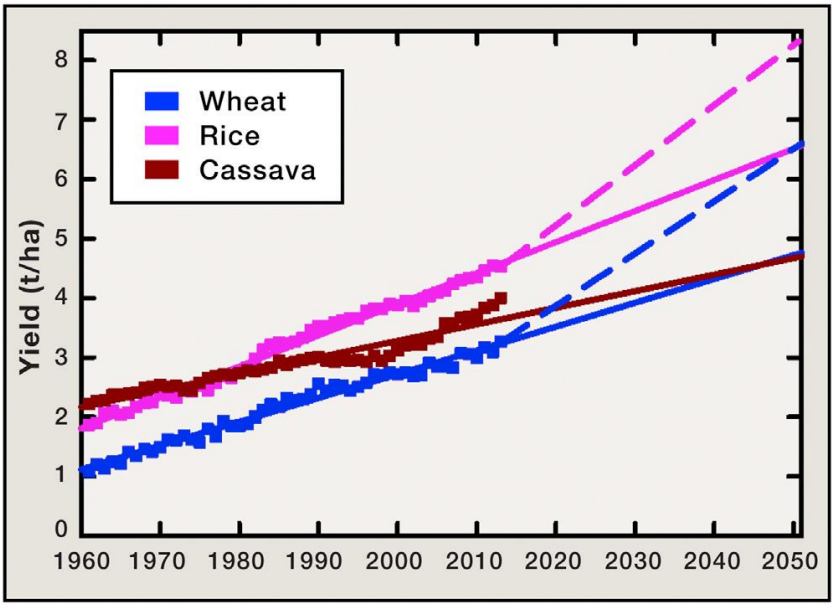

Figure 1 Global average annual yields of wheat, rice, and cassava from 1961 to 2013. Unbroken lines are projected increases in yields for wheat, rice, and cassava, while broken lines show the projected demand for increases in yields for wheat and rice (Long et al., 2015).

1.1 Wheat cultivation and fungal pathogens

Wheat (*Triticum aestivum*) is one of the 15 staple crops that cover 90 percent of the world's energy intake, along with rice (*Oryza sativa)* and maize (*Zea mays)*. The three together represent 60 percent of the world's food energy intake (FAO, 1994), and wheat alone provides 18 percent of the global human calorie intake (Savary et al., 2019). Wheat belongs to the family Gramineae, along with several other important grasses, like barley (*Hordeum vulgare*), rye

(*Secale cereale*), triticale (*Triticosecale*), and oat (*Avena sativa*), and is cultivated all over the world (CGIAR). *T. aestivum* evolved into the hexaploid species it is today through two subsequent hybridization events. This resulted in the wheat we know today, with the AABBDD genome (Matsuoka, 2011). The origin of wheat is hypothesized to be from the fertile crescent in what is known today as Syria, Turkey, and Iraq (Matsuoka, 2011).
Since the domestication of wheat, it quickly dispersed and is now represented in many countries. The main growing areas are in the temperate climate zones of the northern hemisphere, spanning from Northern America, Europe, Asia to Northern Africa (Savary et al., 2019). Yields vary depending on the growing area and the cropping system. The highest yields are attained in the North-Western parts of Europe and New Zealand (Savary et al., 2019).

Like any other crop, wheat is exposed to several stress factors during its growth period. These are either abiotic (drought, waterlogging, heat, and salinity) or biotic stresses (weeds, animals, insects, and pathogens) (Oerke, 2006). The biotic ones, including weeds and animals, can often be controlled via mechanical measurements, whereas the insects' and pathogens' primary control heavily relies on chemical inputs. Several pathogens infect the host crop (wheat), and depending on the location, climate, growth conditions, and crop diversity, some pathogens might be more prevalent than others. Some of the most important wheat diseases are caused by pathogens such as *Z. tritici*, *Fusarium* spp., *Puccinia* spp., *Blumeria graminis, Pyrenophora tritici-repentis, Parastagonospora nodorum, Tapesia* spp., *and Gaeumannomyces graminis* (Savary et al., 2019). *Z. tritici, P. tritici-repentis,* and *P. nodorum* form the leaf blotch complex in wheat (Ponomarenko et al., 2011). The global estimated yield losses of wheat as a result of pathogens are listed in order, going from highest to lowest: leaf rust (3.3%), fusarium head blight (2.9%), septoria tritici blotch (2.4%), and stripe rust (2.1%) (Savary et al., 2019). The percentage losses are out of a total estimated wheat yield loss of 21.5%, made up of all yield loss aspects. To maintain high yields and keep losses at a minimum, farmers employ chemical fungicides. Global wheat yield response to fungicide treatments is in the order of 2.5 t per ha increase (Torriani et al., 2015). Under Danish conditions, the yield increases from fungicide use vary between 5 and 15 dt per ha (Jørgensen et al., 2014).

Importance of *Z. tritici*

Z. tritici is the most important disease on wheat in Europe, which is reflected in the fungicide market share, where over 2.4 bn USD were spent on fungicides in Europe. Out of this amount, 1.7 bn USD were used to control wheat diseases, and thereof 70% (1.2 bn USD) were estimated for control of *Z. tritici* in 2014 (Torriani et al., 2015). The amount of fungicides bought and used to control *Z. tritici* is reflected in the estimated percentage losses of the total loss caused by diseases in wheat, which under North-western European conditions can vary between 39 and 75 % (Jørgensen et al., 2014).

Z. tritici is one of the most important fungal pathogens on wheat. It causes the disease septoria tritici blotch (STB) (Ponomarenko et al., 2011; Quaedvlieg et al., 2011). *Z. tritici* can, on susceptible cultivars during severe epidemics, cause up to 50% yield reductions, if not controlled (Eyal et al., 1987), but is often between 5 to 20%, depending on the cultivar grown and the local environment (Fones & Gurr, 2015). Estimations of fungicide use in Europe, suggests that up to 70% of the total use is spent on controlling *Z. tritici* (McCorison & Goodwin, 2020). STB is prevalent in the humid temperate climate zones, specifically the "maritime zone" encompassing the major wheat-growing regions of North-Western Europe (Fones & Gurr,

2015). The conditions in these areas are marked by high precipitation and humidity combined with temperatures in the range of 15 °C to 25 °C (Ponomarenko et al., 2011).

Taxonomy

Zymoseptoria tritici, formerly known as *Septoria tritici* (anamorph/imperfect), and *Mycosphaerella graminicola* (teleomorph/perfect), is the causal agent of septoria tritici blotch (STB) on wheat (Steinberg, 2015). It was formerly referred to as *Septoria tritici*, with the sexual stage being referred to as *Mycosphaerella graminicola* (Sanderson, 1972). This was replaced when Quaedvlieg et al. (2011) proposed a novel genus *Zymoseptoria* that included all Septoria-like species, which infected graminicolous hosts. The fungus resides in the family of Mycosphaerellaceae in the order of Capnodiales, under the class Dothideomycetes in the phylum Ascomycota (Stukenbrock et al., 2012). It is believed that the origin of the pathogen is in the Middle East, deriving from the fertile crescent, which coincided with the origin of domesticated wheat (Stukenbrock et al., 2007). It shares its taxa with closely related important plant pathogenic fungi such as those residing in the *Ramularia* and *Cercospora* genus (Stukenbrock et al., 2012). Species residing in the *Zymoseptoria* genus, all share the characteristics of yeast-like growth in culture and up to three different types of conidia (pycnidial conidia, phragmospores, and microcyclic conidiation) (Quaedvlieg et al., 2011).

Table 1 Taxonomy of *Z. tritici* after Stukenbrock et al. (2012).

Domain	Eukaryota
Kingdom	Fungi
Phylum	Ascomycota
Class	Dothideomycetes
Subclass	Dothideomycetidae
Order	Capnodiales
Family	Mycosphaerellaceae
Genus	Zymoseptoria
Species	Zymoseptoria tritici

Disease cycle

Figure 2 Septoria tritici blotch (black spots = pycnidia) on a wheat leaf (Dean et al., 2012).

STB develops symptoms with pycnidia, the fruiting bodies, which produce pycnidiospores, the asexual spores of the infection cycle (figure 2) (Ponomarenko et al., 2011). These characteristic symptoms start to appear on the leaves once the pathogen switches from the biotrophic phase to its necrotrophic phase (Steinberg, 2015). The necrotrophic phase is characterized by the development of dark pycnidia and the encompassing necrotic halo area, which in time alters the fraction of green leaf area (GLA). This in term reduces overall photosynthesis, which has a direct impact on the yield. Once symptoms start to appear, few control measures are available, since most of the available fungicides on the market only provide a preventative to curative level of control. The yield of wheat is measured as the weight of grains harvested. The main benefactor to grain filling (allocation of nutrients to grains) is the three top flag leaves, contributing to more than 50% of total grain filling (Wazziki et al., 2015; AHDB, 2021).
This marks the importance of protecting these upper three leaves, which is achieved by growing resistant cultivars and/or applying appropriate fungicides.

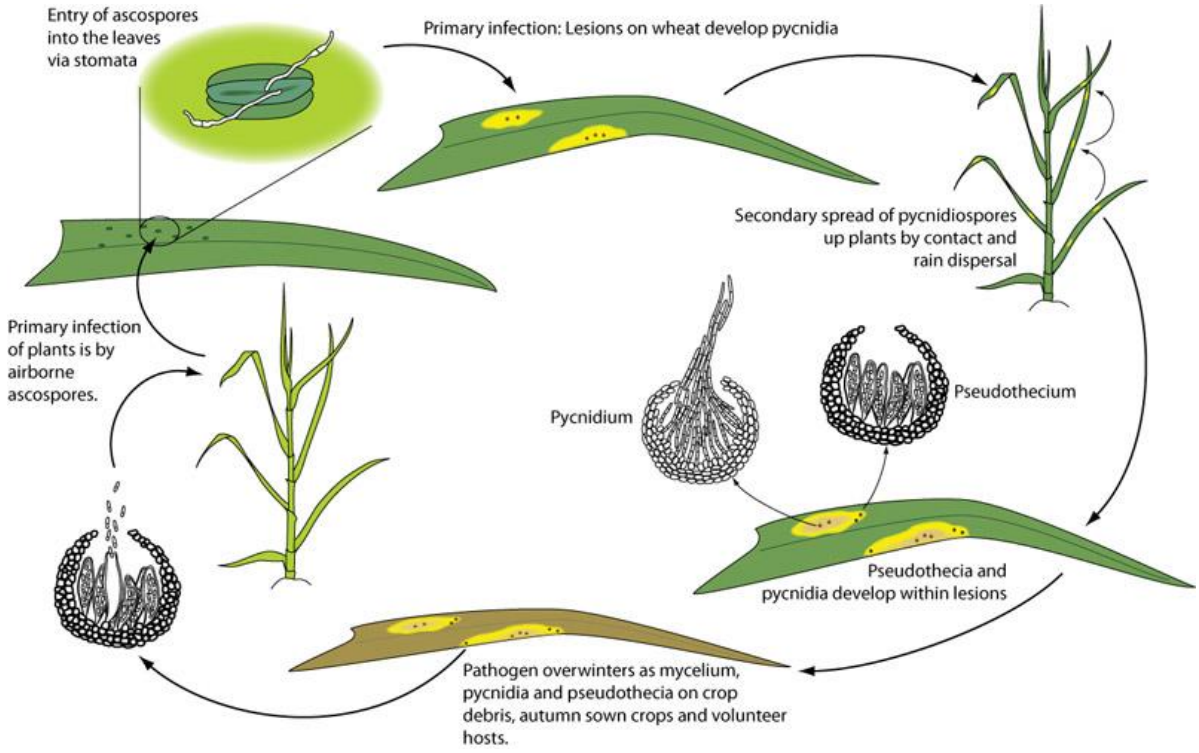

Figure 3 Disease cycle of *Zymoseptoria tritici* (Ponomarenko et al., 2011).

Initial infection of *Z. tritici* is initiated by the release of ascospores (sexual propagules) from pseudothecia, which resides as primary inoculum in leftover wheat debris and volunteer hosts (Ponomarenko et al., 2011). *Z. tritici* ascospores were first identified in New Zealand in 1972 (Sanderson, 1972), and it has been shown that *Z. tritici* forms pseudothecia as a result of mating between two opposite mating types (Kema et al., 1996), identifying it as heterothallic in nature. The primary infections take place during autumn, by both pycnidiospores and ascospores, germinating on leaf surfaces (Shipton et al., 1971; Eyal et al., 1987; Suffert et al., 2011). The spread of ascospores can be local or from long-distance dispersion, as ascospores are transmitted by the wind and have been theorized to travel up to thousands of kilometers (Suffert & Sache, 2011; Steinberg, 2015). The ascospores are formed in the pseudothecia (fruiting bodies), which again are formed due to mating of the two different mating types. The mating of two opposite strains is dependent on the coalescence of lesions on the leaf surface (Cowger

et al., 2000). This signifies the impact severe epidemics can have on the formation of pseudothecia, and subsequent sexual reproduction and genetic diversity (Zhan et al., 2003). Pseudothecia also acts as a survival mechanism of *Z. tritici*, as it can be identified in the field after the formation of pycnidia (Eriksen & Munk, 2003). Pseudothecia continues to be viable even after harvest, on debris that serves as a source of ascospores. In winter wheat cultivation in the Northern hemisphere, the release of ascospores follows a seasonal pattern, that first peaks in late autumn and later again at the end of the growing season (Eriksen & Munk, 2003; Duvivier et al., 2013; Morais et al., 2016). Once the primary infection has taken place, secondary infection proceeds by the release of pycnidiospores from pycnidia (Ponomarenko et al., 2011). The spores are dispersed by water droplets, most commonly as a result of rain or irrigation (Steinberg, 2015). Specific criteria have been observed that must be met for a successful infection by pycnidiospores. Leaf wetness in the time span of a minimum of six hours to four days is essential for successful germination and subsequent infection (De Wolf, 2008). Once the infection has taken place, a long latency period is observed (17-28 days) (Eyal et al., 1987), followed by the development of the characteristic symptoms of pycnidia, which are the black fruiting bodies shown in figure 2. STB is a polycyclic disease, which is shown by the continuous formation of pycnidia and pseudothecia, followed by the subsequent release of pycnidiospores and ascospores, respectively, throughout the growing season (Kema et al., 1996). In the case of polycyclic pathogens, the primary inoculum does, in most cases, not play an important role in terms of the disease severity of an epidemic (Suffert & Sache, 2011). Morais et al., (2016) showed that the amount of primary airborne inoculum in wheat fields did not pose as a limiting factor when considering the onset and severity of an epidemic. This identifies the main source of primary infection being caused by wind-dispersed ascospores, from either distant sources or local sources, such as wheat debris, while the secondary infection is initiated by splash-dispersed pycnidiospores from neighboring wheat plants or debris (Suffert et al., 2011). The infection will follow the plant upwards and spread to neighboring wheat plants until the end of the growing season (Shaw, 2006).

Infection process

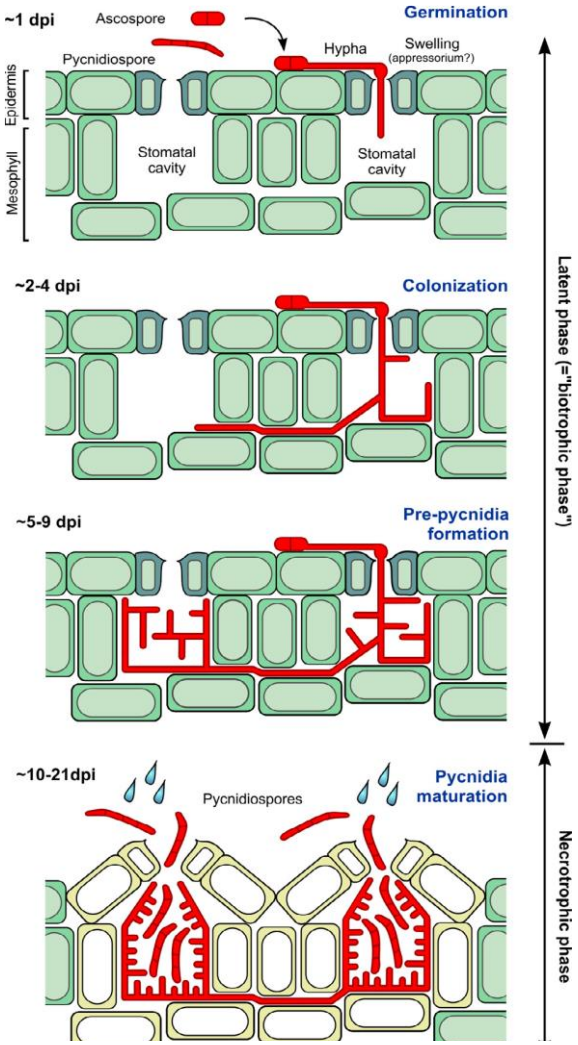

Figure 4 Infection of *Zymoseptoria tritici* via hyphal growth into the stomatal openings of wheat leaf (Steinberg, 2015).

Infection occurs by the germination of either ascospores or pycnidiospores (Eyal et al., 1987). Once the spores come in contact with a leaf, germination takes place, which is enabled by growing hyphae that enter the leaf through substomatal openings (Kema et al., 1996). The germination takes place without the utilization of an appressorium, which is the case for other pathogenic fungi (Cousin et al., 2006). The infection process of *Z. tritici* is illustrated to be separated into two stages; a biotrophic and a necrotrophic stage (figure 4). This mechanism is used to classify *Z. tritici* as a hemibiotrophic pathogen (Ponomarenko et al., 2011).

The biotrophic phase is often referred to as the latent phase since germination, colonization, and pre-pycnidia formation are symptomless (Steinberg, 2015). During the latent phase, hyphae penetrate and grow within the mesophyll tissue, while acquiring nutrients from the host's apoplast (Ponomarenko et al., 2011). It has, however, been hypothesized that the pathogen relies on its lipids and fatty acids as a primary energy source (Kettles & Kanyuka, 2016).

The colonization of the mesophyll cells and acquisition of nutrients transpire without the activation of the host plant's defense mechanisms. *Z. tritici* is theorized to evade the recognition and defense mechanisms by secretion of proteins that dissolve the plant cells' proteins and starch, while protecting the pathogen from hydrolytic enzymes, which are typically secreted by the plant (Steinberg, 2015). The non-activation of the host's immune-response is theorized to be a result of two effectors found in *Z. tritici*, namely Mg1LysM and Mg3LysM (Marshall et al., 2011). These effectors have been shown to prevent chitin-triggered immunity, and subsequently are essential for infection (Marshall et al., 2011). Following the latent phase, *Z. tritici* shifts to a more necrotic lifestyle, for which the trigger is unknown (Ponomarenko et al., 2011). Several hypotheses have been suggested for the transition to the necrotic lifestyle, among these accounts, the upregulation of the “Necrosis-Inducing Proteins” ZtNIP1 and ZtNIP2 identified by Kettles and Kanyuka (2016).

Shetty et al. (2007) investigated the impact of hydrogen peroxide (H_2O_2) on the biotrophic and necrotrophic phases of *Z. tritici*. It has been reported that biotrophic pathogens are inhibited by the accumulation of H_2O_2 (Mellersh et al., 2002), while favored by necrotrophic pathogens (Able, 2003). Since *Z. tritici* is a hemibiotrophic pathogen, they posed the question of how a hemibiotrophe, such as *Z. tritici*, reacts to radical oxygen species (ROS) like H_2O_2, during different phases of growth. They observed that H_2O_2 is harmful to *Z. tritici* during the disease

cycle, but can be tolerated. In another study Shetty et al. (2008) found that resistant wheat cultivars produce oxidative bursts in the early pathogen infection phase. The oxidative burst coincided with the arrested growth of STB in resistant cultivars, while susceptible cultivars did not accumulate ROS until STB sporulation, in which the pathogen degraded surrounding tissues.
The necrotic phase is characterized by the appearance of chlorotic/necrotic areas on infected leaves, and the formation of pycnidia (Steinberg, 2015). The symptoms appear as a result of programmed cell death (PCD) of the surrounding leaf cells, which releases nutrients that enable rapid growth and development of the pathogen's structures (Kema et al., 1996). The maturation of pycnidia is followed by the release of pycnidiospores, which proliferate through adjacent leaves by rain splash (Steinberg, 2015).

Genetics and population diversity

In 2011, the entire genome of *Z. tritici* was sequenced. The genome is comprised of 21 chromosomes, eight of which were identified as non-essential dispensable chromosomes (Goodwin et al., 2011). The *Z. tritici* genome was found to constitute very few genes encoding cell wall degrading enzymes (Goodwin et al., 2011). The presence of the dispensable chromosomes is theorized to progress the adaptation of *Z. tritici* to environmental changes, fungicidal selection pressure offered from different modes of action groups, and cultivar genetics, since these chromosomes are often lost during meiosis and are comprised of high repetitive elements (Steinberg, 2015). In field populations of *Z. tritici,* it has been discovered, that 90% of the entire genetic pool of *Z. tritici* is represented within a single field (Zhan et al., 2003). This illustrates the versatility of *Z. tritici* in the genetic makeup of a field population's adaptability during adverse climatic conditions. Eriksen et al. (2001) showed that up to 30% of the *Z. tritici* population within a field derived from sexual reproduction. The combination of these factors explains the adaptive nature of *Z. tritici,* even under high fungicidal conditions.

1.2 Disease management in wheat

To ensure high and stable yields, farmers rely on several methods to control diseases, that may reduce the yields and quality of harvested products (Oerke, 2006). The most prevailing control method is the use of fungicides, while cultural practices, breeding of resistant cultivars, and mechanical mechanisms tend to play a less significant role. Some agricultural practices, such as delayed/earlier sowing, crop rotation, no-till or tillage, and cultivar mixtures might have an impact on the severity of pathogen outbreaks. Overall most agricultural practices, except for organic farming, rely heavily on pesticides in the case of fungal pathogens, fungicides (Suffert & Sache, 2011). IPM (integrated pest management) describes the most effective, environmentally friendly, and sustainable way of controlling pests in the field illustrated as a pyramid (figure 5). The pyramid illustrates the different aspects of IPM, the importance of each aspect, and how much of an impact it might have on the environment (toxicity). In the following sections, each of the aspects will be elaborated.

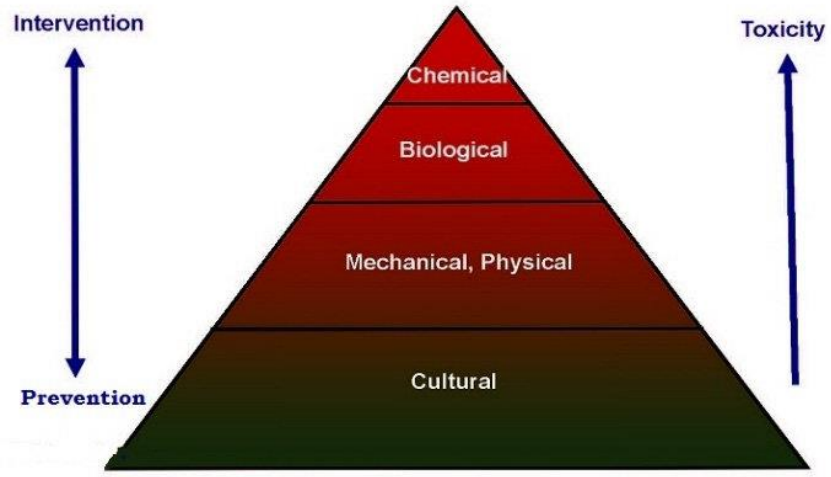

Figure 5 Integrated pest management illustrated as a pyramid (Singh et al., 2017).

Cultural methods

In general, when aiming to control pathogenic fungi that survive on crop residues in the field, the cultural practices aim to lower the amount of the primary inoculum, for instance, by incorporating the residues into the soil by tillage practices. This will, in general, reduce the intensity of succeeding diseases (Eyal et al., 1987). This has been shown to be the case for tan-spot in wheat, in which disease severity was shown to be positively correlated with the initial amount of debris and ascospores of *Pyrenophora tritici-repentis* (Adee, 1989). Concerning STB control, Morais et al. (2016) showed that this did not apply. They reported that the local presence of *Z. tritici* infected wheat residues had no effect on neither the amount of airborne *Z. tritici* ascospores, nor the earliness of the epidemic. Removal of *Z. tritici* infected wheat debris is thereby not a significant management strategy to control STB under Western European conditions (Morais et al., 2016). *Z. tritici* is only affected to a low degree by tillage. Crop rotation has also been shown to follow the same trend, as the release of ascospores that can traverse long distances often in areas with high intensity of wheat cultivation neglects the effect of crop rotation. This leaves crop rotation and tillage as poor methods of control (Gladders et al., 2001; Eriksen & Munk, 2003). The earliness of STB epidemics is mostly determined by the sowing date, as shown by several authors like Gladders et al., (2001), Suffert and Sache, (2011) and Morais et al. (2016). The sowing date poses a dilemma, since early and late sowing dates might favor the development of different pathogens. This has been highlighted by cases of early autumn sowing of winter wheat, which increases the risk of diseases such as STB and eyespot, while late sowing increases the risk of powdery mildew and yellow rust (Jørgensen et al., 2014). Since early sowing favors STB epidemics, recommendations on control of STB would be a delay of the sowing date, and the inclusion of cultivars with varietal resistance, which may mitigate the build-up of primary inoculum in the season and decrease or delay STB disease severity/epidemics in the subsequent year (Morais et al., 2016; Heick et al., 2017a; Kristoffersen et al., 2020; Mäe et al., 2020). High input of nitrogen fertilizer has also been identified as increasing the development of STB (Simón et al., 2003), while low sowing densities are favored by *Z. tritici* due to greater splash-dispersal from rain penetrating the canopy (Bjerre et al., 2006). Early sowing and minimum tillage favor STB epidemics. Varietal resistance and delayed sowing help mitigate the primary inoculum at the beginning of the season, thereby reducing disease severity the following year (Mäe et al., 2020).

The main issue when relying on cultural methods as control options of one or more diseases such as *Z. tritici* is that one option might show positive effects on one disease, but favor another (Jørgensen et al., 2014b)

Resistance breeding and cultivar mixtures

A key control method of limiting the impact of *Z. tritici* on wheat is the adaptation of wheat cultivars that show phenological resistance to the pathogen. The resistance to *Z. tritici* is achieved by identifying and implementing genes within the wheat genome that suppress infection and subsequent colonization of the pathogen (Brown et al., 2015). Plants respond to the foreign presence within the cells by recognition of pathogen-associated molecular patterns (PAMPs), for instance, chitins, β-glucans, mannans, and ergosterol (Kettles & Kanyuka, 2016). Detection is enabled by transmembrane pattern recognition receptors (PRRs) within plant cells (Dangl & Jones, 2001). Once the immune system is triggered, PAMP-triggered immunity (PTI) is acquired, and this subsequently leads to hypersensitive cell death response (HR) in host plants (Kettles & Kanyuka, 2016).

Brading et al. (2002) showed that *Z. tritici* and wheat interact based on gene-to-gene-interaction. Gene-to-gene interaction is characterized by host resistance genes and pathogen a-virulence genes (Brading et al., 2002). This type of resistance is qualitative, since the resistance is highly effective, if the matching a-virulence gene in the *Z. tritici* strain and host resistance gene is present. As of date, 21 qualitative resistance genes have been discovered and mapped (Brown et al., 2015). Apart from qualitative resistance genes, the wheat genome also consists of quantitative resistance genes, which are genes that are not as impactful as qualitative genes, but when bred in a pyramid scheme, in which quantitative gene traits are stacked, can promote durable, long-lasting resistance (Brown et al., 2015; Vagndorf et al., 2017). Control of fungal pathogens is thereby attainable with resistance breeding, but has often been associated with a yield penalty (Brown et al., 2015).

In more recent times, breeders have overcome this penalty and today provide competitive high-yielding resistant cultivars (www.Sortinfo.dk). Even so, resistant cultivars still tend to show positive yield responses when treated with fungicides, which could be explained by lack of resistance to all diseases, or/and that fungicides offer some benefits in terms of the physiological effects on cultivated crops (Bartlett et al., 2002). Aside from growing resistant cultivars, integrating cultivar resistance in mixtures offers another great option for *Z. tritici* control, along with several other important diseases on wheat. Most fields around the world today are grown in monocultures and, in that aspect, mono-cultivars (Long et al., 2015). This type of cropping system aims to maximize yields, by ensuring simultaneous ripening, simple harvest, and reliable yields, but results in a less resilient cropping system (Oerke, 2006). Harvested grains are often used by companies that rely on specific criteria of the grains, be it bakers (gluten and protein content) or brewers (protein, germination, and malting qualities) (Borg et al., 2018). Cultivar mixtures are not as uniform as their mono-culture counterparts. The date of ripening might be variable in the field. The end product may be very diverse in terms of qualities mentioned earlier, or the growth of some cultivars in the mix might supersede the others, thereby out-growing them (Kristoffersen et al., 2020). Cultivar mixtures do, however, offer a more stable and resilient harvest.

There are five mechanisms in which mixtures can mitigate disease epidemics, as opposed to pure stand cultivars. (a) Dilution effect, which prevents the pathogen from spreading as rapidly

within a field. (b) A barrier effect, preventing the spread from susceptible, infected cultivars to other susceptible cultivars. (c) Premunition involves the signaling between crops in the field. Once a plant is infected, it signals non-infected plants, which induces resistance. (d) Cultivar mixtures including several different cultivars with a plethora of diversity of resistance genes, cause disruptive selection on the pathogens, as no single strain is favored. (e) Compensation, in which the downfall of one cultivar enables the resistant cultivar to take the place of the susceptible one (Borg et al., 2018). Growing cultivar mixtures or resistant cultivars will likely reduce the need for disease control by one, as described by Kristoffersen et al. (2020).

Chemical control using fungicides

Fungicides are the group of pesticides, which are used to control fungal pathogens on cultivated crops. Fungicides account for 36% of the total pesticide use in cereals (Mcdougall, 2017), signifying the importance of this group on a global scale. Fungicides can be classified into three categories, according to their efficacy on application timing. (i) Protectants are fungicides that prevent pathogenic fungi from infecting host plants, thereby blocking the fungi from infiltration and initiation of the disease cycle. The protectants are for this reason applied prior to infection. (ii) Curative fungicides are effective post-infection, by limiting the growth of the pathogen already present in the crop. (iii) Eradicant fungicides prevent pathogens in the sporulation. The protectants are non-mobile and not translocated in the plant, while curative and eradicant fungicides can be mobile and transported throughout the plant (Balba, 2007). Protectant fungicides that are immobile are referred to as contact fungicides and include multi-site inhibitors such as folpet, mancozeb, and chlorothalonil (Oliver & Hewitt, 2014).

Fungicides are also classified based on the mode of action (MOA). The MOA can be based on inhibiting a single biochemical process (single-site) or several biochemical processes (multi-site) (Oliver & Hewitt, 2014). Four major groups of fungicides play or have played a role in the control of STB - QoIs, DMIs, SDHIs, and multi-site inhibitors. Each of these will be shortly described, while SDHIs will be further elaborated, due to their significance to this thesis.
Previously, methyl benzimidazole carbamates (MBCs) were an important fungicide group (Driscoll et al., 2014), but have been discontinued due to resistance (FRAC). As of yet, the fungicide groups available for STB control are demethylation inhibitors, quinone-outside inhibitors, succinate dehydrogenase inhibitors, and multisite inhibitors.

Quinone-outside inhibitors (QoI) showed great prospects back when they were first introduced at the beginning of the 2000s. The group, however, experienced a great efficiency decline, as *Z. tritici* strains that harbor the G143A mutation, which resulted in resistance development, quickly spread across the world, and in turn, made QoIs unfit for control of *Z. tritici* (Fraaije et al., 2005). QoIs were highly used following the introduction to the fungicide market, reflected in the market share QoIs had in the global market (10%) back in 1999 (Mcdougall, 2001). QoIs, often referred to as strobilurins, might not offer sufficient control of STB (Jørgensen, 2006), but still proves effective in the control of important diseases such as rust diseases in cereals (Anderson, et al., 2014). QoI fungicides inhibit fungal respiration by blocking electron transport in complex III at the Qo-site of cytochrome bc1 complex, of the electron transport chain (Bartlett et al., 2002; Balba, 2007; FRAC, 2019).

DMI fungicides mainly constitute the azole group, in which the most important are the triazoles and triazolinthione (only prothioconazole) (Oliver & Hewitt, 2014). The azoles are the major group used to control STB and several other important foliar diseases (Fraaije et al., 2007;

Jørgensen et al., 2018). DMI fungicides inhibit the C14-demethylase (CYP51) protein, which is an intermediate enzyme in the biosynthesizes of ergosterol, a major constituent of the fungal cell wall (Yang et al., 2011). Ergosterol provides membrane integrity of the cell wall, which in the lack thereof, disrupts the membrane and results in electrolyte leakage (Kettles & Kanyuka, 2016). DMI fungicides block the enzyme P450 monooxygenase, an enzyme encoded by the *Cyp51* gene, which results in a build-up of precursor sterols and low production of ergosterol (Cools et al., 2011).

The SDHIs offer excellent broad control of many of the major pathogens (Gold et al., 2009; Sierotzki & Scalliet, 2013). This is reflected in the use pattern in the United Kingdom, in which close to 60% of cereal farmers used at least one SDHI containing product per season in 2012 (Sierotzki & Scalliet, 2013). The same tendency was observed in Germany, which experienced an increase in SDHI treatments from 15 to 25% in the same year (Rehfus et al., 2016). SDHI and DMI fungicides constitute the majority of fungicides used on a global scale (Sierotzki & Scalliet, 2013; Mordor Intelligence, 2018; Heick et al., 2020).

Multi-site inhibitors are fungicides that do not have a single mode of action. These fungicides inhibit several biochemical processes, which makes them very effective in controlling several pathogens. Since the MOA is not a single target, pathogens acquiring resistance towards this group of fungicides rely on the accumulation of several mutations, which confer resistance. This is highly unlikely to occur in a single strain, and therefore, no resistance to this group of fungicides has been observed yet (Hobbelen et al., 2014). The multi-site inhibitors offer a low-to-intermediate level of control of several pathogens while being at a very low risk of developing resistance, which makes them very relevant in terms of anti-resistance management strategies (Heick et al., 2017). Unfortunately, many of the multi-site inhibitors affect non-target organisms in the environment, and therefore pose a high level of toxicity, hence, many of the multi-site inhibitors have been banned in Europe.

Succinate dehydrogenase inhibitors (SDHIs)

The SDHIs show a broad spectrum of control of several pathogens among ascomycetes and basidiomycetes, with the exception of oomycete control (Sierotzki & Scalliet, 2013). SDHIs as a group were formerly known as carboxamides, until the formation of the Fungicide Resistance Action Committee (FRAC) in 2009, which renamed it (Stammler et al., 2015).
SDHIs were first introduced in 1966 (carboxin and oxycarboxin), which did not show high efficacy against foliar diseases (Sierotzki & Scalliet, 2013), instead, SDHIs were used as seed treatment against basidiomycetes (Scalliet et al., 2012). Several other compounds of the group followed (mepronil, flutolanil, furametpyr, and thifluzamide), however, the activity spectrum of these compounds continued only to act against basidiomycetes, offering control of e.g. *Rhizoctonia* spp., and *Ustilago* spp. The aforementioned first wave of SDHIs is often referred to as the first generation SDHI fungicides, due to their limitations and low mobility in plants (Glättli, Grote, & Stammler, 2011). SDHI use grew since the introduction of boscalid (BASF) in 2003, which was the first foliar fungicide, which also controlled STB, and since then a steady supply of new products has made its way onto the global market (Rehfus et al., 2018). The introduction of boscalid was achieved by replacing the 1,4-oxathiin ring with a pyridine moiety combined with the establishment of a phenyl group at the 2' position of the anilide ring (Stammler et al., 2015).

Following the introduction of boscalid, the second generation of SDHIs soon followed pursuit, as companies opened their eyes to the versatility and possibilities offered by this group of fungicides. Several compounds have since been introduced, or are on the verge of being introduced to the market. These include benzovindiflupyr, bixafen, fluopyram, fluxapyroxad, isofetamid, isopyrazam, penflufen, penthiopyrad, and sedaxene. SDHIs have, since their introduction, expanded on the structural complexity of the molecules, but some characteristics of the compounds are still shared within the group. These include: a central amide moiety, which is crucial for hydrogen bond interaction at the ubiquinone binding site of the SDH an aromatic ring in the aniline part is in place to secure optimal hydrophobic contacts, and finally a nitrogen-containing heterocycle (pyridine or pyrazole) is often to be found in newer compounds, as it increases the binding affinity (Stammler et al., 2015). The amide bond is common for all SDHIs (figure 6).

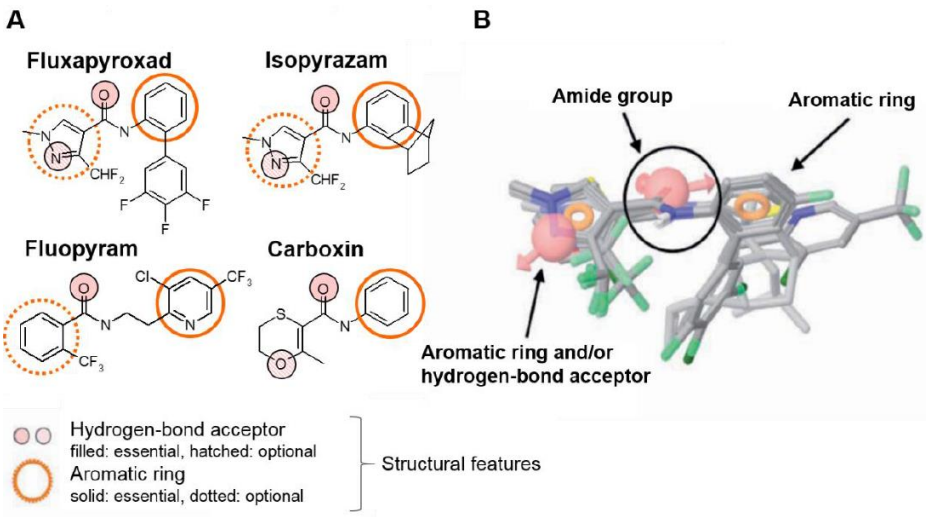

Figure 6 (A) Chemical structure of four SDHIs and their common features that interact with the amino acid residues at the ubiquinone-binding site. (B) The structural alignment and interaction sites of various SDHIs. (Rehfus, 2018).

SDHI fungicides disrupt the mitochondrial tricarboxylic acid cycle (TCA), by inhibiting the succinate dehydrogenase (SDH) enzyme. The SDHIs work by binding to the ubiquinone binding site (Qp), thereby inhibiting the reduction of ubiquinone and subsequently fungal respiration. Four polypeptides form the SDH enzyme, these subunits are named according to a letter scheme (A, B, C, and D). Subunit SDHA and SDHB compose the soluble catalytic dimer, facing the matrix, while SDHC and SDHD shape the integral membrane, which fastens the enzyme to the internal membrane of the mitochondria. SDHA is a flavoprotein that catalysis the oxidation of succinate to fumarate, while SDHB is an iron-sulfur protein that meditates the electron transfer from succinate to ubiquinone (figure 7). The Qp site of the SDH mediates a 2 step electron transfer, in which electrons are transferred from the iron-sulfur cluster (SDHB) to the ubiquinone substrate (Scalliet et al., 2012). SDHC and SDHD fasten the complex to the membrane of the mitochondria. The central amide moiety of the SDHIs forms hydrogen bonds to the conserved tryptophan of SDHB and tyrosine of SDHC. The site of action of the SDHIs was hypothesized to be in the ubiquinone binding pocket, which was confirmed by Stammler et al. (2015).

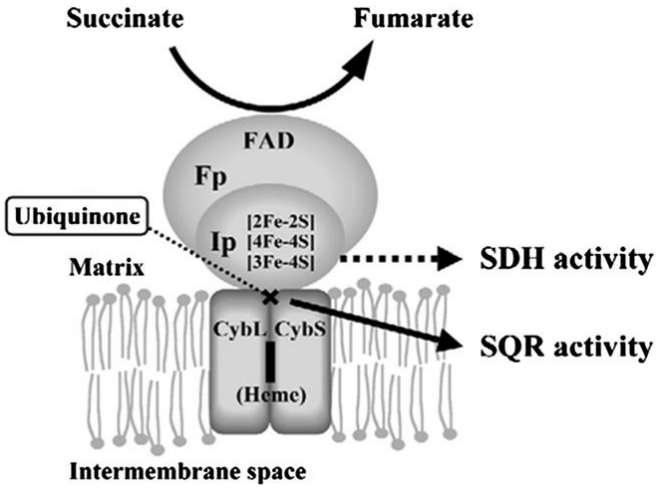

Figure 7 Structure of the SDH enzyme (Avenot & Michailides, 2010)

New and even more potent SDHIs are in the pipeline and could lead to the third generation of SDHIs. These new actives have proven to be very effective and thus strengthening the profile of this group further. These include pydiflumetofen (adepidyn) which provides high control of both STB and fusarium head blight (Sierotzki et al., 2017) as well as isoflucypram (Bartholomaeus et al., 2021), which has a strong profile on most cereal pathogens.

Table 2 List of all SDHIs from (FRAC).

phenyl-benzamides	benodanil, flutolanil, mepronil
phenyl-oxo-ethyl-benzamide	isofetamid
pyridinyl-ethyl-benzamide	fluopyram
furan-carboxamides	fenfuram
oxathiin-carboxamides	carboxin, oxycarboxin
thiazole-carboxamides	thifluzamide
pyrazole-carboxamides	benzovindiflupyr, bixafen, fluxapyroxad, furametpyr, isopyrazam, penflufen, penthiopyrad, sedaxane
pyridine-carboxamides	boscalid
N-methoxy-(phenyl-ethyl)-pyrazole-carboxamides	pydiflumetofen

Fungicide use pattern in different countries

Fungicide use in the European Union (including the UK) is very diverse, both in terms of fungicides used (MOA) and pathogens targeted by them, as well as the number of sprays in a growing season, which varies between 0-4 times depending on climate, region and dose applied. Commonly two treatments are applied. Sales of agrochemicals in 2006 and 2007 and national surveys, showed that the highest use of fungicides was within the UK, while Denmark had the lowest compared to Germany and France (Jørgensen et al., 2014).
Countries that have a very high frequency of high dose sprays (like the UK), are some of the first places in the EU to discover novel mutations in the field conferring resistance, indicating a West-to-East gradient of fungicide resistance-conferring mutations (Heick et al., 2017b; Jørgensen et al., 2021).

The general fungicide strategy in European grown winter wheat, involves an average of two fungicide applications, per season, not including seed treatment. This may be higher, as intensive grown areas that encounter high disease pressure, such as the UK, require a higher input to attain equivalent levels of control. The first fungicide application in winter wheat is usually applied at growth stage 30/31 (BBCH scale), during stem extensions. This is to protect the base of the stem and control early foliar pathogens, like eyespot and *Z. tritici*. At growth stage 37-39 (flag leaf emergence), a second fungicide application is performed, to protect the crop against foliar diseases, with the main goal of protecting the flag leaf (Oliver & Hewitt, 2014). In high-risk scenarios, a later treatment might be applied for topping up the control on the upper leaves and protection of the ear from fusarium head blight during flowering (GS 61-69).

Differences in spray patterns as the ones mentioned above, also apply to neighboring countries such as Sweden and Denmark (Wieczorek et al., 2015). As this study focuses mainly on the differences in fungicide use, and frequency of *Z. tritici* strains carrying mutations in either the *Sdh* or *Cyp51* genes, in Danish and Swedish *Z. tritici* populations, the dissimilarities between the two countries will be further elaborated. Sweden previously did not authorize the use of any SDHI fungicides, to control STB (Wieczorek et al., 2015; Heick et al., 2017b). Since 2018 this changed, and now bixafen, fluopyram, fluxapyroxad, boscalid, and benzovindiflupyr are allowed in mixtures for use in cereal crops (Berg, 2018). In Denmark, boscalid has been available to Danish farmers since 2006 (Middeldatabasen) used in mixtures with epoxiconazole and pyraclostrobin, and fluopyram has been available since 2017 in mixtures with prothioconazole (Propulse) (Middeldatabasen). In the DMI fungicide spectrum, Sweden has authorized the use of difenoconazole, propioconazole, prothioconazole and tebuconazole (Berg, 2018), while Denmark relies heavily on prothioconazole and epoxiconazole (Wieczorek et al., 2015), but has tebuconazole, difenoconazole and metconazole available (Heick et al., 2017b). As of 2021 epoxiconazole will no longer be authorized in Denmark. Apart from different authorizations on fungicides, the number of applications and dose rates vary between the two countries. Danish farmers usually apply reduced rates (30-50%) of the standard rates in 2 to 3 treatments, while Swedish farmers apply 50-80% rates of the standard rate in 1 to 2 treatments per season (Wieczorek et al., 2015).

1.3 Evolution of fungicide resistance

When there is little variation, in terms of the MOA of the fungicides used, the conditions for resistance development are very often met. Fungicide resistance is a pathogen's response to the extreme selective pressure, sanctioned by the high input of fungicide. The environment under which these organisms flourish is in most cases agricultural areas, grown with the corresponding hosts for each of the known pathogens (Barrès et al., 2016). Fungicides act by favoring fungal strains, that can cope with the selective pressure (Deising et al., 2008).

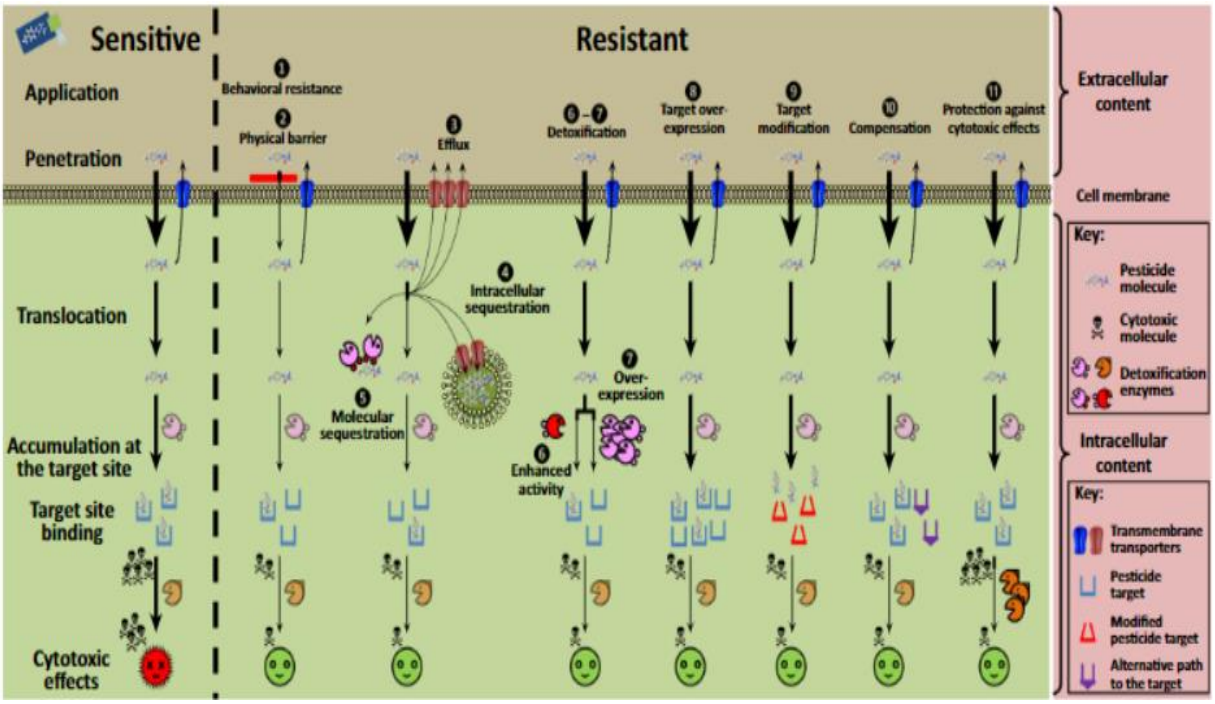

Figure 8 Pathogens' different mechanisms of developing resistance to a toxic substance i.e. a fungicide (Barrès et al., 2016).

The different mechanisms, upon which resistance to a given fungicidal active ingredient might be acquired for a given pathogen, are shown in figure 8. The mechanism behind resistance development can be distinguished by the process that results in resistance. Qualitative resistance is based on mutations figure 8 (9, target modification), which are randomly introduced in the fungal population during reproduction, or for instance by UV-radiation (Deising et al., 2008). Mutations can either be detrimental, neutral, or positive, but in most cases, mutations will have neutral effects on the organisms, or more specifically the enzyme or enzymatic process in which the gene has mutated (Loewe, 2008). However, natural selection drives organisms to adapt to their environment. Adaption may result from sexual reproduction, in which genes are mixed to produce offspring superior to the parents. In agricultural fields, natural selection takes place, but has been subsided to follow a more artificial selection, put forth by man, and the use of pesticides.

A mutation can be positive, as is the case with acquiring e.g. SDHI resistance via target-site mutation, thereby promoting the mutation in an environment, in which this mutation gives an evolutionary advantage. A mutation can be obstructive, resulting in an impact on the fitness of the organism, for instance, a mutation in an important enzyme, which then disrupts biochemical processes in the organism. In most cases, however, the mutation is neutral, not having any or very limited impact on the organism´s biochemical processes. An example could be a mutation that does not result in an amino acid substitution (Loewe, 2008). Fungicides target specific enzymatic processes of the pathogens, which are no less prone to mutations than any other part of the genome (Deising et al., 2008). Depending on the fungicides used, the selective environment might be that of favoring mutations in the genes encoding the synthesis of ergosterol (DMIs), the oxidation of succinate to fumarate (SDHIs), or the electron transfer of complex III (QoIs).

Once a strain acquires a mutation conferring resistance, that lowers the sensitivity to a certain fungicide, the strain then possesses an evolutionary advantage over other strains within that population (Bradley et al., 2012). It might not compete well in a natural environment, but that lack is compensated for, by the actual environment of fungicides. As time progresses, more strains might acquire these or similar mutations, either by mutations in their genome, by sexual recombination, or by migration, as is the case with many pathogens (Hawkins et al., 2019).

The gradual shift in a pathogen population, exposed to a given fungicide over time, which shows the increasing ratio of resistant to sensitive strains in the population is given in figure 9. Resistance can develop in either a stepwise manner, as the one observed for DMI resistance as a result of the accumulation of mutations in the *Cyp51* gene, which gradually has shifted the *Z. tritici* population from sensitive to more and more resistant (Fraaije et al., 2007; Cools et al., 2011; Jørgensen et al., 2021), or it can occur in a disruptive manner, as was remarked when resistance to QoI fungicides was observed in *Z. tritici* populations, shortly following the introduction (Bartlett et al., 2002; Balba, 2007). The QoI resistance was mainly a result of the single amino acid substitution G143A, which rendered all strobilurins ineffective in controlling *Z. tritici* and many other pathogens. The case of DMI resistance in *Z. tritici* populations could be identified as the second mechanism of resistance, being a result of quantitative resistance (figure 9,b), which are not a direct result of single-site mutations. The lower sensitivity observed may be due to overexpression of the target enzyme (figure 8,8) or detoxification by efflux pumps (figure 8,3), which have been observed in *Z. tritici* populations concerning both DMIs and SDHIs (Cools et al., 2012; Omrane et al., 2015; Sang et al., 2018; Kildea et al., 2019).

In *Z. tritici* populations, resistance to all single-site fungicides has been detected, which has been targeting control of STB. The level of resistance and type of resistance varies both on a global scale, and even local scale (Jørgensen et al., 2017,2018).
Cross-resistance is the term used when lower sensitivities are observed within a group of fungicides, as opposed to a single fungicide (Barrès et al., 2016). This mainly applies to fungicides with the same MOA, since the target enzyme is the same. Therefore mutations in the gene encoding the protein may affect several fungicides within the respective group, however, lower-to-moderate sensitivities have also been observed to fungicides of different MOA (Omrane et al., 2015, 2017; Sang et al., 2018), as a result of efflux transporters (figure 8,3). The term preselection is used when a fungicide, selects strains that are resistant to other fungicides within its class (Sierotzki & Scalliet, 2013), impacting future fungicides within the group, before these are even developed, marketed, and applied in the field (Fan et al., 2015). In the case of DMI resistance, a gradual shift has been observed the last decade, but a particular amino acid substitution (S524T) in combination with other mutations in the field populations of *Z. tritici* has started to emerge and is increasing (Leroux et al., 2007; Jørgensen et al., 2021). This mutation confers resistance to prothioconazole and epoxiconazole, which in recent years have been most widely used for control of STB in Europe (Cools et al., 2011). The newly introduced azole, mefentrifluconazole, although showing cross-resistance to other azoles (Heick et al., 2020), still provides significantly better control of STB compared with older azoles (Jørgensen et al., 2020).

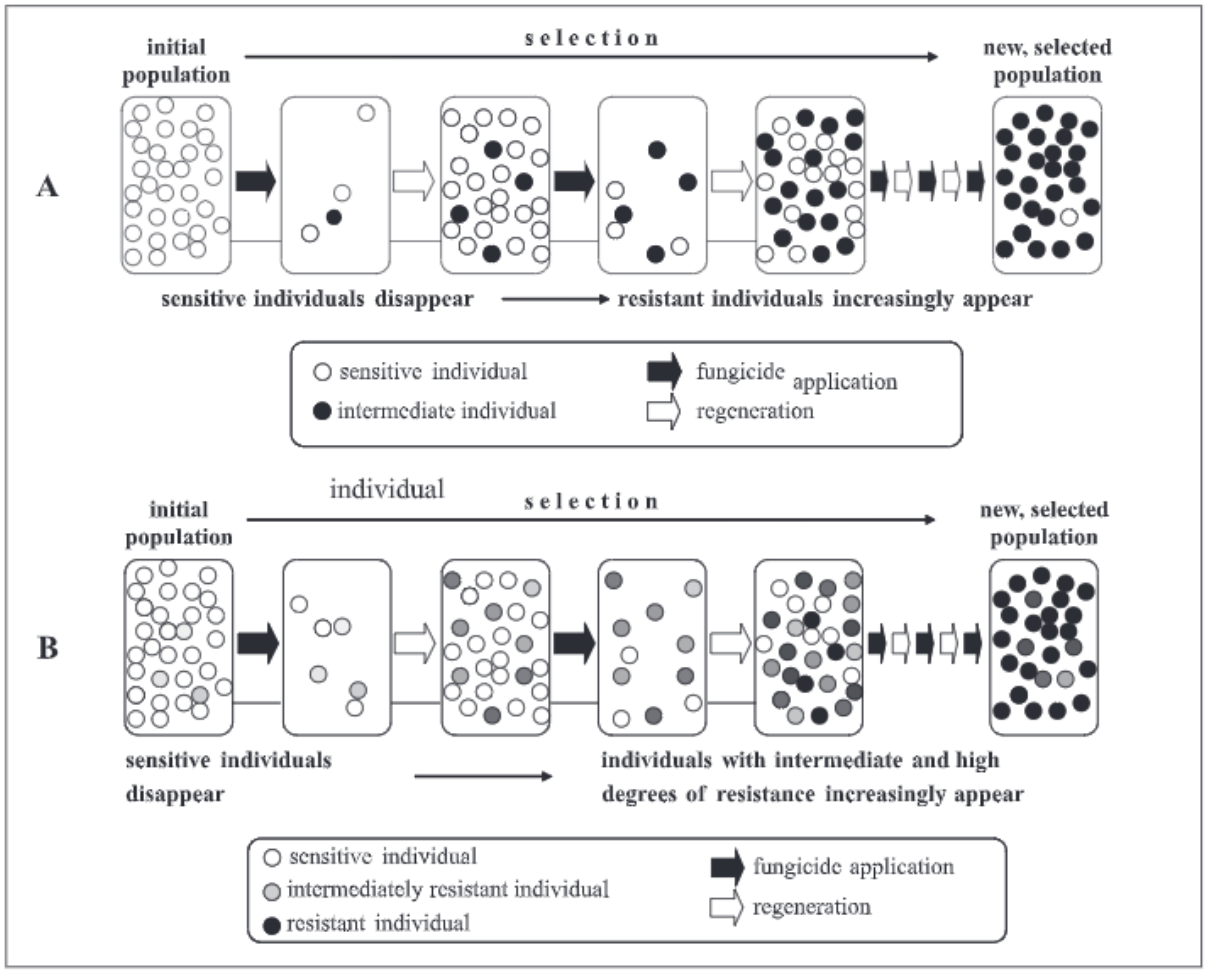

Figure 9 Fungicide resistance development process (modified after Oliver & Hewitt, (2014) by Deising et al. (2008).

Resistance to succinate dehydrogenase inhibitors

There are two molecular mechanisms, which explain the lower sensitivity observed towards SDHI fungicides in *Z. tritici* (figure 10). The main being the result of mutations in the *SdhB, SdhC, and SdhD* genes, which impact the binding affinity of SDHI fungicides (Sang & Lee, 2020). The second mechanism is the overexpression of efflux transporters. This overexpression reduces the intracellular concentration of SDHI fungicides, which reduces the sensitivity to more than just one group of fungicides (Oliver & Hewitt, 2014; Sang et al., 2018; Sang & Lee, 2020). This is often referred to as multi-drug resistance (MDR) as several compounds may be affected by this type of resistance. As shown by Omrane et al. (2015, 2017) the overexpression of efflux transporters, is the result of inserts in the promotor regions of important families of transporters, like the major facilitator superfamily *MFS1*. Yamashita & Fraaije (2018) reported the first case of SDHI resistance not being a result of target-site mutations.

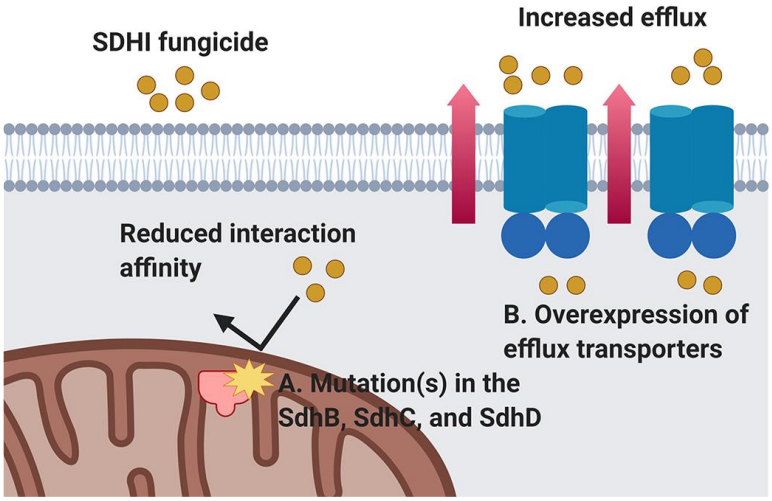

Figure 10 Succinate dehydrogenase inhibitor resistance mechanisms in plant pathogenic fungi. (A) Decreased SDHI binding affinity because of mutation(s) in the subunits of the SDH enzyme SDHB, SDHC, and SDHD. (B) Increased efflux of SDHI fungicides, due to overexpression of efflux transporters, leading to lower accumulation rates of SDHI fungicides (Sang & Lee, 2020).

Resistance towards SDHIs was first observed 5 to 7 years post the introduction of carboxin (Stammler et al., 2015). The first cases of mutations conferring resistance were observed in the 1970s in strains of *Ustilago maydis* and *Aspergillus nidulans.* It was discovered that mutations in the *Sdh* gene accounted for resistance observed in these strains. It was noted that the resistance factors were differing in resistant strains, when treated with analogs of carboxin (Sierotzki & Scalliet, 2013), indicating an unclear level of cross-resistance. Resistant strains might therefore be controlled with other SDHIs.

In a study, a total of 27 amino acid substitutions in the three subunits of the ubiquinone binding site of the SDH enzyme were identified, occurring at 18 different positions (Scalliet et al., 2012). All the identified substitutions showed to harm the efficacy of actives significantly. Very low enzyme activity did not impact the survival of the cell, stipulating no/low fitness penalties. FRAC, (2021) lists the following SDHI mutations identified in *Z. tritici*;
Subunit-B: N225T/I/M, T268I/A, R265P, C266G.
Subunit C: T79N/I, N86S/A, H152R, R151S/T/M, N33T, N34T, T168R, A84F, W80S, V166M, P127A, L184W.
Subunit D: I50F, M114V, D129G.

Z. tritici isolates carrying mutations that confer resistance towards SDHIs are either identified in the laboratory or the field. The laboratory mutants are often generated by the use of UV mutagenesis on SDHI amended media (Sierotzki & Scalliet, 2013). UV mutagenesis can generate a plethora of mutants, carrying distinct mutations in the three subunits of the SDH enzyme (Yamashita & Fraaije, 2018). The mutations will perhaps later be observed in the field or never be identified. The same can be stated for field isolates, which carry mutations not previously described in the lab (Birr et al., 2021). The explanation can be a high “super-selection” under laboratory conditions, which can emit isolates carrying "moderate" mutations (Brown et al., 2015). It should also be noted that mutants generated in the lab can carry mutations, which are not favorable in the field. The mutants that carry mutations that confer high levels of resistance, may also offer a fitness penalty, proving detrimental to the survival of that strain (Hawkins & Fraaije, 2018). An example of a strain carrying a mutation first

discovered in the field was the C-T79N mutation in SDHC of *Z. tritici*, which had not previously been shown during mutagenesis screening in the lab (Sierotzki & Scalliet, 2013). Stammler et al. (2015) suggested that, since some laboratory mutants and field isolates carry mutations that confer complete loss of sensitivity to all current SDHIs on the market, while other mutations indicate no complete cross-resistance, it can be asserted that cross-resistance between SDHIs are in the general present, but that some exceptions might occur. It has been proposed that an explanation for the different resistance patterns that have been observed across the SDHI fungicide group, including negative cross-resistance, is that the array of SDHI compounds vary in their binding properties to the SDH enzyme (Scalliet et al., 2012). Preselection has also been suggested to occur for SDHI fungicides, where the application of one SDHI, pre-selects for strains that are insensitive to other SDHI fungicides (Sierotzki & Scalliet, 2013). Cross-resistance was observed in *Z. tritici* before the introduction of bixafen (2010), which showed decreased sensitivity based on the cross-resistance to boscalid, the first SDHI that was used for control of *Z. tritici* since its introduction in 2003 (Yamashita & Fraaije, 2018; Birr et al., 2021). Apart from cross-resistance, negative cross-resistance has also been observed. In the case of the amino substitution of H272Y in SDHB, decreased sensitivity is observed towards boscalid, isopyrazam, and bixafen, while fluopyram indicates hypersensitivity (Sierotzki & Scalliet, 2013). Hypersensitivity is the term used for strains of pathogens, which show higher sensitivity towards a certain fungicide, than other sensitive isolates.

As SDHI fungicides have been used throughout Europe to control *Z. tritici* for some years now (approx. 15 years), SDHI resistant strains have started to emerge in the European *Z. tritici* population. Various mutations have been detected in the population, including mutations in *SdhB* (N225T and T268I) and *SdhC* (T79N, W80S, N86S, H152R, and V166M) (Sang & Lee, 2020). The most frequent mutations in Europe are the C-T79N and C-N86S mutations (FRAC, 2021), which confer low-to-moderate resistance levels to SDHI fungicides. However, these mutations are only detected in few cases in Scandinavia each year (Mäe et al., 2020). Compared to the DMI resistance observed in *Z. tritici*, in which haplotypes carrying more than a single mutation in the *Cyp51* gene have been identified, *Z. tritici* haplotypes that carry more than one mutation in the *Sdh* gene are rarely detected in nature as of yet (Mäe et al., 2020). The C-H152R mutation observed in *Z. tritici* has been identified as the most important mutation so far, providing high resistance towards many of the major SDHI fungicides on the market (Fraaije et al., 2012; Scalliet et al., 2012; Rehfus et al., 2018; FRAC, 2021) and constitute major importance in the coming years in terms of SDHI efficacy and guidelines. If C-H152R in time becomes dominant in the European *Z. tritici* population, this mutation could lead to a complete control failure, just as the one observed for the QoIs. Alternatively, the evolution of SDHI fungicide resistance could be a gradual decrease in sensitivity. In a step by step course, in which the build-up of several mutations with lower impact on fungicide efficacy might steer the general *Z. tritici* population into a more resistant one (Dooley et al., 2016). The C-H152R mutation confers resistance to the major part of the newer SDHIs. The mutation was shown for the first time in *Z. tritici* field populations in Ireland in 2016 (Dooley et al., 2016) and has also been found in UK (BASF intern communication). Even though fitness penalties might have an impact on the spread and severity of *Z. tritici* populations carrying the mutations, the frequency of the alleles conferring high resistance is likely to increase, as a combination of strong selection pressure and good control of the pathogen will increase in the following years (Dooley et al., 2016). As Fan et al. (2015) showed in their study of fitness of *A. alternata*, in the case of SDHI resistance, the amino acid substitutions B-H227Y (SDHB) and C-H134R (SDHC), did not impact the isolates´ overall fitness (growth, spore production, osmotic and oxidative sensitivity

and pathogenicity). This was in accordance with what Bauske and Gudmestad, (2018) found for *Alternaria solani*, where five different mutations in the *Sdh* gene, did not cause fitness penalties, and where one mutation (D123E) gave rise to even more aggressive strains of *A. solani* on tomato leaves compared to sensitive strains.

Resistance development and impact of control strategies

Van den Bosch et al. (2011) described the evolution of fungicide resistance as having three stages (figure 11). (i) The emergence phase, wherein the first strains harboring a mutation occur, either through random mutation or via migration. The proportion of the resistant strain is very low at this point and might either die out due to random events, or progress to the next stage. (ii) The selection phase precedes the emergence phase. Once the resistant strain has grown into a sufficiently high frequency in which it won't perish due to random events. The selection phase's progress depends on the intensity and frequency of the fungicide group use and distribution.

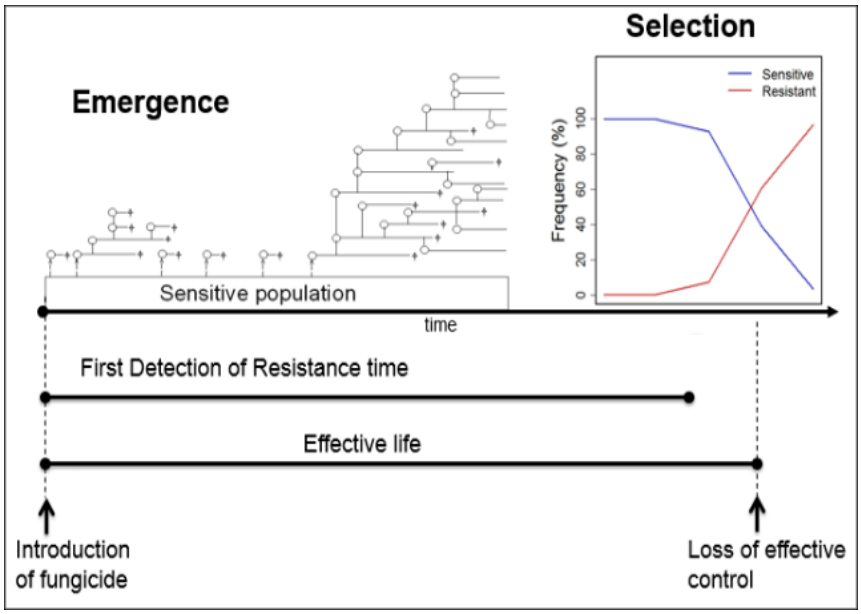

Figure 11 Phases of resistance evolution (Rehfus, 2018) modified after Van den Bosch et al. (2011).

Since fungicides present high selective pressure on *Z. tritici* populations, the strains that have an evolutionary advantage over the sensitive population are favored. The increase in the ratio of resistant strains versus sensitive strains in the population is expressed by the rate of increase and determines the selection phase. Once the resistant population has progressed into a substantially high frequency, and the fungicide(s) field efficacy in question is undermined, the (iii) adjustment phase follows. In the final phase, disease management practices have to be adjusted (Dooley, et al., 2016), to maintain a proficient control of the pathogen, either by introducing novel MOA fungicides within the class/group or by introducing fungicides with different MOA. The emergence of fungicide resistance can occur across different geographical areas, independent of one another. This was the case with QoI resistance and has also been shown for the DMIs and SDHIs (Sierotzki & Scalliet, 2013). In general, fungicide resistance development in the *Z. tritici* population in Europe originate in the North-Western part, mainly Ireland, where a combination of high disease pressure and high fungicide input favors a habitat in which high selection pressure is present and for the *Z. tritici* strains to adapt (Jørgensen et al., 2020). Fungicide-adapted strains are often discovered first in these ´high risk´ areas, due to

the conducive climatic conditions for fungal pathogens (Jørgensen and Wieczorek, 2018). This leads to a gradient of fungicide resistance, both in terms of emergence and prevalence, from the West to the East of Europe (Jørgensen et al., 2018). The mechanisms behind resistance development are mainly mutations in the target genes of fungicides, or alterations within the fungal cell. These alterations, however, may come with fitness penalties (Hawkins & Fraaije, 2018), where the alteration might prove beneficial in a fungicide intensive environment, but shown to be limiting in a non-selective environment. The mutations conferring resistance are often in the target site of fungicides. These targets are, as mentioned earlier, often biochemical enzymatic processes, which are essential for the pathogen. When mutations in such genes occur, they may impact the efficacy of the enzymatic processes of the cells. The impact of these mutations depends on the evolutionary environment (Bauske & Gudmestad, 2018; Hawkins & Fraaije, 2018). This has a great impact on resistance management, as is the case when resistance is attained via amino acid substitutions, without suffering fitness penalties. These isolates might compete with the sensitive population, even under a non-selecting environment (no use of the respective fungicide) (Fan et al., 2015).

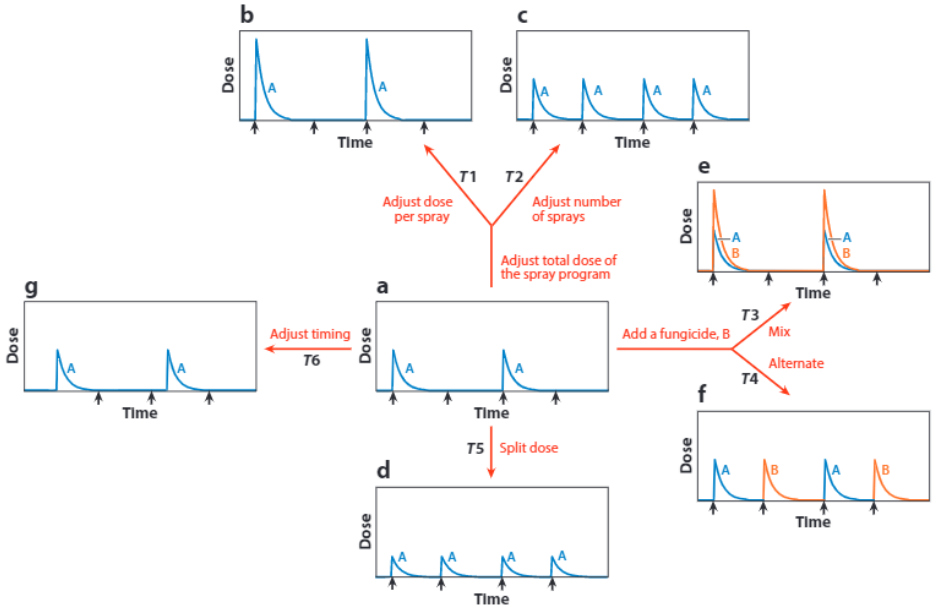

Figure 12 Aspects of resistance management that change one aspect at a given time (Van den Bosch et al., 2014).

Van den Bosch et al. (2014) evaluated and validated the governing principles of fungicide resistance, on the basis of Milgroom and Fry (1988). The overall goal of the governing principle is to reduce the selection coefficient and the exposure time of a pathogen population to a given fungicide. This is given in the generalized equation from Van den Bosch et al. (2014): $sT = (r_R - r_S)T$, in which T is the fungicide exposure time to a given pathogen population. r_R and r_S is the average per capita rate of increase of the resistant fraction (R) and sensitive (S) fraction of the pathogen population.

During the evaluation process, they investigated six different fungicide strategies (figure 12), named T1-T6. Each of the control strategies was evaluated based on modeling and experimental studies collected by the research team. Subsequently, all studies that included a given strategy, were separated into three categories, namely ´Increase selection´, ´No effect´, and, ´Decrease selection´. Each strategy will be shortly summarized in the following, and the results of the study are presented later.

T1: Adjusting the dose impacts the survival of the sensitive strains more so than the resistant strains. By increasing the dose, the fraction of the sensitive strains decreases, thereby increasing selection.
T2: Adjusting the number of applications impacts the time span of which the pathogen population is exposed to the fungicide. Increasing the number of applications thereby increases the time of exposure, which in term increases the selection.
T3: Adding a mixing partner impacts the survival of both the sensitive and resistant strains, since it is expected that the resistant strain is not resistant to fungicides of different MOA. The selection coefficient is thereby reduced, and selection is decreased.
T4: Alternating between fungicides of different MOA has no direct effect on selection since the time span of each fungicide does not overlap. Fungicide A (figure 12, T4) poses a selection pressure when applied, however, the selection does not take place when fungicide B (figure 12, T4) is applied. The selection coefficient is thereby not altered since no selection takes place.
T5: Splitting fungicide applications impacts the selection of the pathogen population by increasing the time span of exposure. Since the dose is split, as opposed to T2, in which the dose remains the same, but is applied at more than one instance, the dose is lowered, which decreases selection (as observed in T1), but increases the exposure time of fungicide, thereby increasing selection.
T6: Adjusting spraying timing may impact selection. Crop-pathogen interactions are crucial to establish when spray timing becomes relevant. Targeting stages of the pathogen life cycle, in which the pathogen is most vulnerable (i.e. pre-infection), might reduce selection impact.

Table 3 Fungicide practices and the impact on resistance selection (modified after van den Bosch et al., 2014).

	Increase selection	No effect	Decrease selection
Increase dose	24	1	2
Increase spray number	8	0	0
Split dose	12	0	1
Add mixture partner	1	5	43
Alternate (replace sprays)	0	3	15
Adjust timing	3	1	4

According to Van den Bosch et al. (2014), several fungicide practices can have a direct impact on the subsequent increase or decrease in terms of selection pressure (table 4). Increasing doses to label rate or even higher further selection to a very high degree, as opposed to half or lower rates of fungicide applied. Increasing the number of applications with the same fungicide also proves to impact selection in favor of resistant phenotypes. This is expected, due to the increased time span, in which the selection takes place. Furthermore, using split-dose strategies as opposed to single-dose strategies increases selection to a degree comparable to increased doses. This strategy is contradictory, since split-dose strategies distribute the dose, thereby decreasing the dose used at each application, however, as described above, the frequency of applications is increased, which is in favor of higher selection. When evaluating advice concerning strategies that decrease selection, thereby prolonging efficacy of present and future fungicides (fungicide effective life), adding a mixture partner, i.e. an SDHI and a DMI fungicide, will decrease selection to a very high degree. Alternatively, alternating different fungicides between applications also provides a decrease in the selection, and decreasing the dose also decreases the selection (Van den Bosch et al., 2014).

1.4 Monitoring for fungicide resistance

Monitoring fungicide resistance is carried out to survey the fungicide resistance development to react promptly when resistance arises. The monitoring is carried out by national research institutions (independent) and companies that need to prove that their products still provide sufficient control. FRAC collects the results from each year's monitoring and publishes the status of resistance development, frequency of mutations, and efficacy of different fungicides against the most important fungal pathogens on the major grown crops worldwide. The methods mentioned below are relevant for identifying lower sensitivity in the *Z. tritici* populations, as well as an explanation for the observed decrease in field control. However, as the observed sensitivities only rely on a small proportion of the total *Z. tritici* population, the data obtained from these measurements are not to be taken to the extreme, when interpreting the results. Resistance might be observed in the laboratory, but it is essential to conduct field trials, and evaluate the level of control for a given fungicide attained in the field, before concluding on the resistance levels of a given population, in a given area (Brent & Hollomon, 2007).

EC_{50}

Fungicide resistance is an arbitrary expression and interpretation of the measured fungicide sensitivity observed in single isolates and field efficacy. A method used to monitor resistance development is monitoring isolates' half-maximal effective concentration (EC_{50}) for different compounds. An EC_{50} value is defined as the concentration at which 50% of fungal growth is inhibited in *in vitro* studies (Oliver & Hewitt, 2014). Fungal pathogens that are non-obligate are grown and harvested from agar plates. The spores are then suspended in a liquid before being added to 96-well microtiter plates, in which increasing concentrations of the respective fungicide are present. The plates are then inoculated for a period, before the growth of each isolate at each concentration is measured on a microplate reader, which measures the absorbance. The data produced can then be used to calculate the EC_{50} values for each isolate, to give an estimate of the degree of inhibition offered by the respective fungicide. Since the EC_{50} values only offer a concentration of which 50% of the fungal growth is inhibited, resistance factors (RF) are often calculated, to provide a better interpretation of the resistance levels. The RF are calculated as the ratio of EC_{50} of a ´resistant´ isolate to that of a sensitive isolate, usually an isolate known to have high sensitivity to the fungicide (Oliver & Hewitt, 2014). The resistance factor is arbitrary but can be divided into low (>5), moderate (5-20), and high (>20). Resistance is deemed, when an isolate exceeds the moderate-to-high RF values, while lower resistance factors can be interpreted more as variation and different tolerance of the strain to the fungicide.

Molecular methods

EC_{50} values verify if tested fungicides are effective in controlling the tested strains of *Z. tritici*. When an explanation for the observed decrease in sensitivity is needed, molecular methods offer several methodologies that explain the mechanism of resistance. These include the search for target site mutations, efflux, over-expression, etc.

PCR and qPCR

Polymerase chain reaction (PCR) is the amplification of defined sections of DNA to an amount that can be sequenced or visualized on agarose gel. Quantitative-real-time PCR (q-RT-PCR) enables the user to view the amplification process in real-time. Amplicons are the designated

part of the DNA that the user wants to amplify, using specific primers designed to anneal to the target sequence. Probes, which consist of a fluorophore and a quencher, are then used which anneal to the specific sequence. The probe recognizes the sequence of the gene of interest on single-stranded DNA (ssDNA) and binds to it. During elongation, when the DNA polymerase starts to produce double-stranded DNA (dsDNA), the probe is degraded, which releases the fluorophore from the quencher, causing it to emit light. A qPCR machine then measures the gradual increase in light emitted from the consecutive reactions, which take place during each cycle. The cycle time (Ct) value is an estimate of the point at which DNA is amplified exponentially. The lower the value, the higher the starting concentration of DNA was and vice versa. PCR takes place in a thermocycler working in three steps. (i) Heating splits the dsDNA (denaturation of hydrogen bonds). (ii) Cooling allows for annealing, which takes place when the primer (18-30 n) binds to the specific sequence from the 3' side of the sequence (from 5'->3' synthesis). (iii) DNA elongation, where the DNA polymerase starts to synthesize the new DNA strand, starting point indicated by the primer. This method can be used to screen *Z. tritici* DNA for mutations, that are known to confer resistance, based on the sequenced genome of *Z. tritici*.

2 Aim of study

The aims of the thesis were (i) to monitor the frequency of the *Sdh* mutations C-T79N and C-N86S and the *Cyp51* mutation S524T in Danish and Swedish *Z. tritici* isolates from 2019/20. (ii) To estimate sensitivity (EC_{50}) of *Z. tritici* single isolates from Denmark and Sweden from 2019/20 to prothioconazole-desthio and fluxapyroxad, and (iii) to estimate cross-resistance of fluxapyroxad x fluopyram x boscalid. (iv) To measure the level of multidrug resistance in *Z. tritici* populations. (v) To assess the selection of the mutations based on different fungicide schemes from field trials.

Hypothesis

1. Since 2018, Swedish farmers have gained access to more SDHI fungicides than Danish farmers. Do the access and use of more SDHI products lead to an increase in cases of SDH mutations?
   It is known that frequent use and high dosages can lead to resistance development.
   - Different SDHI and DMI fungicides select differently in the *Sdh* and *Cyp51* genes of *Z. tritici* under field conditions.
2. Denmark and Sweden are two very similar countries regarding climate and agronomical approach, but with different history and fungicide uses. Is there a difference in the multidrug resistance development in the two countries, and can this be due to the use of different fungicides?
3. Fungicide schemes that rely on solo acting fungicides split treatments, and high doses select to a higher degree for mutations conferring resistance towards the respective group of fungicides.

3 Emerging SDHI resistance in the *Zymoseptoria tritici* populations in Denmark and Sweden – what drives the selection?

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Abstract

Zymoseptoria tritici (*Z. tritici*) is one of the major fungal pathogens and the causal agent of septoria tritici blotch (STB) on wheat. Farmers rely to a major extent on the use of fungicides to control the disease and limit yield losses. The main fungicide classes used are the demethylase inhibitors (DMIs) and the succinate dehydrogenase inhibitors (SDHIs). Frequent use of fungicides commonly leads to the development of resistance in the *Z. tritici* population. DMIs have been used for STB control for the last 40 years and a gradual sensitivity shift has been observed as a result, due to the accumulation of *Cyp51* target site mutations, conferring low-to-high resistance levels. In recent years, prothioconazole has been the most used DMI in Denmark and Sweden, and its use has led to an adopted *Z. tritici* population. SDHI fungicides have been used for less than ten years for the control of STB, and mutations conferring resistance to this group have been detected in several countries, however, to a varying degree. This study monitored the frequency of the two target site mutations C-T79N and C-N86S in the SDH enzyme, the target site of SDHI fungicides, and the target site mutation S524T in the CYP51 target enzyme of DMI fungicides, in *Z. tritici* single isolates collected in 2019 and 2020 from Denmark and Sweden. The same target site mutations were also monitored from leaf samples from field trials carried out in 2020 using different control strategies. The S524T mutation was found in varying (6-31 %), but increasing degrees across Denmark and Sweden, from 2019 to 2020. C-N86S and C-T79N were only rarely detected in the single isolates and field samples, indicating that the *Sdh* mutations so far only occur at low frequencies in Danish and Swedish *Z. tritici* populations. The field trials indicated that the use of the most potent SDHI containing compounds, which include fluxapyroxad, imposed selection of the C-N86S mutation, and to some extent also the C-T79N mutation when compared to other SDHIs. The treatments that included azoles showed a selection of S524T with a tendency of prothioconazole selecting more so than mefentrifluconazole. Split treatments did also select more compared to solo control strategies. Overall, this study confirmed that, under field conditions, current anti-resistance strategies, including lower doses, limiting the number of treatments, mixing and alternating fungicides of a different mode of action, are essential to prolong the efficacy of current and future fungicides and to delay resistance development in the *Z. tritici* population.

Keywords: Fungicide resistance, septoria tritici blotch, demethylase inhibitors, succinate dehydrogenase inhibitors

1. Introduction

Wheat (*Triticum aestivum*) is cultivated worldwide and accounts for 18 % of the global population's calorie intake (Savary et al., 2019). With a growing world population, the demand for higher crop yields becomes ever more pressing, with wheat not being an exemption (Long, et al., 2015). A necessary increase of global wheat yields of 50-70% is estimated to accommodate the population of 2050 (Driscoll, et al., 2014).

Several fungal pathogens play an important role in reducing wheat yields across the world. The yield losses due to various fungal pathogens vary depending on climatic conditions, farming practices, and grown cultivars (Savary et al., 2019). While *Puccinia striiformis* is the most devastating pathogen on wheat in America, *Zymoseptoria tritici* (*Z. tritici*), the causal agent of septoria tritici blotch (STB), is the most important under North-Western European growing conditions (Dean et al., 2012; Torriani et al., 2015).

Under favorable conditions, yield losses attributed to *Z. tritici*, with low levels of control and non-resistant cultivars, have been reported as high as 50 % (Eyal, et al., 1987). To control the disease, farmers heavily rely on chemical fungicides, which form the backbone of management control strategies of the pathogen.

Currently, the main groups of fungicides in wheat production are the quinone outside inhibitors (QoI), the demethylation inhibitors (DMI), and the succinate dehydrogenase inhibitors (SDHI) (Oliver & Hewitt, 2014), and multisite inhibitors such as folpet. QoI fungicides, however, no longer offer sufficient control of *Z. tritici* in many parts of the world since the emergence of the G143A mutation, which confers complete resistance to all compounds within this fungicide group (Bartlett et al., 2002; Jørgensen et al., 2017). Therefore, *Z. tritici* control mainly relies on spray programs with a combination of DMI and SDHI fungicides (Jørgensen et al., 2014; Lucas et al., 2015) and, to some extent, on multisite inhibitors (Lucas et al., 2015).

DMIs act by disrupting the CYP51 enzyme, an essential part of the biosynthesis pathway of ergosterol, which is a crucial compound in the fungal cell wall (Cools & Fraaije, 2013). The DMI fungicides have been used for control of STB for more than 40 years (Russell, 2005) and are at moderate risk for developing fungicide resistance (FRAC, 2021). Despite this moderate risk, DMI fungicides have experienced a significant decrease in control levels during the last decade (Leroux, et al., 2007; Cools & Fraaije, 2013; Heick, et al., 2020; Mäe, et al., 2020). This shift can be explained by the accumulation of mutations in the *Cyp51* target gene of DMIs, along with overexpression of CYP51 and efflux transporters (Cools et al., 2012; Omrane et al., 2015, 2017). Over 30 mutations have been reported conferring DMI resistance at varying levels. The S524T amino acid substitution, a relatively recent change, has been found to decrease sensitivity to all DMI fungicides and indicate the adaption of DMI resistance to the currently most widely used DMIs (Jørgensen et al., 2021).

SDHI fungicides have been on the market since 1960, with the earliest compounds of the group, carboxin, and oxycarboxin (Sierotzki & Scalliet, 2013). These older SDHI had a narrow target spectrum and did not attain high usages prior to introducing the first broad-spectrum fungicide, boscalid (Glättli et al., 2011). Since the introduction of boscalid in 2003 (Stammler et al., 2015), several new generation SDHIs have either reached the market or are on the way (Rehfus et al., 2018). SDHI fungicides act by inhibiting the fungal respiration, blocking the quinone at the SDH or complex II of the electron transport chain, which also inhibits the reduction of succinate to fumarate, a vital constituent of the Krebs-cycle (Sierotzki & Scalliet, 2013). Decreased sensitivity to fungicides of this group has been detected in several countries and pathogens (Stammler et al., 2015; Rehfus et al., 2018; Birr et al., 2021). As with the DMI fungicides, the main resistance mechanisms derive from amino acid substitutions in the three subunits *SdhB, SdhC,* and *SdhD* of the target gene *Sdh* (Sierotzki & Scalliet, 2013). Several mutations have been identified (FRAC, 2021) with low-to-moderate resistance impact, among those, C-T79N and C-N86S. However, the most recent mutation C-H152R has been shown to cause a loss of sensitivity to all SDHI class fungicides (Dooley et al., 2016; Hellin et al., 2020).

Resistance monitoring is important, in order to implement relevant control strategies in the field, to both ensure crop yield security and prolong the efficacy of fungicides for as long as possible (Barrès et al., 2016). In recent years rapid and easy detection of resistance has been made available by assessing specific target site mutations on either single isolates or leaf samples. Using specific primers for the target mutations, based on sequenced data on the *Z. tritici* genome and Real-Time polymerase chain reaction (RT-PCR/qPCR), the dynamic of changes can be examined following different control strategies' and scenarios' impact on selection.

The aims of this study were (i) to determine EC_{50} sensitivity values of *Z. tritici* to prothioconazole-desthio and fluxapyroxad. (ii) To estimate cross-resistance levels between three widely used SDHI fungicides (boscalid, fluopyram, and fluxapyroxad) in the presence of the Sdh mutations C-T79N and C-N86S. (iii) To monitor the frequency of the *Sdh* mutations C-T79N and C-N86S, the *Cyp51* mutation S524T, and promotor insert in the *MFS1* gene, in Danish and Swedish *Z. tritici* isolates from 2019 and 2020. (iv) To measure the selection conferred by different fungicide schemes on the specific mutations.

Material and methods

Field trials

A total of six field trials were conducted during the growing season of 2019 and 2020, four of which were located at AU Flakkebjerg Research Center, and one of which was situated in Jutland near Horsens, in Denmark. One trial was carried out in Southern Sweden by the Swedish Board of Agriculture ‘Jordbruksverket’ (table 2). All the trials were designed as block trials

with randomized plots and four replicates containing listed treatments and untreated control (tables 1,2). The plot size varied from 12.5 to 22 m^2, depending on the site. Each trial was sprayed according to the trial plans shown in tables 4-7. Application rates and combinations of products varied depending on the specific protocol. In most cases, either half or full-label rates were used. All fungicides were applied using a plot sprayer in 200 L ha^{-1} water at low pressure with flat fan nozzles. In terms of other management control strategies, the crop was treated using standard cultural practices. A list of the fungicides used in this study, with their respective formulation names, standard-dose rate, and active ingredients is given in table 1. The hypothesis of each specific trial is given in table 2.

Disease and yield assessments

Each trial was assessed for the percent severity of STB in 10-day intervals during the growing season. The two upper leaves were chosen as representatives for the overall disease severity in each trial. The assessments were carried out in accordance with EPPO guideline 1/26 (4). Disease assessments at growth stage (GS) 75 were included as the key assessment in the statistical analyses. All trials were harvested and grain yield measured per plot, adjusted to 15% moisture content, and converted to dt/ha.

Table 1 List of fungicides, the treatment numbers, producer, and their respective active ingredients and standard-dose rate.

Treatment	Fungicide	Company	Dose – standard rate l/ha	Active ingredient per l
1	Revystar	BASF	1.5	50 g fluxapyroxad + 100 g mefentrifluconazole
2	Imtrex	BASF	2.0	125 g fluxapyroxad
3	Luna	Bayer	0.2	500 g fluopyram
4	Thore	Bayer	1.0	125 g bixafen
5	Silvron Xpro	Bayer	0.75	100 g bixafen + 100 g fluopyram
6	Elatus plus	Syngenta	1.0	100 g benzovindiflupyr
7	Revysol	BASF	1.5	100 g mefentrifluconazole
8	Prosaro EC 250	Bayer	1.0	125 g prothioconazole + 125 g tebuconazole
9	Propulse SE 250	Bayer	1.0	125 g fluopyram + 125 g prothioconazole
10	Balaya	BASF	1.0	100 mefentrifluconazole + pyraclostrobin
11	Amistar gold	Syngenta	1.0	125 g difenoconazole + 125 g azoxystrobin
12	Folicur Xpert	Bayer	1.0	120 g tebuconazole + 80 g prothioconazole
13	Entargo	BASF	0.7	500 g boscalid
14	Univoq	Corteva	1.5	50 g fenpicoxamid + 100 g prothioconazole
15	Proline 250EC	Bayer	0.8	250 g prothioconazole
16	Ascra xpro	Bayer	1.5	130 g prothioconazole + 65 g fluopyram + 65 g bixafen
17	Sulfur product	UPL	5 kg	825 g sulfur

Table 2 List of trials, the treatments, the cultivars, the location, and the respective goal of each trial.

Trial	Treatment	Cultivar	Location	Goal
(1)	2-7	Hereford	Flakkebjerg, Denmark	To test the efficacy, yield potential, and mutation frequencies from different SDHI fungicides and mefentrifluconazole application at GS 37.
(2)	1-2, 10, 14-17	Torp Torp	Flakkebjerg, Denmark Jordbruksverket, Sweden	To test the efficacy, yield potential, and mutation frequencies from different solo or coformulations of DMI and SDHI fungicides used alone (GS 39) or in split applications (GS 37 and 55).
(3)	8-13, 2	Hereford Torp	Flakkebjerg, Denmark Horsens, Denmark	To test the efficacy, yield potential, and mutation frequencies from different coformulations of DMI and SDHI fungicides used in a split application strategy applied at GS 37 and 55.
(4)	1	Hereford	Flakkebjerg, Denmark	To test the efficacy, yield potential, and mutation frequencies from half and full dose rate of Revystar (mefentrifluconazole and fluxapyroxad) at GS 37 and a half dose split application at GS 37 and 55.

Isolation of Z. tritici isolates

Single pycnidium isolates were produced from leaf samples collected in 2019 and 2020. The leaves were kept in Petridishes on moistened filter paper at high humidity for 24 hours. With a sterile needle, cirrhi from single pycnidium were transferred to Potato Dextrose Agar supplemented with 0.01% streptomycin, before incubation at 20 °C in a 12 hour white light, 12-hour darkness cycle for five days. A total of 740 *Z. tritici* isolates were produced. *Z. tritici* isolates were stored at -20 °C degrees until further use. The distribution of *Z. tritici* isolates from Denmark and middle/southern Sweden 2019-20 is given in table 3. Since sensitivity change is a gradual process, which does not necessarily develop from one year to another, the sensitivity data on prothioconazole-desthio and fluxapyroxad for *Z. tritici* from 2016-18 were included in the data analysis.

Table 3 The number of pycnidial isolates of *Z. tritici* used to analyze EC_{50} values for the two fungicides, the presence of inserts in the *MFS1* gene, and mutation detection using qPCR.

Year x region	Denmark	Middle Sweden	South Sweden
2019	173	189	93
2020	118	93	75
Total	291	282	168

In vitro sensitivity test and determination of EC_{50} values

All single isolates from 2019 and 2020 were *in vitro* screened for sensitivity to fluxapyroxad and prothioconazole-desthio (both Sigma-Aldrich, St. Louis, MO, USA) with microtitre assays. The spore suspensions were made by scraping six-day-old *Z. tritici* spores, which were transferred to sterile demineralized water. The suspensions were then vortexed in 10 mL Falcon tubes for 10 min to homogenize the suspensions. The spore concentrations were adjusted to 2.5 x 10^4 spores mL^{-1}. Prothioconazole-desthio and fluxapyroxad were mixed with 2 x Potato Dextrose Broth to obtain the following fungicide concentrations (mg $^{L-}$1): 10, 3.33, 1.11, 0.37, 0.12, 0.041, 0.014, 0. A total of 100 µl of spore suspension and 100 µl of fungicide solution were added to nunc™ 96-deep well microtitre plates (ThermoFisher, Roskilde, Denmark). Every isolate was made in duplicates, and the sensitive Dutch isolate IPO323 and OP15.1 were used for both fungicides as references. The microtiter plates were then covered in aluminum foil and incubated in the dark at 20 °C for six days. During the six days, plates were visually assessed for bacterial or fungal contamination before the analysis, which was performed in an iMark™ Microplate Absorbance Reader (Bio-Rad, Copenhagen, Denmark) at wavelength 620nm.

The fungicide sensitivities were calculated as the concentration of fungicide, which inhibited fungal growth by 50% (EC_{50}) by non-linear regression (curve-fit) using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Following EC_{50} calculation and qPCR detection of the three mutations and multi-drug resistance, 30 isolates were chosen for further testing, based on either high EC_{50} values (prothioconazole-desthio or fluxapyroxad), promotor insert in the *MFS1* gene, presence of investigated mutations, or a combination of the mentioned. The 30 isolates were examined for their sensitivity to fluopyram and boscalid (both Sigma-Aldrich, St. Louis, MO, USA), in the same procedure as described above.

C-N86S, C-T79N, and CYP51-S524T mutation frequencies

Single isolates from Denmark and Sweden

Single isolates from 2019 and 2020 collected from trials in Denmark and Sweden were analyzed for the *Sdh* mutations C-T79N and C-N86S, along with the *Cyp51* mutation S524T using qPCR.

DNA was extracted using a 'rapid DNA extraction' protocol. *Z. tritici* isolates were cultured on PDA, harvested, and transferred to 96-well microtiter plates and stored at -20 °C until use. A total of 50 µl of NaOH (250nM) was added to each well, covered with aluminum foil, and incubated for 2 min at 96 °C in a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA). After incubation, 100 µl of Tris-HCL (500 nM, pH9) were added to each well and incubated as previously described. The samples were then centrifugated at 2.500 rpm for 2 min in an Eppendorf Centrifuge 5430 (Eppendorf, Hørsholm, Denmark). Samples were diluted 1:10 with DEPC-water in a separate 96-well microtiter plate and stored at -20 °C until further use.

Mutation detection was carried out using specific primers for each of the three mutations, using primers and probes designed by Hellin et al. (2020; submitted). All qPCR reactions were carried out on a ViiA7 Thermocycler (ThermoFisher Scientific, Roskilde, Denmark). The cycling conditions were: initial heating at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 sec, and annealing at 60 and 62 °C for SDH and CYP51 alterations respectively, for 1 min. The reactions were prepared with 10 µl of Takyon™ Low Rox Probe Master Mix (Eurogentec, Seraing, Belgium), 6.3 and 6.7 µl DEPC water, 0.4 and 0.6 µl forward and reverse primers (both 10µM), for C-N86S/S524T and C-T79N respectively, 0.25 µl of the respective probe pair for each of the mutations (both 20 µM), and 2 µl of DNA template.

Leaf samples from field trials

Leaf samples (5x10 leaves per replicate) with STB symptoms from six field trials were collected at GS 75-77. In each trial, leaves were picked from each of the four replicates, kept separate, dried at room temperature, and stored until further use. The leaves were cut into discs using a Whatman™ biopsy puncher. DNA was extracted from a bulk of leaf samples (50 discs from 10 leaves). The samples were then transferred to Eppendorf tubes, lyophilized for 24 hours before being ground in the presence of two steel balls (ø 5mm) for 4 min at 1.500 rpm on a Geno/Grinder™ 2010 (Spex SamplePrep, New Jersey, USA). Genomic DNA was extracted from the pulverized leaf/fungal material using Sbeadex™ mini plant kit (LG Genomics, Teddington, UK) according to the manufacturer's protocol on a KingFisher™ Flex Purification System (Thermo Fisher Scientific, Roskilde, Denmark) and eluted in 50 µl elution buffer.

The three mutations (C-T79N, C-N86S, and S524T) were detected using qPCR as previously described, and every reaction was performed in duplicates. The quantitative analysis was based on dual-labeled probes (FAM and VIC) to estimate a ratio between the wild-type allele and mutation, based on ct values (Hellin et al., 2020). The frequency of sensitive allele and resistant allele were calculated as described by Rehfus et al. (2016) with the formula:

Frequency of $allele_1 = 1/(2^{\Delta Cq} + 1)$

ΔCq = (Cq of $allele_1$ specific PCR) – (Cq of $allele_2$ specific PCR)

Multi-drug resistance

Multi-drug resistance in *Z. tritici* was detected in this study, using polymerase chain reaction (PCR) with specific primers for the *MFS1* gene for efflux transporters as described by Omrane et al. (2015). The DNA amplicons were mixed with 6x DNA loading dye (Thermo Scientific, Roskilde, Denmark), transferred onto 1% agarose gel, with 5 µl SYBR® Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA), and run on a gel electrophorese system for 30 min at 100 V. DNA amplicons were visualized on a gel imaging system (Bulldog-Bio, Portsmouth, NH, USA).

Statistical analysis

Analysis of sensitivity and EC_{50} values along with field data on STB disease severity and yield was performed in the Agricultural Research Management (ARM) software. Data from 2006 to 2009 and 2016 to 2018 was included in the data analysis to get a greater grasp on the sensitivity changes.

Results

EC_{50} values for the tested fungicides

The number of *Z. tritici* isolates tested varied between years and country (Table 3). All isolates were screened for sensitivity to fluxapyroxad and prothioconazole-desthio. Prothioconazole-desthio had EC_{50} values between 0.01 and 6.00 ppm, and between 0.003 and 3.00 ppm for Denmark and Sweden respectively. Fluxapyroxad had EC_{50} values between 0.004 and 3.00 ppm, and between 0.005 and 1.5 ppm for Denmark and Sweden respectively. EC_{50} values for both prothioconazole-desthio and fluxapyroxad showed significant differences between the years for both Sweden and Denmark. Figure 1 shows the average log-transformed EC_{50} values for prothioconazole-desthio and fluxapyroxad from the tested *Z. tritici* isolates of Denmark and Sweden. EC_{50} values for prothioconazole-desthio remained stable between the growing years 2016 and 2020, both in Sweden and in Denmark, however with some fluctuations between the years. A clear shift in sensitivity was observed from the EC_{50} values of 2006 to 2009 and 2016. The EC_{50} values for fluxapyroxad did not change significantly in the time span of 2006 to 2020, neither in Denmark nor Sweden. Considering the effects of years, the sensitivity of *Z. tritici* isolates did change significantly to both fungicides between 2016 and 2020.

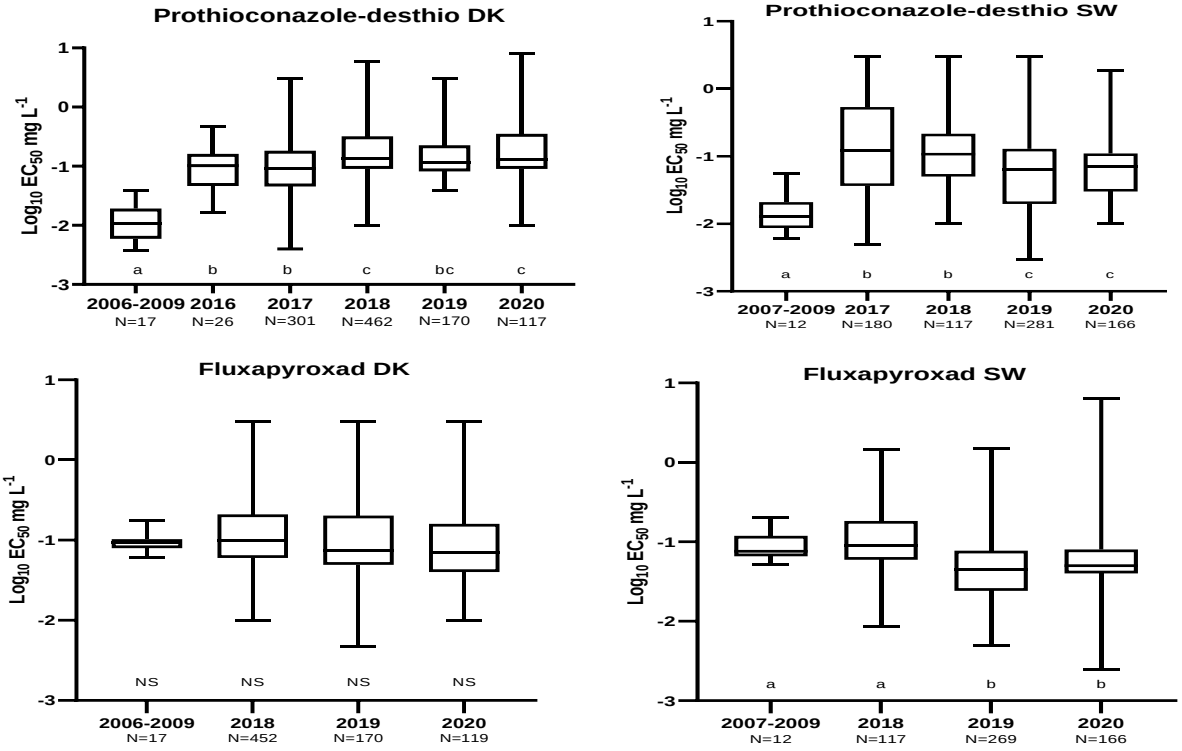

Figure 1 Box-whisker plots of log-transformed EC_{50} values for prothioconazole-desthio and fluxapyroxad from Denmark and Sweden, 2016 to 2020. The horizontal line represents the median, while the outer box indicates the 5th and 95th percentile. Whiskers indicate lower and upper limits. Letters indicate a significant difference between years and country.

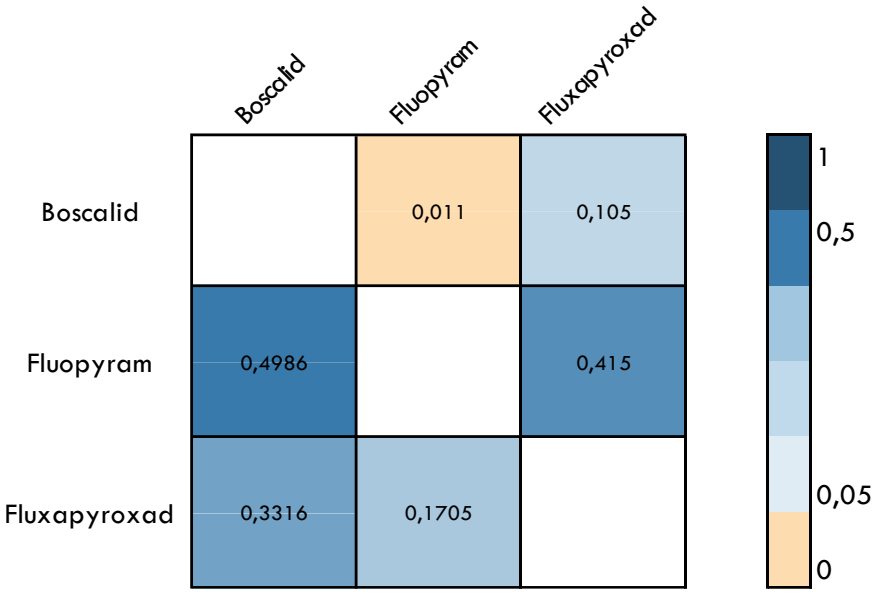

Figure 2 Correlation matrix between boscalid, fluxapyroxad, and fluopyram fungicide sensitivity in Danish and Swedish *Z. tritici* populations. Below the diagonal are Pearson correlation coefficient (r) values represented, while p-values are above the diagonal. Based on 30 selected isolates from Denmark and Sweden.

Following microtiter tests with fluxapyroxad and prothioconazole-desthio, 30 isolates with high EC_{50} values or confirmed presence of mutations tested, were further investigated for their sensitivity to boscalid and fluopyram. The EC_{50} values for boscalid, fluxapyroxad, and fluopyram were then further analyzed through Pearson correlation. The analysis showed that boscalid and fluopyram sensitivity was positively correlated (r = 0.499, p = 0.011) (figure 2). No significant correlation was found between boscalid and fluxapyroxad nor fluxapyroxad and fluopyram.

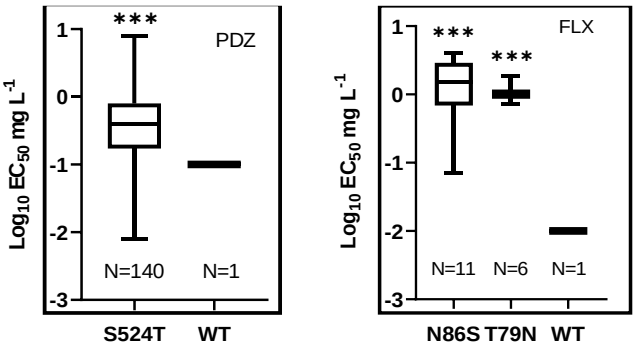

Figure 3 Average log-transformed EC_{50} values for isolates carrying each of the respective mutations S524T, C-N86S, and C-T79N to prothioconazole-desthio (PDZ) and fluxapyroxad (FLX). * indicate significant difference.

The average log-transformed EC_{50} values for isolates that carried the same mutation, for fluxapyroxad or prothioconazole-desthio are shown in figure 3. *Z. tritici* sensitivity to fluxapyroxad decreases when the strain harbors either of the two amino acid substitutions (C-T79N and C-N86S). The highest decrease in sensitivity was observed for the C-N86S mutation but it was not significant. The same was observed for the isolates carrying the S524T mutation when exposed to prothioconazole-desthio.

CYP51 mutation S524T in single isolates

All 740 *Z. tritici* isolates were investigated for the presence of the S524T mutation in Danish and Swedish *Z. tritici* isolates. A mutation frequency was then calculated, based on the number of isolates carrying mutation out of the total number of isolates for each respective region and year. Isolates from Sweden were grouped in two categories, either "south" or "middle" depending on their geographical origin. Overall, an increase was observed in all locations from 2019 to 2020, except for middle Sweden. In Denmark, the frequency of S524T increased by around 7% points. An increase from 13% to 31% in the frequency of isolates carrying the S524T amino acid substitution was observed from 2019 to 2020 in the south Swedish *Z. tritici* population. For the middle Swedish *Z. tritici* population, no apparent change was detected in the investigated populations from 2019 to 2020 (figure 4).

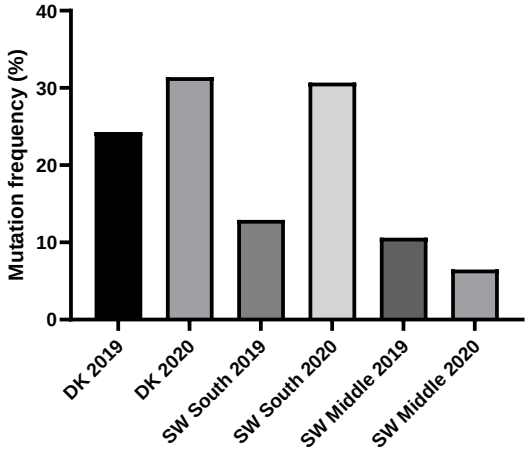

Figure 4 Frequency (%) of the S524T mutation in *Z. tritici* collected in 2019 and 2020 in Denmark (DK) and the south and middle regions of Sweden (SW).

SDH-C mutations T79N and N86S in single isolates

Out of 740 *Z. tritici* single isolates, only a few isolates harbored either of the two *Sdh* mutations tested in the qPCR assays. C-N86S was more prevalent than C-T79N, as shown in figure 5. In Denmark, single cases of either C-T79N and C-N86S were observed in 2019, while an increase was observed in 2020, of 1.3% and 3.9% respectively. C-T79N was not detected in isolates from Sweden in 2019, and only a single case was observed in 2020, still at an occurrence rate below 1 %. The C-N86S mutation increased slightly from 0.4% to 1.2% in Sweden from 2019 to 2020.

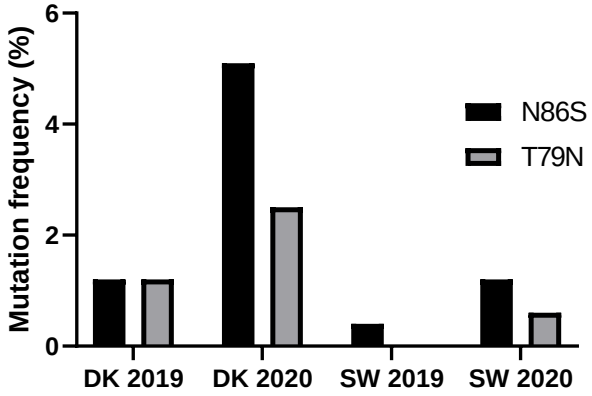

Figure 5 Frequency of the C-N86S and C-T79N (%) for single *Z. tritici* isolates from Denmark (DK) and Sweden (SW) in 2019 and 2020.

Multi-drug resistance

Furthermore, all isolates were screened for the presence of promotor inserts in the *MFS1* gene. All promotor inserts described by Omrane et al. (2017) (Type I, II(a/b), and III) were detected in this study. Type I and III promotor inserts were only detected in two separate single isolates from Denmark in 2019. The detected frequency of type II/a (3%) and type II/b (1%) was seen in Danish isolates from 2019. In Danish isolates from 2020, the detected frequencies were 1% for type II/a and 3% for type II/b. Out of all Swedish isolates from 2019, only a single isolate harbored an insert, the type II/a. In 2020 3% of the Swedish isolates carried the type II/b insert.

Control of STB and yield responses from STB control

Infection of STB developed in all trials, however, disease pressure varied depending on site and cultivar. Overall the infection level was moderate due to climatic conditions of the growing season 2020. Even so, attacks on 2nd leaves (F-1) varied in untreated plots between 50 and 80% severity, while the disease severity on flag leaves varied between 5 and 60%, the latter assessed in the most susceptible cultivar. The two cultivars Hereford and Torp, have high and moderate susceptibility scoring, respectively, according to Sort-info.

All treatments provided significant and good control of STB, and only in a few cases, inferior control was assessed for specific solutions.

All treatments in the six trials provided significant yield responses, increasing the range of 5-15 %. The increase typically reflected the obtained degrees of disease control. More details on the specific plans are given in the following section.

Field trials

In this section, specific data from each trial is summarized, including both mutation frequencies of the three mutations analyzed from leaf samples collected from the field trials, and data on the efficacy of STB control and yield responses.

Table 4 The effect of different SDHI fungicides and one azole (mefentrifluconazole) on mutation frequencies (%), level of STB control (%) on the flag leaf (leaf 1), and the second leaf (leaf 2) and yields (dt/ha). Different letters represent statistically significant differences (Trial 1).

	Mutation frequency (%)			STB (%)		Yield (dt/ha)
Treatment	S524T	T79N	N86S	Leaf 1	Leaf 2	
Untreated	20	0 b	0 b	61,3 a	85,0 a	99,9 c
125 g fluxapyroxad	9	3 a	29 a	2,5 d	20,0 cd	112,0 a
100 g fluopyram	11	0 b	3 b	17,3 c	66,3 b	105,2 b
125 g bixafen	20	0 b	4 b	4,5 d	19,8 cd	110,0 a
100 g bixafen + 100 g fluopyram	19	0 b	12 b	1,0 d	7,8 e	112,6 a
100 g benzovindiflupyr	14	0 b	8 b	5,0 d	26,8 c	111,5 a
150 g mefentrifluconazole	18	0 b	1 b	2,0 d	9,8 e	112,4 a
Treatment prob(F)	0.5	0.001	0.01	0.0001	0.0001	0.0001
LSD 95	NS	1.2	15.2	6.3	6.8	3.4

Trial 1 tested the effect of different SDHI fungicides (Table 4), it was observed that the most potent SDHI fungicide, fluxapyroxad, resulted in the highest registered mutation frequencies of both C-T79N and C-N86S (3% and 29% respectively), followed by the coformulation of the two SDHIs (bixafen + fluopyram) with 12% mutation frequency of C-N86S. The level of the S524T mutation frequency was around 20% in all treatments, even in the treatment with mefentrifluconazole, indicating that this azole did not increase the mutation frequency of the *Cyp51* S524T mutation. Control of STB varied among the treatments, with the highest levels of control achieved by the coformulation of bixafen and fluopyram, as well as the mefentrifluconazole treatment. Yields increased significantly compared to untreated, but responses to treatments were not significantly different, except for the sole floupyram treatment, which yielded significantly lower compared to the other treatments in the trial.

Table 5 The effect of different co-formulations of SDHIs and DMIs used as solo treatments or as split treatments on mutation frequencies (%), level of STB control (%) on the flag leaf (leaf 1), and the second leaf (leaf 2) and yields (dt/ha). Different letters represent statistically significant differences. Leaf 1 was only assessed from the Danish trial, while leaf 2 was assessed in both SW and DK trials and the average (avg.) was estimated (trial 2).

Treatment	Mutation frequency (%)			STB (%)		Yield
	S524T	T79N	N86S	DK Leaf 1	Avg. Leaf 2	(dt/ha)
Untreated	30.9 ab	0	0.8	9.5 a	39.4 a	114.9 b
GS 39 0.8 Proline	48.3 a	0	0.5	3.5 b	22.0 c	120.1 a
GS 39 1.5 Balaya	33.0 ab	0.3	0.4	0.1 d	1.8 e	121.5 a
GS 39 1.5 Ascra Xpro	37.2 ab	0	1.9	0.1 d	2.4 e	121.0 a
GS 39 2.0 Imtrex	30.6 ab	1.3	7.5	0.1 d	3.6 e	121.7 a
GS 39 1.5 Univoq	35.9 ab	1.4	1.6	0.1 d	8.8 d	120.5 a
GS 39 5.0 sulphur	18.3 b	0	0.3	2.3 c	27.8 b	115.5 b
GS 32 0.75 Ascra Xpro + GS 55 0.75 Revystar	17.8 b	0.1	17.0	0.1 d	1.2 e	123.8 a
GS 32 0.75 Ascra Xpro + GS 55 0.75 Univoq	18.6 b	0.4	4.8	0.2 d	2.5 e	123.4 a
Treatment prob(F)	0.005	0.32	0.0006	0.0001	0.0001	0.0002
LSD 95	16.0	NS	7.6	0.89	3.8	3.0

Trial 2 compared different coformulations and split treatment (table 5), which showed that Proline (prothioconazole) yielded the highest mutation frequency of S524T (48%), followed by Ascra Xpro (37%) and Univoq (36%), which also contain prothioconazole. The highest degree of mutation frequency of C-N86S was detected in the split treatment (Ascra Xpro and Revystar), both of which contain an SDHI in the coformulation (17%), followed by Imtrex (8%) and Ascra Xpro/Univoq (5%), both treatments containing one SDHI active ingredient. Only the double treatment with SDHI increased the mutation rate significantly. The level of control for all treatments did not vary, except for the sole Proline or sulfur treatment, which performed significantly lower compared to the control. With the exception of the sulfur treatment, all treatments increased yield significantly compared to untreated. The yield responses from the specific treatments did not vary significantly, however, the superior yield response derived from the split treatments of Ascra Xpro and Revystar.

Table 6 The effect of different co-formulations of DMIs and SDHIs used as split treatments (GS 37 and 55) on mutation frequencies (%), level of STB control (%) on the flag leaf (leaf 1), and the second leaf (leaf 2) and yields (dt/ha). Average of 2 trials (Flakkebjerg and Horsens) (trial 3).

Treatment vs 37/55	Mutation frequency (%)			STB (%)		Yield
	S524T	T79N	N86S	Leaf 1	Leaf 2	(dt/ha)
Untreated	13 ab	0 b	2 b	5.1 a	38.1 a	97.9 d
0.75 Prosaro/0.5 Prosaro	33 a	0 b	0.3 b	1.1 b	6.4 bc	106.0 c
0.75 Propulse/0.5 Prosaro	32 a	0 b	0.6 b	1.2 b	6.8 b	106.9 bc
0.75 Balaya/0.5 Amistar Gold	17 ab	0 b	0.5 b	1.3 b	3.0 d	108.6 abc
0.75 Balaya/0.75 Balaya	21 ab	0 b	0.4 b	0.5 c	2.0 d	109.9 a
0.75 Balaya/0.35 Propulse + 0.15 Folicur Xpert	12 ab	0 b	0.4 b	0.5 c	3.0 d	109.7 ab
0.5 Balaya + 0.18 Entargo/0.35 Propulse + 0.15 Folicur Xpert	7.5 b	0 b	2.5 b	0.5 c	2.3 d	110.1 a
1.0 Imtrex/1.0 Imtrex	31 a	15 a	25 a	0.3 c	2.0 d	110.9 a
Treatment prob(F)	0.16	0.17	0.003	0.0001	0.0001	0.0001
LSD 95	22.7	12.1	12.6	0.6	3.0	2.7

Trial 3 compared fungicide coformulations and split applications (table 6), which showed that treatments with prothioconazole (Prosaro and Propulse) yielded the highest mutation frequency of S524T (32-33%), followed by a split application of Imtrex (31%). The split application with Imtrex produced the highest mutation frequencies of both C-T79N and C-N86S (15% and 25% respectively). The levels of control did not vary significantly between the treatments, however, the split applications with Prosaro/Prosaro and Propulse/Prosaro resulted in the lowest control levels. Yield responses did not vary significantly between treatments, apart from Prosaro/Prosaro and Propulse/Prosaro, which had the lowest yields.

Table 7 The effect of dose and split application of Revystar on mutation frequencies (%), level of STB control (%) on the flag leaf (leaf 1), and the second leaf (leaf 2) and yields (dt/ha) (trial 4).

	Mutation frequency (%)			STB (%)		Yield
Treatment	S524T	T79N	N86S	Leaf 1	Leaf 2	(dt/ha)
Untreated	14	0	1	45.0 a	70.0 a	102.0 b
GS 37-39 1.5 Revystar	10	0	4	1.0 b	3.8 b	112.2 a
GS 37-39 0.75 Revystar	11	0	1	3.3 b	8.5 b	112.3 a
GS 37-39 0.75 Revystar + GS 55 0.75 Revystar	21	2	6	1.0 b	2.5 b	111.9 a
Treatment prob(F)	0.37	0.18	0.33	0.0001	0.0001	0.0011
LSD 95	NS	NS	NS	7.7	5.8	6.24

Trial 4 compared the effect of dose and split application of Revystar (table 7), which showed that the highest mutation frequencies of all three mutations, S524T, C-T79N, and N86S, was produced by the split-half dose Revystar treatment (21%, 2%, and 6% respectively), followed by the full dose treatment (1.5 l/ha Revystar). None of the differences were significant compared with untreated. However, the increase in mutation frequency from the full dose treatment was only observed for C-N86S and it was low and not significant compared to the untreated plots. STB control levels and yield responses of treated and untreated plots were significantly different, while the three treatments did not vary significantly amongst each other.

Discussion

Chemical control of fungal diseases on wheat remains the primary method of ensuring and maintaining high yields in the wheat-growing regions of the world where intensive and high-yielding production takes place. Control of *Z. tritici* mainly relies on coformulations of DMI and SDHI fungicides and fungicide treatments are commonly applied between 1 and 4 times per season (Jørgensen, et al., 2017). As a result of the intensive use, the resistance development towards the main groups of fungicides is a rising concern in European wheat production. A reliable control can still be reached in most regions. However, due to shifting sensitivity in Ireland, Great Britain, and parts of Western Europe, the efficacy of DMI fungicides is compromised.

Despite the problems with resistance development, DMI and SDHI fungicides are still the first choices for controlling STB and other wheat pathogens in European winter wheat crops. The observed changes in sensitivity call for change in the current control strategies to minimize selection and erosion in efficacy. Implementation of IPM and anti-resistance strategies are critical elements if resistance development is to be kept down.

In Denmark and Sweden, the current fungicide application practices consist of one to three sprays per season depending on the cultivars' susceptibility and the disease pressure (Jalli et al., 2020). Only one application of SDHI fungicides per season is recommended, typically applied in mixtures with DMIs. DMIs are often included in two or three of the applications carried out, but it is recommended not to use the same DMI more than twice per season (Heick et al., 2020). The dose applied is nearly permanently reduced and adjusted depending on the specific risk to optimize net return (Jørgensen et al., 2017).

In the presented *in vitro* studies, isolates of *Z. tritici* confirmed that the sensitivity levels towards prothioconazole-desthio and fluxapyroxad have remained stable since 2016, which was also observed by Heick et al. (2020). However, the level of control obtained in the field trials showed that the efficacy of prothioconazole, ranked among the lowest of the tested fungicide treatments, which is also in line with previous findings (Heick et al., 2020; Jørgensen et al., 2021). Furthermore, the tendency of a sensitivity decrease for the DMIs has been observed to follow a west-to-east gradient (Jørgensen et al., 2018; Jørgensen et al., 2021). This has been suggested to be explained by the high disease pressure and intensive fungicide use, which takes place in areas such as Ireland and Great Britain, and leads to quick local adaptation of the *Z. tritici* populations. In terms of DMI resistance development, the CYP51 target site mutation S524T has been found in several European countries and has been shown to confer higher levels of resistance to all DMIs (Cools et al., 2011; Kildea, et al., 2019). The S524T mutation often appears in combination with other target site mutations like D134G and V136A (Cools & Fraaije, 2013; Kildea et al., 2019). The gradual decrease in sensitivity observed for the DMI fungicide group is explained by haplotypes of *Z. tritici* harboring several target site mutations, conferring increasing levels of resistance (Huf et al., 2018).

This study confirmed an increase in the presence of S524T in the *Cyp51* gene in the Danish and Swedish *Z. tritici* population, both in individual isolates from Denmark and Sweden (*in vitro*) and leaf samples collected from field trials. Furthermore, an increase in single isolates frequency was observed in Denmark and Sweden by >5 percentage points from 2019 to 2020. This suggests that the DMIs used in Denmark and Sweden are favoring the selection for *Z. tritici* haplotypes carrying this mutation.

When the Swedish isolates were divided into middle and southern Sweden, a high increase of the S524T mutation was observed in the southern part of Sweden (>15 percentage points). In comparison, a minor decrease in frequency was seen in the middle of Sweden (approx. 5 percentage points). Both, the fungicide use and the crop distribution in southern Sweden are similar to those in Denmark. In middle Sweden on the other hand, the fungicide use tends to be lower, and crops are grown less intensively, which might explain the geographical distribution observed in this study. However, according to Vagndorf et al. (2018), no significant difference was observed between Danish and Swedish *Z. tritici* populations, which was attributed to the short distance between the two countries and the wind-blown ascospores. Therefore, the shown decrease of S524T mutations in middle Sweden was not significant and might be due to the smaller sample size between 2019 and 2020. On the other hand, the increase observed in the southern part of Sweden might also be due to a lower sampling size. However, the tendency observed throughout Europe, is that the frequency of the S524T mutation is increasing (Jørgensen et al., 2021).

The development of fungicide resistance in a given population is a gradual process, in which the resistant fraction of the population replaces the sensitive population, the main factor is then at what time span this occurs. The difference in S524T mutation frequency between the southern and middle part of Sweden, observed in this study, could be an example of a gradual replacement of the sensitive population within a country, as opposed to between countries. This has previously been described by Garnault et al. (2019). They found that a fungicide resistance gradient existed in France. The gradient was established by analyzing the spatiotemporal distribution of different *Z. tritici* phenotypes (StrR, TriR6, and TriR7-8) as described by Leroux and Walker, (2011). The frequency of phenotypes was measured across France in the years 2004 to 2013. They found that the fungicide resistance frequency followed a North-to-South gradient (North having the highest frequency), which correlates to the intensity of the area grown with wheat, following the same trend. The same could be argued in the case for the middle and southern parts of Sweden, in which the intensity of wheat cultivation and fungicide use, follows a similar gradient, in this case, the South-to-North gradient.

SDHI fungicides still provide effective control of STB in most of Europe. Several target site mutations have been identified (FRAC, 2021), which confer low-to-high levels of decreased sensitivity towards this group (Rehfus et al., 2018). The target site mutation C-H152R is especially significant, as it has been demonstrated to decrease the sensitivity to all fungicides within the SDHI group drastically (Scalliet et al., 2012; Rehfus et al., 2018). This mutation has not been found in Denmark or Sweden so far, and the frequency of SDHI resistant strains in Europe, in general, is still low (Garnault et al., 2019; Hellin et al., 2021). Still, in recent years, the frequency of strains harboring this mutation has been rising in Ireland and Great Britain (Dooley et al., 2016; Hellin et al., 2021). *In vitro* resistance testing confirmed high sensitivity towards fluxapyroxad of the Danish and Swedish *Z. tritici* populations from 2019 and 2020, with resistance factors around 1. In single isolates collected from 2019 and 2020 in Denmark and Sweden, and leaf samples from field trials, very few cases of the two mutations C-T79N and C-N86S were detected. Dooley et al. (2016) showed that isolates harboring mutations in the SDH complex had reduced sensitivity towards SDHI fungicides. This was also confirmed by Rehfus et al. (2018), who furthermore demonstrated that isolates with these mutations (C-T79N and N86S), were observed to have a reduced sensitivity (figure 3) towards SDHIs in isolates with several mutations in the SDHI target gene *Sdh*. In the current study the highest decrease in sensitivity was observed for the C-N86S mutation, compared to the C-T79N mutation, which was not significant, but still in accordance with FRAC (2021) and Mäe et al. (2020), who found that the two mutations confer moderate resistance levels.

In the current study, three isolates were observed to have decreased sensitivity towards fluxapyroxad, which could not be explained by either of the two investigated mutations. FRAC (2021) has listed several other amino acid alterations in Denmark and Sweden, which may impact SDHI sensitivity. Among these, B-T268I/A, B-N225I, B-R265P, C-T168R, C-T79N/I, C-R151S/T/M, C-N86S/A, C-W80S, C-V166M, D-I50F, and D-M114V have been shown to decrease sensitivity towards some of the most commonly used SDHIs in European countries. The lower sensitivity towards fluxapyroxad could be explained by the presence of either of these mutations. This still needs to be verified.

The *Z. tritici* isolates collected from Sweden in 2019 and 2020, showed a very low number of both *SdhC* substitutions. This indicates that these mutations have yet to emerge in Sweden, or that other mutations are favored by the *Z. tritici*

population in Sweden by different SDHI use. The low level likely also reflects the short history of using SDHI fungicides in Sweden, which has only had these products authorized since 2019.

The cross-resistance analysis based on 30 isolates revealed that a positive correlation exists between SDHI actives, which is in accordance with previous findings (Yamashita & Fraaije, 2018). In the current study fluopyram and boscalid, indicating some level of cross-resistance, which might be due to a general trend but could also indicate that the presence of mutations in the *Sdh* gene, is affecting both fungicides. The degree of cross-resistance among SDHIs for several fungal pathogens has been highly debated. Avenot and Michailides (2010) found that a highly boscalid resistant strain of *Alternaria alternata* carrying the B-H134R target site mutation also showed decreased sensitivity towards fluopyram. In contrast Fan et al. (2015) stated the opposite. Veloukas et al. (2013) showed that mutations in the SDH-B subunit in isolates of *Botrytis cinerea* conferred varying degrees of sensitivity towards several SDHI fungicides, among them, boscalid, fluopyram, fluxapyroxad, and bixafen. The degree of cross-resistance in several fungal pathogens is therefore highly debatable for fungicides within the same MOA group. It could be argued, that resistance levels observed in this study of both fluopyram and boscalid, stems from the fact, that the only SDHI fungicides available on the Danish market are these exact two, which could be speculated to select for mutations conferring resistance to both fungicides, apart from the mutations examined in this study. Overall the presented study indicates that the *Z. tritici* populations of Denmark and Sweden can still be regarded as fully sensitive to SDHI fungicides based on both sensitivity and the development of target site mutations.

To evaluate effects on the selection for the amino acid alterations of different fungicide treatments, intensity, and dose leaf samples from each trial and treatment were screened. C-T79N, C-N86S, and S524T were used as marker mutations for SDHI-resistance and DMI resistance, respectively.

All treatments showed low frequencies of both C-N86S and C-T79N, except for the two-time treatment with fluxapyroxad (Imtrex), which yielded C-T79N and C-N86S mutation frequencies of 15% and 25% respectively. This confirms that very potent solo SDHI products like fluxapyroxad promotes the selection for mutations in the *Sdh* gene. It also gave indications that the more potent SDHIs select more than less effective SDHIs (table 4). Not all the mutation changes measured were significantly different but overall similar trends were seen in the conducted trials.

The trials lead to the following conclusions. (i) High doses of very active compounds, such as fluxapyroxad, impose a higher selection pressure than lower dose alternatives or less potent actives. (ii) The frequency of treatments has an impact on the selection of resistance-conferring mutations - multiple treatments offer higher selection. (iii) Solo fungicides promote higher mutation frequencies, as opposed to mixing or alteration, as was observed for prothioconazole and fluxapyroxad.

These results are in conjunction with the advice that several researchers have been advocating. The guidelines include the mixing/alteration of fungicides with a different mode of action, lowering the dose of active ingredients during the application, and decreasing the frequency of applications. Several studies (Van den Bosch et al., 2011; Hobbelen, et al., 2013; Van den Bosch, et al., 2014; Gutiérrez-Alonso, et al., 2017) indicated that lowering the dose of active ingredients, has the potential to decrease the selection pressure of the fungicides. The dose rate has mainly been a topic of debate (Van den Bosch et al., 2011; Van den Berg, et al., 2016) since some studies have found a positive correlation between high dose rate and resistance development (Gutiérrez-Alonso et al., 2017), few have found no effect of lower doses (Van den Bosch et al., 2014) while most studies have found negative correlations to lower dose rates (Ayer et al., 2020).

Conclusion

The resistance development in the *Z. tritici* populations to the main fungicide groups, SDHIs and DMIs, was monitored in Denmark and Sweden in the growing years 2019 and 2020. Since a shift in azole sensitivity 5-10 years ago the situation appears to be stable. So far the sensitivity to SDHIs has not changed in Denmark and Sweden, but the first target site mutations were found in Denmark and very few in Sweden. Field trials indicated that the anti-resistant strategies, including mixtures/alternations, lower doses, and decreasing number of treatments are valid since treatments that deviated from these strategies, resulting in higher frequencies of the investigated mutations S524T, C-T79N, and C-N86S. The fungicide strategies which impacted selection least still provided good disease control and yield responses.

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4 Discussion

This master thesis aimed to monitor the *Z. tritici* populations of Denmark and Sweden, to estimate the frequencies of important mutations in the SDH and CYP51 target sites of SDHI and DMI fungicides, respectively. Apart from the mutation frequencies, sensitivity levels to fluxapyroxad and prothioconazole-desthio were established to estimate, if the level of control offered by these compounds has drifted or shifted across seasons. From the total population of isolates investigated, 30 isolates were chosen for further sensitivity testing (fluopyram and boscalid) to identify possible cross-resistance between the three SDHI fungicides. The total of isolates was evaluated for frequency of multidrug resistance, by identifying inserts in the promotor region of *MFS1*. Finally, field trials were carried out to evaluate different fungicide scheme levels of control (STB severity and yields) and the impact of mutation selection.

As discussed in the article, the two SDHC mutations (C-T79N and C-N86S) were rarely found in the single isolates from Denmark and Sweden. This correlates with the EC_{50} values calculated for fluxapyroxad, which did not show a significant decrease in sensitivity from 2019 to 2020, neither from the historic isolates from 2016 to 2018, nor for Danish nor Swedish isolates. It was hypothesized that, since Sweden has gained access to more SDHI fungicides than Danish farmers, perhaps an increase in frequencies of the two SDHC mutations would be observed, or a decrease in sensitivity levels could be detected. This was, however, not the case. The frequencies of the two SDH mutations and EC_{50} values between the two countries did not vary significantly, which shows that the availability of more SDHI fungicides has not yet furthered selection in the Swedish *Z. tritici* populations. Further monitoring in the coming years will be essential to evaluate, if current anti-resistance practices are sufficient, and to identify, if the access to more potent SDHI fungicides, like fluxapyroxad, furthers resistance development.

The target site mutation S524T in the CYP51 enzyme of *Z. tritici* was found in varying but increasing degrees in Denmark and Sweden from 2019 to 2020. EC_{50} values for prothioconazole did not decrease significantly from 2019 to 2020, even with the increase of the S524T mutation observed in this study. The mutation has been observed to increase in recent years (Jørgensen et al., 2021). With the significant use of DMIs in Europe this increase is expected to continue, which will further the gradual decrease in sensitivity towards DMI fungicides, as more diverse *Z. tritici* haplotypes accumulate more mutations in the *Cyp51* target gene. As shown by Mäe et al. (2020), the S524T substitution was always found in combination with V136A and I381V in Estonian and Lithuanian samples, and in some cases also included L50S and D134G. This shows that more complex haplotypes carrying diverse mutations in the *Cyp51* gene is continuously developing. The build-up of these point mutations, which have been found to confer lower sensitivity in *Z. tritici* isolates, furthers the gradual shift in DMI resistance. It could be theorized that since S524T was always found in combination with V136A and I381V, this could be the case for the isolates found in this study, that showed the presence of this point mutation. This again could explain the difference observed in EC_{50} values for isolates carrying this amino substitution.

Kildea et al. (2019) investigated the reduced azole sensitivity in the Irish *Z. tritici* population. They found that a combination of target site mutations in the *Cyp51* gene, inserts in the promotor region of CYP51 and enhanced efflux as a result of inserts in the MFS1 promotor region, explained the reduced sensitivity towards the DMI fungicides epoxiconazole, metconazole and tebuconazole in particular. In a sub-sample of the isolate collection, they

found 25 different CYP51 haplotypes, in which the majority of the population carried a combination of target site mutations, namely V136A, I138V and S524T. In the Irish *Z. tritici* population, a 16x decrease in mean sensitivity has been detected towards epoxiconazole. Mutation frequencies and subsequent decline in field control of several fungicides, is often first detected in Ireland. This outcome is a result of the ideal growth conditions for STB, namely temperate humid climate, which leads to high disease pressure. The response is the application of frequent, near label-rate fungicide doses. This has led to a continuous decrease in mean sensitivity to the most common fungicides in a stepwise manner.

With the ongoing evolution of more complex CYP51 haplotypes, as a result of continued high selection pressure, it has become more and more difficult to classify the haplotypes based on their respective accumulated mutations. So far, the most commonly used classification system has been the ´tri R-types´ system. To accommodate the growing complexity Huf et al. (2018) proposed a new nomenclature, to enable easy classification of complex CYP51 haplotypes. The classification system is based on a combined letter and number scheme. The letter indicates the number of amino acid alterations in the CYP51 enzyme, while the number identifies the specific combination of already defined amino acid alterations.

When estimating EC_{50} values for single isolates, which is done in several studies, extremely high EC_{50} values might be detected for certain compounds, however, these compounds might still show very high efficacy in the field. This is indicative of single isolates that have a polymorphism in the target site of fungicides, compared to using field populations, which increases the probability to detect a decrease in sensitivity of *Z. tritici* populations in the field (Birr et al., 2021).

Several cases have shown that there exists a lack of cross-resistance between boscalid and fluopyram (Sang & Lee, 2020). It has been hypothesized that this lack of cross-resistance is because SDHIs, such as benodanil and fluopyram, are benzamide derivatives, which might result in better binding affinity in the Q-pocket of the *Sdh* complex in strains with tyrosine at the 272 codon (Veloukas et al., 2013). However, in this study, a positive correlation between boscalid and fluopyram was detected, indicating cross-resistance between the two SDHI fungicides in *Z. tritici* populations of Denmark and Sweden, where there still is a low occurrence of SDHI mutations.
No clear pattern was observed in the screening of inserts in the *MFS1* promotor region of *Z. tritici* populations of Denmark and Sweden from 2019 to 2020. The frequency of the three types of inserts varied between 2019 and 2020 and between the two countries. The hypothesis of different fungicide use between Denmark and Sweden to yield a difference in levels of multi-drug resistance could not be assessed, since no clear pattern was observed. Multidrug resistance is an important aspect in terms of anti-resistance strategies since, if an increase in cases of this type of resistance was observed, strategies would have to be re-evaluated, as this mechanism in principle impacts all types of fungicides. A better understanding of how multidrug resistance is impacting control and sensitivity would need to be clarified.

Resistance development is the response of a given pathogen population when selection pressure is imposed in the form of fungicides. EPPO (European and Mediterranean Plant Protection Organization) defines the term ´practical resistance´ as when a loss of field control is observed due to a shift in sensitivity (EPPO, 1988). Delaying the development of resistance is in the interest of the whole agricultural sector since the loss of field control for a given fungicide limit the farmer's fungicidal arsenal, which can be costly if a heavy reliance is given on the particular fungicide. Furthermore, this arrest possibilities for using mixtures and alternating between fungicides of different MOA, which increases fungicide resistance selection. The producers of fungicides (companies) are directly impacted by the loss of income from the given fungicide, and indirectly, by selection taking place within a given fungicide MOA group, impacting several fungicides at the same time. Developing, manufacturing, and registering a novel fungicide is very costly for the companies, which emphasizes the importance of prolonging the efficacy of fungicides for as long as possible.

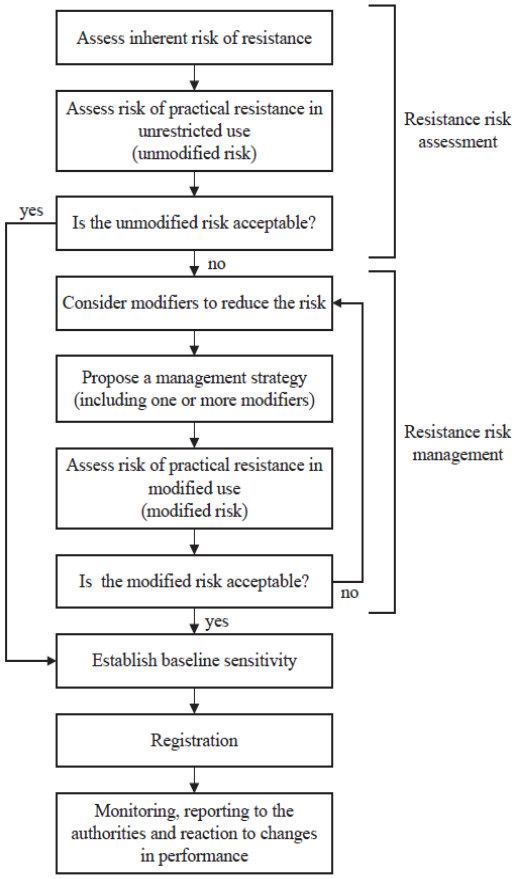

Figure 13 Process of resistance risk analysis and registration (EPPO, 2015).

EPPO (2015) states the following standard to indicate the obligations of registration authorities and registration applicants to assess and manage the risk of practical resistance: the concepts of resistance, assessing resistance risk, managing resistance, data necessary to support resistance risk analysis, supplementary data on resistance and concluding of registration decision regarding the risk of resistance (figure 13).

As of yet, control of STB still relies heavily on fungicides. To achieve efficient control and enable anti-resistance strategies to work, an arsenal of compounds with different modes of action becomes essential (Scalliet et al., 2012). This study confirmed that, the more diverse the fungicide scheme was, the higher level of control was achieved, and less selection took place. When mixing two fungicides it is advised that the individual compound is applied at a rate that is effective in control when applied alone (Dooley et al., 2016).

As previously mentioned, the latent phase of *Z. tritici* is symptomless, making it difficult to assess, when to treat the field with appropriate fungicides. This proves a problematic issue when deciding on when the application of fungicides is relevant (Marroni et al., 2006). This is one of the aspects mentioned by Van den Bosch et al. (2011).

A key factor in the debate concerning delaying resistance development is preserving the efficacy of the few available groups of fungicides. Developing fungicides with novel MOA or expanding the present groups is time and resource consuming, due to the limitations of discovery, development, and registration (Sierotzki & Scalliet, 2013), especially in Europe, in which the EU directive (2009/128/EC) focus lies on risk assessment, as opposed to the previous one on hazard assessment. The SDHIs fungicides pose a paradoxical dilemma. They are essential in resistance management, by offering a different MOA, that compliments fungicide

groups such as the DMIs, however, they are at risk of resistance development (Sierotzki & Scalliet, 2013).

As has been described previously, fungicides are essential in global crop yield security. The use of fungicides, and pesticides in general, however, has posed a threat to both human health and ecosystems (Hepperly, 2009), which has led to strict regulations of which pesticides are authorized within Europe, especially with the transition from a hazardous to a risk assessment based chemical authorization (The European Parliament and The Council of The European Union, 2009). This has led to a shrinking availability of fungicides on the market, since new fungicides and novel MOA fungicides rarely enter the market due to the strict regulations and the very high cost of discovering, producing and authorization of new chemical compounds, averaging around 215 million euro (Phillips McDougall, 2016).
Some countries, including Denmark, enforce even stricter regulations of which active fungicide ingredients are allowed within the country (Ministry of Environment and Food of Denmark, 2017). The key criteria of approval for a given active ingredient are efficacy and no/low toxicity towards non-target organisms, human health, the groundwater, and the environment. For a fungicide to be approved in Denmark, it must first be approved by the European Union and then by The Danish Environmental Protection Agency (EPA).
Given these circumstances, the availability of different fungicides within the same classification and fungicides with different MOA are limited, which results in low variation when it comes to the given fungicides used in fields within the EU, and to a higher degree, fungicides used within Denmark.

The final hypothesis was whether solo acting fungicides, split treatments, and higher doses select to a higher degree for mutations conferring resistance towards the respective group of fungicides. This study confirmed the current anti-resistance advice since solo acting fungicides were selecting to a higher degree for the three mutations investigated in this study. This was especially prevalent for the very potent SDHI fungicide fluxapyroxad. Split treatments were as well identified as increasing the frequencies of the investigated mutations. Finally, higher doses also showed indications of increased selection.

5 Additional experiments

In the following section, experiments that were conducted during the work of this thesis, but not included in the article, will be explained.
Following the extraction of genomic DNA from the leaf/fungal material, derived from the leaf samples collected from the field trials, qPCR detection of the three mutations (C-T79N, C-N86S, and S524T) was to be carried out. In the initial process, however, the qPCR runs did not work properly since no DNA amplification was identified. Therefore, the first step to identify the problem was to measure the DNA concentration. The basis for this procedure was, that if DNA extraction had been successful, it should be possible to measure the DNA concentration of the extracted DNA, thereby validating the extraction process. Another important aspect was, that the concentration gives an estimate of how much starting material (DNA) forms the base for the qPCR amplification process.

5.1 Qubit Fluorometric Quantitation

The DNA concentration was measured using Qubit™ Fluorometric Quantitation (Qubit™ 4 Fluorometer, Thermo Fisher Scientific, Roskilde, Denmark). The procedure was as follows: 2 µl of DNA were mixed with 200 µl of Qubit™ buffer (Qubit™ dsDNA HS Assay Kit, Thermo Fisher Scientific, Roskilde, Denmark) in 500 µl thin-walled polypropylene tubes (Qubit™ Assay Tubes, Thermo Fisher Scientific, Roskilde, Denmark). The tubes were then closed and vortexed for 1 min. Post vortex, the tubes were left to settle for 2 min. Finally, the tubes were inserted, one at a time, in the Qubit™ 4 Fluorometer. The machine was set to run ´dsDNA,´ and the volume of DNA was set to ´2 µl´. Shortly after running the samples, DNA concentrations were established for each of the samples.
The assessment confirmed that DNA was present in sufficient concentrations for running qPCR reactions.
This type of DNA concentration verification is based on linear regression. With the assay kit comes two DNA standards with known DNA concentrations. These two standards are used to calibrate the machine. The machine calculates a linear regression based on the two DNA standards; one has a low DNA concentration, while the other has a high DNA concentration. The assay kit buffer contains dyes, which bind to dsDNA and emit fluorescence only when bound to DNA. The dyes will bind to DNA (if present) and emit fluorescence; the higher the DNA concentration, the more fluorescence is emitted, and the machine then estimates a DNA concentration based on emitted light and known DNA standard concentrations.
To establish if DNA extraction had been successful, two subsequent qPCR assays were conducted that would identify if the problem derived from DNA extraction.

5.2 HOR assay

Nicolaisen et al. (2008) developed an assay with specific primers for amplification of wheat genomic DNA (appendix, table 7). This assay was carried out to establish if DNA extraction had been successful, which the assay could verify, since if amplification occurred, sufficient plant DNA was extracted to be amplified and viewed during a qPCR amplification. The procedure was as follows: 24 samples were chosen, based on relatively high DNA concentrations, measured with the Qubit analysis. For each sample, 6.25 µl of POWER SYBR GreenPCR Master Mix (Thermo Fisher Scientific, Roskilde, Denmark), 0.375 µl of forward and reverse primers, 2.375 µl of DEPC water, 0.625 BSA was mixed with 2.5 µl DNA template.

The cycling conditions were as follows: 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, and annealing at 62 ◦C for 1 min, along with melting curves. Negative H_2O control was included, along with a standard curve from previously diluted wheat DNA standards (1:5, 1:25, 1:125, 1:625, 1:3,125, 1:15,625).
The assay verified that genomic wheat DNA had been successfully extracted in the initial DNA extraction of leaf material. This confirmed that the DNA measured with the Qubit and subsequently amplified during the HOR qPCR assay indeed stemmed from the leaf samples.

5.3 ZYM assay

Once verification of the DNA extractions' successfulness had been carried out, the last step in the process was to verify, if genomic *Z. tritici* DNA had been extracted as well. Since leaf samples were collected based on subjective assessments of STB symptoms and subsequently air-dried for an extended period, a risk of no *Z. tritici* being present on the leaf samples was indeed valid. To verify the presence of *Z. tritici* DNA and validate the DNA extraction process, the ZYM assay, developed by Bearchell et al. (2005), was conducted. The assays consisted of primers specific to the regions of *Z. tritici* DNA, thereby enabling amplification of the matching primer sequences in the presence of DNA. The procedure was as follows: Each sample was prepared with 7.5 µl TaqMan Universal PCR Master Mix, 1.35 µl of forward and reverse primer, 2.05 µl DEPC water, 0.75 µl probe (5µM), and 2 µl DNA template. The cycling conditions were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Included in the run were a negative H_2O control and a *Z. tritici* DNA standard curve (dilutions as for the HOR assay).
The ZYM qPCR assay verified that *Z. tritici* was present in all 24 tested samples, however, with relatively high ct values, which implied that the overall DNA extraction had been successful, except the high ct values observed from the ZYM assay indicated that the starting material (*Z. tritici* DNA) was in low quantities. This could be explained by STB developing in every field trial, but it was observed that during the collecting of leaves, few had developed prominent STB symptoms. Treatments were very successful in limiting the development and spread of *Z. tritici*, which could also explain the lack of STB symptoms.

6 Troubleshooting

During most experimental work, several constraints will present themselves, which need to be solved to move forward and obtain the results required for the study. In the following, the constraints that occurred during this study and the subsequent troubleshooting and solving of these problems will be delved upon.

6.1 Rapid DNA Extraction

The DNA extracted from the ´rapid´ DNA extraction, was hypothesized to be of questionable quality since the process does not include separation of DNA from fungal material. Several dilution steps were made to establish the dilution grade necessary for the different experiments conducted. DNA diluted 1:7 was sufficient for running standard PCR reactions, which was in connection to the amplification of DNA segments in the *MFS1* gene of transporters. The 1:7 dilution, however, proved inefficient when used in qPCR reactions, for which a 1:10 dilution proved more successful.

6.2 qPCR reactions

From the start of the project, qPCR reactions proved unsuccessful in several instances. To elucidate the problem, several steps were taken, to figure out how to make the reactions work. The first step in the troubleshooting process was establishing the optimal primer and probe concentrations, which was conducted following the primer/primer, primer/probe concentration matrix (manufacture protocol).

Each of the different primer concentrations (both forward and reverse) combinations, along with the different probe concentrations, were tested on reference isolates, harvested, and extracted using Sbeadex Mini Plant Kit, and run on the thermocycler. Following the screening of optimum primer and probe concentrations. The DNA concentration was adjusted as previously mentioned, in which it was found, that the optimum DNA dilution was of the order 1:10. The next step was to establish if the settings for the software on the ViiA7 were at an optimum. The cycling conditions were tested until an optimum had been found. It was also noted that the ViiA7 machine did not allow ´Fast´ cycling conditions, which had previously been tested. In the option of ´Reagents,´ the option that proved to be correct was the ´Other´ option, as the Takyon™ master mix did not belong in the other categories ´TaqMan´ nor ´SYBR Green´. Finally, after further investigation, it was found that the original master mix used (Takyon™ No Rox MasterMix) did not support the thermocycler (ViiA7) which was used in this project, and subsequently, the master mix that was approved to use on this thermocycler (Takyon™ Low Rox MasterMix) was ordered an put to use. In the end, the cycling conditions, along with optimum primer concentrations and settings, were the ones provided by Hellin et al. (2020).

7 Additional Results

The following section presents results, that were neglected or briefly mentioned in the article.

7.1 Sensitivity test

The number of *Z. tritici* isolates across the years and country, along with the calculated average EC_{50} values and resistance factor is given in table 5.

Table 5 Number of pycnidial isolates of *Z. tritici* and calculated EC_{50} and resistance factor (RF) values from Denmark and Sweden.

Country	Year	Prothioconazole-desthio			Fluxapyroxad		
		No. isolates	Average	RF	No. isolates	Average	RF
Denmark	2006/09	17	0.034	3	17	0.09	1
	2016	26	0.13	13	NA	NA	NA
	2017	301	0.32	32	NA	NA	NA
	2018	452	0.31	31	452	0.2	2
	2019	170	0.26	26	170	0.23	2.3
	2020	117	0.5	50	119	0.32	3.2
Sweden	2007/09	12	0.017	2	12	0.09	1
	2017	180	0.55	55	NA	NA	NA
	2018	117	0.26	26	117	0.16	1.6
	2019	281	0.17	17	269	0.09	0.9
	2020	166	0.15	15	166	0.14	1.4

7.2 Multidrug resistance

From the screening of *Z. tritici* isolates from Denmark and Sweden (2019 and 2020), the frequency of the type of inserts in the *MFS1* gene is given in table 6.

Table 6 Number and distribution of insert in the MFS1 gene from single isolates 2019 and 2020.

Year x Region	Denmark	Sweden
2019	Type I: 1	Type I: 0
	Type II(a): 5	Type II(a): 1
	Type II(b): 1	Type II(b): 0
	Type III: 1	Type III: 0
2020	Type I: 0	Type I: 0
	Type II(a): 1	Type II(a): 0
	Type II(b): 4	Type II(b): 5
	Type III: 0	Type III: 0

8 Conclusion

This study was conducted to determine the sensitivity to DMI and SDHI fungicides, as well as the frequency of three mutations (C-T79N, C-N86S, and S524T) in the SDH-C and CYP51 complexes of the *Z. tritici* populations of Denmark and Sweden. Furthermore, the frequency of promotor inserts in the major facilitator superfamily, *MFS1*, was investigated in the same *Z. tritici* populations. Lastly, the impact of different fungicide strategies, including solo and mixing of fungicides, alternating, and frequency of applications on the selection of previously mentioned mutations was investigated. The study concluded that the frequency of the S524T mutation is increasing in both the Danish and Swedish *Z. tritici* populations as a response to current fungicide practices. It can, therefore, be assumed that haplotypes harboring this mutation (among others) will further increase, as seen in other countries. The two SDH-C mutations were only found in low and varying degree, which suggests that in the present, these mutations have not yet established in the *Z. tritici* populations, but are in the emergence phase, which emphasizes the caution of future SDHI use to guarantee the longevity of fungicides within this group and to delay fungicide resistance development. Inserts in the promotor region of *MFS1* were found in both Denmark and Sweden, however, in low and varying degrees, which suggests that there is no clear evidence of selection taking place towards *Z. tritici* populations harboring this type of mutation. Finally, the investigated impact of different fungicide schemes in the field trials confirmed present advice concerning fungicide use. Single treatments with a solo fungicide, such as prothioconazole or fluxapyroxad, favored increased frequency of the Cyp51 mutation and SDH-C mutations, respectively. Double treatments with fungicides of similar MOA or, more pronounced, the same fungicide favored, increased mutation frequencies to a high degree. The dose-effect was varying but was majorly found to increase mutation frequencies when the highest dose was applied.

This suggests that current advice concerning fungicide use, namely lower and economically adjusted doses, alternating/mixing fungicides of different MOA, and reduce the frequency of applications, is highly relevant to maintain the efficacy of current fungicides and delay resistance development in the *Z. tritici* populations.

9 Future perspective

Future anti-resistance management strategies must consider the risk of resistance development from frequent use of solo acting fungicides, full dose treatments, and split treatments with fungicides of similar MOA.
Other strategies besides chemical fungicides must be implemented, as not to rely solely on chemical control. As has been stated previously, in the case of *Z. tritici*, cultural practices such as crop rotation have low to no impact on the severity of STB epidemics. Delayed sowing has been shown to delay STB outbreaks and the decrease severity of STB epidemics. In areas where there is a known history of STB outbreaks, delayed sowing may be a method to employ to delay and reduce the impact of STB, according to IPM strategies.
Breeders have overcome the yield penalty often associated with cultivars bred for *Z. tritici* resistance, by breeding for resistant wheat cultivars, which can negate the detrimental effect *Z. tritici* can have on the yield and quality of the harvest. There is an emphasis on including cultivars that carry relevant resistance genes to reduce the need for chemical control. This could be done by the stacking of resistance genes into one cultivar, or by growing cultivars with different resistance genes. Apart from growing resistant cultivars, cultivar mixtures are an important cultural approach to increase resilience in an otherwise non-resilient monocrop. Cultivar mixtures offer several aspects which could have a positive impact on the reduction of chemical inputs.
Future breeding will probably rely more on genetic editing tools such as CRISPR/Cas9. Breeders achieved complete resistance to powdery mildew (*Blumeria graminis*) in barley, by traditional breeding. The same advances will be difficult to achieve in wheat by traditional breeding, due to the major difference in ploidy levels (barley = diploid, wheat = hexaploid). The resistance obtained in barley was achieved by the complete knockout of the *mlo* genes, which have homolog counterparts in the wheat genome. To knockout the three homolog pairs in the hexaploid genome of wheat seem like an impossible barrier and has so far not been accomplished through traditional breeding. In 2014, however, Wang et al. (2014) overcame this barrier by simultaneous editing of the three *mlo* homoalleles, by the use of the CRISPR/Cas9 technology. The European Union enforces heavy restrictions on the development and cultivation of genetically modified organisms (GMO), which explains the modest cultivation of GMO crops. It could be theorized, that knowledge on the genome of both *Z. tritici* and wheat, combined with the use of genome editing technologies, would open up the possibility of breeding cultivars, that stack both qualitative and quantitative resistance genes, which would further reduce the reliance on chemical fungicides, to control STB.

As an alternative to chemical fungicides, research, and development into non-chemical solutions, such as biopesticides, have gained interest during the last few years. Biopesticides (biological pesticides) are compounds that are not chemically synthesized but derive from naturally occurring substances. Biopesticides, however, show very diverse efficacy in terms of levels of control. Future disease control may rely to a larger extent on biopesticides, or the biopesticides may prolong the longevity of existing fungicides, as an addition to the farmers' toolbox of disease control options, while offering a decent level of control.

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11 Appendix

11.1 Technical devices

Technical device	Origin
2720 Thermal Cycler	Applied Biosystems, Foster City, USA
ViiA7 RT-qPCR	Thermo Fisher Scientific, Roskilde, Denmark
Gel electrophorese system	Bio-Rad, Copenhagen, Denmark
Kingfisher 24 Magnetic Particle Purification System	Thermo Fisher Scientific, Roskilde, Denmark
Kingfisher™ Flex purification system	Thermo Fisher Scientific, Roskilde, Denmark
Qubit Fluorometric Quantitation	Thermo Fisher Scientific, Roskilde, Denmark
SCANVAC COOLSAFE	LABOGENE A/S, Lillerød, Denmark
Geno/Grinder™ 2010	SPEX SamplePrep, New Jersey, USA
epMotion® 5070	Eppendorf, Hørsholm, Denmark
FastGene GelPic LED Imaging System	Bulldog Bio, Portsmouth, USA
Eppendorf Centrifuge 5430	Eppendorf, Hørsholm, Denmark

11.2 Chemicals and consumables

Consumables	Manufacturer
96well microtiter plates	
6x DNA loading dye	Thermo Fisher Scientific, Roskilde, Denmark
Tris-HCL	
NaOH	
DEPC-water	
Difco™ Potato Dextrose Agar	Thermo Fisher Scientific, Roskilde, Denmark
EDTA	
SYBR® Safe DNA Gel Stain	Thermo Fisher Scientific, Roskilde, Denmark

11.3 Enzymes and kits

Name	Manufacturer
Takyon™ Low Rox Probe MasterMix dTTP	Eurogentec, Seraing, Belgium
Sbeadex mini plant kit	LGC Group, Teddington, Great Britain
GoTaq® G2 Flexi DNA Polymerase	Promega, Mannheim, Germany
POWER SYBR GreenPCR Master Mix	Thermo Fisher Scientific, Roskilde, Denmark
TaqMan Universal PCR Master Mix	Thermo Fisher Scientific, Roskilde, Denmark
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific, Roskilde, Denmark

11.4 Buffer and solutions

Name	Composition	Notes
TAE buffer	x M TRIS-base x M acetic acid 5 mM EDTA	

Tris-HCL buffer	x M TRIS-base

11.5 Oligonucleotides

The oligonucleotides listed below served as primers and probes for the standard PCR, in terms of multidrug resistance and qPCR for mutation detection and quantification.

Species	Name	Sequence (5'->3')	Target	T_M[℃]	Reference
Zymoseptoria tritici	MDR_2F	GCAAGGATTCGGACTTGACG	SdhC		(Omrane et al., 2017)
	MDR_4R	CTGCCGGTATCGTCGATGAC	SdhC		(Omrane et al., 2017)
	SdhC-79-86-F	confidential	SdhC		Hellin et al., (under review)
	SdhC-79-86-R		SdhC		
	SdhC-79T-probe		SdhC		
	SdhC-79N-probe		SdhC		
	SdhC-86N-probe		SdhC		
	SdhC-86S-probe		SdhC		
	CYP524-F	GGTGGGTACGGATTACAG	Cyp51	56.0	(Hellin et al., 2020)
	CYP524-R3	CCACCTATGGTTCTGCATAC	Cyp51	57.3	
	Cyp524S-probe	TTTGTTCAGCCGGCC	Cyp51	50.6	
	Cyp524T-probe	TTTGTTCACCCGGCC	Cyp51	50.6	

Table 7 Wheat and *Z. tritici* oligonucleotides for qPCR.

TARGET	PRIMER/PROBE NAME	SEQUENCE (5’-3’)	REFERENCE
PLANT EF1A	Hor1f	TCTCTGGGTTTGAGGGTGAC	(Nicolaisen et al., 2008)

	Hor1r	GGCCCTTGTACCAGTCAAGGT	
Z. TRITICI	Zym-F	GCCTTCCTACCCCACCATGT	(Bearchell et al., 2005)
	Zym-R	CCTGAATCGCGCATCGTTA	
	Zym (FAM-MGB)	TTACGCCAAGACATTC	

11.6 Reference isolates

Table 8 Control isolates with relevant mutations for screening.

Strain	Target site mutation	Source
IPO 323	-	The Netherlands
Reference I	MFS1 Promotor insert Type I	
Reference III	MFS1 Promotor insert Type III	
KB4/KB7	C-T79N	Knockbeg, Ireland 2017
M18	C-N86S	Moorepark, Ireland 2017
S18.158	S524T	Ireland 2018

11.7 Software

Name	Provider
QuantStudio™ Real-Time PCR Software v1.3	Applied Biosystems
BLAST	BASF SE, Ludwigshafen, Germany
GraphPad Prism	GraphPad Software, La Jolla, CA, United States