Universität Hohenheim
Institut für Phytomedizin
Fachgebiet Phytopathologie
Prof. Dr. Ralf T. Vögele

Analysis of the emerging situation of resistance to succinate dehydrogenase inhibitors in *Pyrenophora teres* and *Zymoseptoria tritici* in Europe

Dissertation

zur Erlangung des Grades eines Doktors der Agrarwissenschaften

vorgelegt
der Fakultät Agrarwissenschaften
von

Dipl. Agr. Biol. Alexandra Rehfus

aus Leonberg

2018

Diese Arbeit wurde unterstützt und finanziert durch die BASF SE,
Unternehmensbereich Pflanzenschutz, Forschung Fungizide, Limburgerhof.

Die vorliegende Arbeit wurde am 15.05.2017 von der Fakultät Agrarwissenschaften der Universität Hohenheim als „Erlangung des Doktorgrades an der agrarwissenschaftlichen Fakultät der Universität Hohenheim in Stuttgart“ angenommen.

Tag der mündlichen Prüfung:	14.11.2017
1. Dekan:	Prof. Dr. R. T. Vögele
Berichterstatter, 1. Prüfer:	Prof. Dr. R. T. Vögele
Mitberichterstatter, 2. Prüfer:	Prof. Dr. O. Spring
3. Prüfer:	Prof. Dr. Dr. C. P. W. Zebitz
Leiter des Kolloquiums:	Prof. Dr. J. Wünsche

Table of contents

Abbreviations

Commonly used abbreviations and SI units are not listed. Abbreviations that are used only once are explained in the text. Abbreviations for different European countries were used according to ISO 3166 standard.

aa	amino acid
a.i.	active ingredient
amp	ampicillin
APS	adenosine 5´ phosphosulphate (an analogue of ATP)
ATP	adenosine triphosphate
bp	base pair
cq	cycle of quantification
Cyp51	sterole 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*)
d.c.	double concentrated
DMI	demethylation inhibitor
DMSO	dimethylsulphoxide
dNTP	deoxyribonucleotide triphosphate
dpi /hpi	days / hours post inoculation
EC_{50}	effective concentration of 50% inhibition
FRAC	Fungicide Resistance Action Committee
fw	forward
GM	genetically modified
ha	hectare
HRAC	Herbicide Resistance Action Committee
kb	kilo base pair
MAMA	mismatch amplification mutation assay
MDR	multidrug resistance

MFS	Major facilitator superfamily
n	number
NTC	no-template control
OD_{405}	optical densitiy at 405 nm
PCR	polymerase chain reaction
PTM	*Pyrenophora teres* f. *maculata* (spot type)
PTT	*Pyrenophora teres* f. *teres* (net type)
QoI	quinone outside inhibitor
qPCR	quantitative PCR
rv	reverse
rpm	revolutions per minute
SE	standard error
Sdh	succinate dehydrogenase gene
SDH	succinate dehydrogenase enzyme
SDH-B	succinate dehydrogenase subunit B
SDH-C	succinate dehydrogenase subunit C
SDH-D	succinate dehydrogenase subunit D
SDHI	succinate dehydrogenase inhibitor
SNP	single nucleotide polymorphism
spp.	species pluralis
strep	streptomycin
T_m	melting temperature
TAE	tris-acetate-EDTA
WT	wild type

Figures

Tables

Abstract

Phytopathogenic fungi such as *Pyrenophora teres* and *Zymoseptoria tritici* cause destructive diseases of barley and wheat in all major cereal production areas worldwide. The control of net blotch of barley caused by *P. teres* and Septoria tritici blotch (STB) of wheat caused by *Z. tritici* mainly relies on the usage of fungicides. Thereby, three single-site inhibiting fungicide classes, the quinone outside inhibitors (QoIs), the demethylation inhibitors (DMIs) and the succinate dehydrogenase inhibitors (SDHIs) have the highest relevance. In recent years, the QoI fungicide efficacy was significantly reduced due to the occurrence of G143A in cytochrome bc_1 complex (CYTB) of *Z. tritici*. In *P. teres*, G143A has not been found so far, however, F129L in CYTB has been detected, which mediates 'moderate' resistance levels towards QoI fungicides. Furthermore, the population of *Z. tritici* in Western Europe has shown a continuous 'shift' over many years towards an increased DMI tolerance. The class of SDHIs is the most newly introduced fungicide class and inhibits the fungal succinate dehydrogenase complex (SDH) which is a critical enzyme of the respiratory chain and the tricarboxylic cycle. The upcoming SDHI resistance in European populations of *P. teres* and *Z. tritici* was investigated in the present study and resistance mechanisms underlying SDHI resistance were characterised. SDHI resistant isolates of both pathogens were collected in intensive monitoring programmes which covered the major barley and wheat growing areas in Europe.

SDHI resistant isolates showed point mutations in the genes *SdhB*, *SdhC* and *SdhD* which cause amino acid alteration in the subunits B, C and D of the SDH complex. First SDHI resistant isolates of both pathogens were detected in 2012 and showed amino acid alteration, histidine to tyrosine at position 277 in SDH-B (B-H277Y) in the case of *P. teres* and a threonine to asparagine exchange at position 79 in SDH-C (C-T79N) in the case of *Z. tritici*.

In *P. teres*, a significant increase of SDHI resistant isolates from 2012 to 2015 was observed, particularly in countries such as France and Germany. Several target-site mutations leading to amino acid exchanges, namely B-H277Y, C-S73P, C-N75S, C-G79R, C-H134R, C-S135R, D-D124N/E, D-H134R, D-G138V, D-D145G and D-E178K, were identified in those isolates. Sequencing of *SdhB*, *SdhC* and *SdhD* genes of several isolates confirmed that each isolate carried one mutation in the *Sdh* genes, and not two or more in combination. *In vitro* and *in planta* sensitivity tests were performed and revealed that each SDH-variant causes a distinct resistance phenotype towards SDHIs. Commercially available SDHIs were compared and isolates showed cross-resistance towards all SDHIs tested, although some minor differences in the response to different mutations were observed. Most of the SDHI resistant *P. teres* isolates carried C-G79R substitution which was shown to exhibit one of the strongest effects of all detected alterations. In addition to C-G79R, other substitutions, such as C-N75S and D-D145G, were frequently found in the

field. These SDH-variants were shown to confer low to moderate levels of resistance. Glasshouse data demonstrated that SDHIs can still contribute effectively to disease control when applied in a preventative manner at registered dose rates, particularly in case of mutants with 'low' and 'moderate' resistance phenotypes (e.g. B-H277Y, C-N75S, D-D124N and D-D145G). Analysis of multiple resistance to QoI and SDHI fungicides revealed that isolates from 2013 with C-G79R substitution did not simultaneously carry the F129L exchange in CYTB. However, an increase of QoI and SDHI double resistant isolates was observed in the following years.

In contrast to the rapid 'build-up' of resistant isolates in the population of *P. teres* in countries such as France and Germany, the emergence of SDHI resistance in *Z. tritici* did not evolve as fast as observed in net blotch. Here, only a few resistant isolates have been sampled so far (42 resistent of 3431 investigated isolates, 1.2%). An increase of resistant isolates of *Z. tritici* was observed mainly in Ireland, the United Kingdom and the Netherlands, however, still at low levels. SDH-variants B-N225I, B-T268I/A, C-N86S/A, C-T79N/I, C-W80S, C-H152R and C-V166M were detected in SDHI resistant isolates collected in these and other countries such as France and Germany. Four isolates showed two mutations in the *Sdh* genes in combination. These mutations cause alterations B-R240L+C-T79N, B-T268I+C-I29V, B-T268A+C-F23S and C-H152R+D-R47W. *In vitro* and *in planta* sensitivity measurements demonstrated that C-H152R mutants showed the highest resistance level of all investigated SDH-variants collected in the field. C-T79N and C-N86S exchanges which have been detected more frequently in the field than C-H152R, were shown to confer lower levels of resistance compared to C-H152R.

Dual inoculation tests were performed with several SDHI resistant and sensitive field isolates of both pathogens to detect potential fitness costs of SDHI resistance mutations. Quantitative molecular detection methods were established to detect SNPs causing SDHI resistance and were used to examine an increase or a decrease of resistance alleles in mixtures of resistant and sensitive isolates. Mixtures were propagated for several cycles on barley or wheat seedlings without the use of fungicides. Field isolates of *P. teres* revealed a high natural variability, independent of their resistance status. However, when all mixtures were taken together, a slight decrease of resistance alleles was observed in mixtures with sensitive isolates. In field isolates of *Z. tritici*, a significant decrease of B-T268I and C-H152R in mixtures with sensitive isolates was observed. In contrast, C-T79N field isolates showed a high pathogenicity and competitiveness compared with sensitive isolates. Therefore, it could be proposed that fitness costs can vary between different mutations within a species.

Both phytopathogenic species were shown to evolve a range of diverse target-site mutations, which led to different alterations in both pathogen species with exception of C-N75S in *P. teres* and the homologous variant, C-N86S, in *Z. tritici*. This can be explained

by species-specific variation of the SDH enzyme, a different nature of the pathogens (e.g. host plants and disease geographical spread) as well as a different fungicide use pattern (e.g. mode of action diversity and fungicide application intensity). The absence of a dominant major target-site mutation in the case of SDHI resistance in both pathogens is thought to allow SDHIs as effective control agent against both pathogen species also in the future. Nevertheless, anti-resistance management strategies are highly recommended for the usage of SDHIs. These strategies should not only be based on the use of mixtures and alternations of fungicides, but should also implement integrated disease control measurements (e.g. resistant host cultivars).

1 Introduction

1.1 Wheat and barley cultivation and the importance of fungal diseases

Grasses (Poaceae or Graminea) are perhaps the most economically important plant family with the greatest potential to address the needs of mankind for food, feed and fuel (Mak, 2010). Barley (*Hordeum vulgare* L.) is regarded as the fourth most important cereal crop in the world, placed after maize, wheat and rice (Akar et al., 2004). In the last century, barley was mainly cultivated for human food supply. Nowadays, it is primarily grown as animal feed and for the malt production used in the beer industry (Hayes, 1992). Barley has a high ecological compatibility and can be produced also under unfavourable climatic conditions with relatively long periods of drought. Therefore, in some developing countries with arid and semi arid climates, barley is still the only cereal which serves as a staple food resource (Akar et al., 2004).

Wheat (*Triticum aestivum* L.) belongs to the family Graminea and has the widest distribution worldwide within all cereal crops. It is cultivated in some 100 countries with the highest concentration in temperate zones of the northern hemisphere, including major cereal growing areas of North America, Europe, Asia and North Africa (Oleson, 1994). Wheat serves as an important human food source and feed grain for many classes of livestock (Ranhotra, 1994). About 95% of the world production is derived from wheat varieties, which belong to the category of common or bread wheat, whereas the remaining 5% are durum wheat varieties (Oleson, 1994). Global harvests of 705 million metric tonnes were reached in 2013 to 2014 (Gurr and Fones, 2015). Nowadays, Europe is the most productive wheat growing area worldwide, when yields per hectare between different continents are compared (calculations based on http://www.fao.org/faostat/en/). France and Germany are the biggest wheat producers in Europe contributing ~26% and ~17%, respectively (Gurr and Fones, 2015).

Since the green revolution, cereal yields have increased significantly to satisfy global requirement of the ever-increasing global population (Welch and Graham, 2004). A major task of the 21th century is to produce enough food to meet the demand of an increasing population in an environment of climate change, and with requirement of more substainable cultivation systems (Tilman et al., 2002).

Plants are vulnerable to abiotic stress factors, such as heat, cold, drought, salinity and nutrient stress (Wang et al., 2003) and biotic attacks, which include fungi, bacteria, viruses, nematodes and insects (Hammond-Kosack and Jones, 2000). Global potential losses due to pests were calculated to be 50% in wheat, of which the highest losses were caused by weed pests, followed by animal pests and pathogens. Whereas weed pest control can be

managed mechanically and chemically, control of animal pests and diseases are mainly dependent on chemical control measures (Oerke, 2006). Many thousands of plant pathogenic fungi are known. Regarding each crop individually, the number of fungi that is able to attack and cause damage is limited. However, infections of a pathogen in a favourable environment can result in significant economic losses.

Barley and wheat are believed to have been domesticated as crop plants since the settling of humans in the Fertile Crescent around 10,000 years ago (Badr et al., 2000; Özkan et al., 2002). Since that time, intensive cultivation and selection of genetically homogenous crops has put strong selective pressure on natural fungal populations and has led to the emergence of completely new pathogens, which are highly adapted to different crop species (Brunner et al., 2007; Stukenbrock et al., 2007; Friesen et al., 2008a, 2008b). In a recent study, scientists voted in a survey the 'Top 10' of most economic and scientific important fungal pathogens (Dean et al., 2012). Eight of ten nominated pathogens are fungal species, which infect gramineacious hosts. Destructive grass diseases are, for example, the rice blast disease, caused by *Magnaporthe oryzae* (Ou, 1980), rust diseases of cereals (stem rust, yellow rust, brown rust), particularly of wheat, caused by several *Puccinia* species, and *Blumeria graminis*, which causes powdery mildew of grasses, including wheat and barley. In addition, *Fusarium graminearum* senso lato and associated *Fusarium* species, which normally cause 'moderate' yield losses, can lead to mycotoxin-contaminated grain in several cereal species (Kazan et al., 2012). Furthermore, *Zymoseptoria tritici*, which causes Septoria tritici blotch (STB) of wheat, was placed at rank seven in its importance (Dean et al., 2012). Plant pathogens are highly variable and adapt to environmental changes quickly. Since the 1960s, *Z. tritici* has become the most prominent pathogen on wheat in Europe, replacing other pathogens such as rusts, mildews and *Parastagonospora nodorum* (often called *Leptosphaeria nodorum*) (Oliver and Hewitt, 2014).

1.1.1 Net blotch disease in barley caused by *Pyrenophora teres*

Pyrenophora teres Drechsler (asexual morph: *Drechslera teres* [Sacc.] Shoem.) is the causal agent of net blotch of barley. *P. teres* is an ascomycetous, necrotrophic fungus belonging to the class of Dothideomycetes in the order Pleosporales. Net blotch is a major disease in barley, and causes severe yield losses in many barley growing regions worldwide. In temperate climate zones in untreated conditions *P. teres* can cause yield losses of 10-40% and may reach even higher levels up to 100% under favourable environmental conditions for the fungus (Mathre, 1997; Minarikova and Polisenska, 1999; Murray and Brennan, 2010). Furthermore, infection can negatively affect feed and malting quality of barley kernels due to a reduction of kernel size (Mathre, 1997; Grewal et al., 2008). *P. teres* can infect a range of different gramineous species within the genera *Aegilops*, *Agropyron*, *Elymus*, *Hordeum*, *Hordelymus* and *Stipa* (Brown et al., 1993).

1.1.1.1 Taxonomy and relationship to other *Pyrenophora* species

The sexual morph *Pyrenophora teres* was first described by Drechsler in 1923. The asexual morph, *Drechslera teres*, was originally placed in the genus *Helminthosporium*, but was reassigned in the genus of *Drechslera* in the late 1950s (Shoemaker, 1962; Alcorn, 1988). Analysis of mating-type gene sequences confirmed that *P. teres* belongs to the Pleosporales group closely related to *Phaeosphaeria nodorum* (Phaeosphaeriaceae) and *Leptosphaeria maculans* (Leptosphaeriaceae) (Rau et al., 2005, 2007). Two other *Pyrenophora* species, *P. graminea* and *P. japonica*, can cause foliar diseases on barley. The three species can be differentiated by small differences in morphology of ascocarp, conidia and conidiophore, in addition to their typical disease symptoms (Ito and Kuribayashi, 1931; Shoemaker, 1962; Sivanesan, 1987).

Net blotch exists in two forms, *P. teres* f. *teres* (net form of net blotch) and *P. teres* f. *maculata* (spot form of net blotch), which show different disease symptoms, but are undistinguishable by other morphological traits (e.g. conidia) (Smedegård-Petersen, 1971). Disease symptoms of the net form of net blotch are elongated lesions with necrotic areas along leaf veins with occasional transverse striations, whereas the spot form of *P. teres* produces more discrete, rounded lesions, mostly surrounded by a chlorotic zone (Figure 1 A, B). Historically, *P. teres* f. *teres* was regarded as the predominant form of *P. teres* but in recent years epidemics with the spot type have been reported, particularly in Canada and Australia (Tekauz, 1990; McLean et al., 2010). However, in these studies the differentiation of both forms was based on appearance of disease symptoms, which can be inconclusive since the appearance of lesions is also dependent on the stage of infection, pathotype, climatic conditions and host genotype (Smedegård-Petersen, 1971). DNA markers and mating-type gene sequences revealed the close relationship of net type and spot type of *P. teres*. Therefore, the form names *P. teres* f. *teres* and *P. teres* f. *maculata* are widely accepted by plant pathologists and geneticists. However, several studies have shown that the two forms of *P. teres* are divergent genetic groups and phylogenetically independent and could be also regarded as different species (Bakonyi and Justesen, 2007; Serenius et al., 2005; Rau et al., 2007; Lehmensiek et al., 2010). *In vitro* mating of the two forms was possible and resulted in fertile progeny with intermediate disease symptoms and were demonstrated to be stable (Campbell et al., 2002; Campbell and Crous, 2003). However, it remains unclear whether natural hybridisation occurs in nature (Campbell et al., 2002; Leisova et al., 2005). As no clear morphological or life cycle differences between both forms are known, these topics will be explained in the following for both types together.

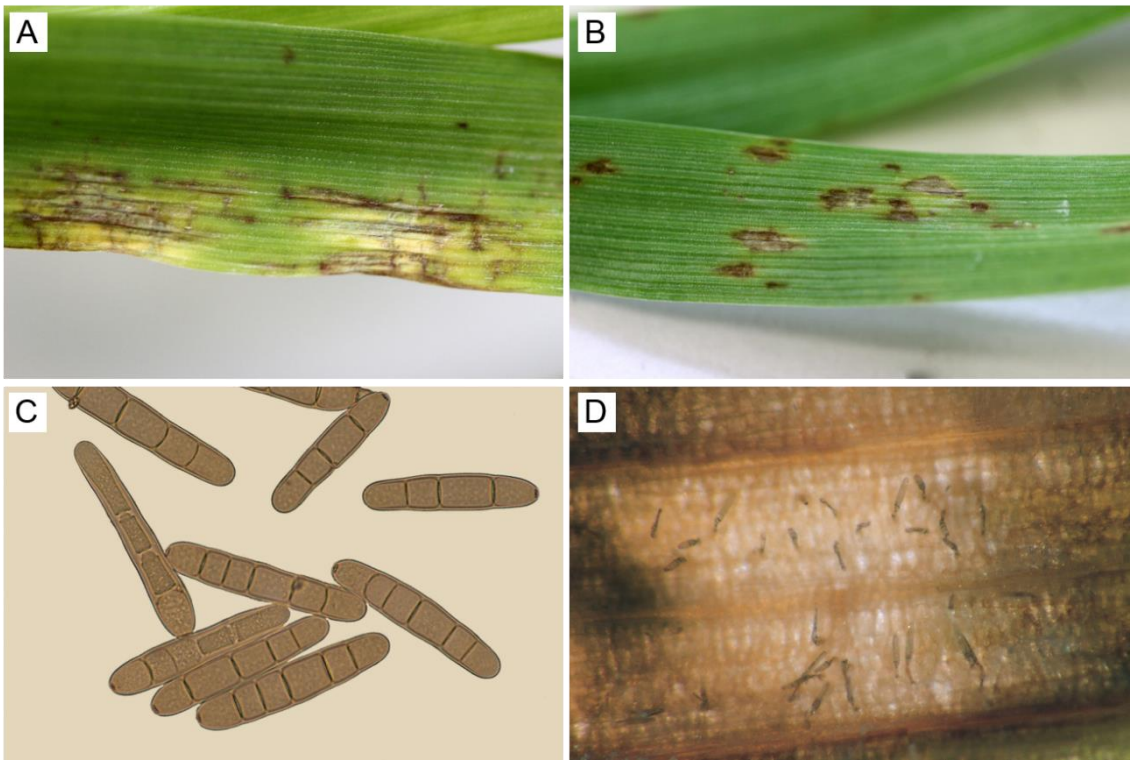


Figure 1: Disease symptoms and conidia of *P. teres*. A: Lesions of net form of *P. teres* on barley leaf (artificially inoculated). B: Lesions of spot form of *P. teres* on barley leaf (artificially inoculated). C: Conidia of *P. teres* (measuring 30-174 µm x 15-23 µm) (BASF SE picture pool). D: Conidiophores on barley leaf (BASF SE picture pool).

1.1.1.2 Life cycle

P. teres is a seed- and stubble-borne pathogen usually produces the ascocarp (pseudothecium), which serves as over-seasoning structure, on barley debris left after harvest. The increase of reduced or no-tillage agricultural practices has probably contributed to a wider presence of net blotch disease and other stubble-borne diseases (Shipton et al., 1973; Mathre, 1997; McLean et al., 2010). The disease cycle of *P. teres* is given in Figure 2. Pseudothecia appear as dark dots on the surface of barley straw in the late summer or at the beginning of autumn. As *P. teres* is a heterothallic pathogen, the development of the sexual stage requires strains of two opposite mating genotypes (Rau et al., 2005). Within mature pseudothecia, asci with generally eight ascospores are formed (Webster, 1951; Mathre, 1997). Ascospores are actively released and dispersed by wind, and lead to primary infections early in the season (Jordan, 1981). In addition, seed-borne mycelium and conidia produced on barley stubble or on an alternative host can also start primary infections (Shipton et al., 1973; Jordan and Allen, 1984; Louw et al., 1996; McLean et al., 2010). The fungus colonizes the plant tissue and produces large numbers of conidia, which serve as secondary inoculum. Production of conidia takes place with several propagation cycles throughout the growing season. Spores are dispersed by wind or rain fall to infect upper leaf layers of the same plant, neighbouring plants or new barley fields in far distance (Jordan, 1981; Mathre, 1997). Successful dispersion, germination and infection of conidia are highly dependent on the relative humidity, temperature and leaf wetness, and

other environmental factors (Jordan, 1981; van den Berg and Rossnagel, 1990). At the end of the growing season, pseudothecia are again produced on senescent tissue.

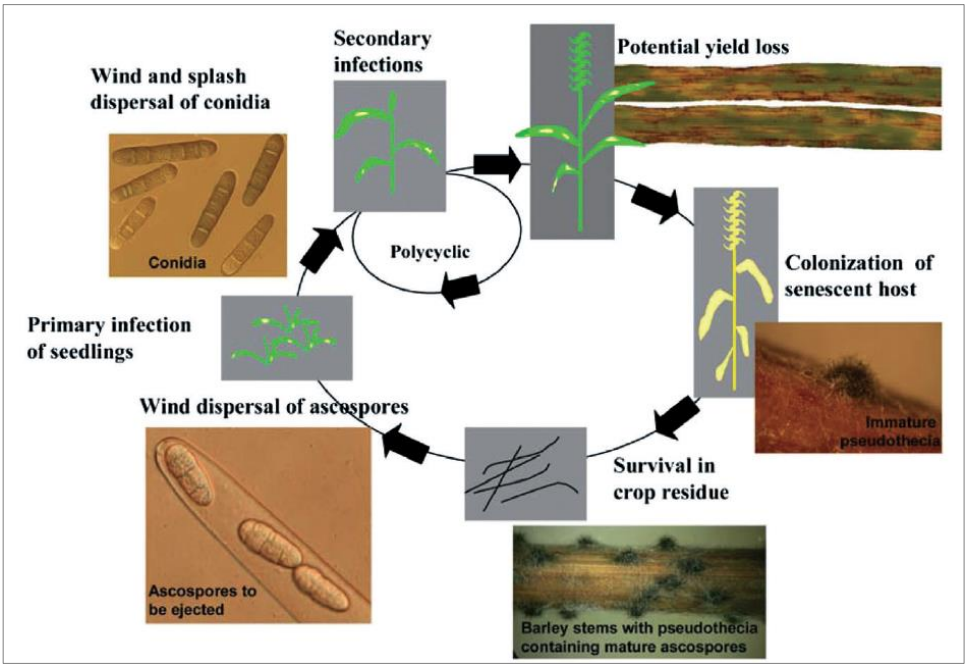


Figure 2: Life cycle of *P. teres.* The picture is taken from Liu et al. (2011) and shows the life cycle over the season. Primary infection of seedlings is mainly driven by wind dispersed ascospores but also stubble-born conidia. Secondary infections (polycyclic) are mainly derived from wind and splash dispersed conidia. Pseudothecia containing ascospores develop within the senescent host tissue.

1.1.1.3 Infection process and toxin production

Conidia or ascospores germinate on the surface of leaves within a few hours in the prescence of water (Shipton et al., 1973; van den Berg und Rossnagel, 1990). *P. teres* directly invades leaf tissue through the cuticle into epidermal cells, between epidermal cells or in rare events by entering through stomata (Van Caeseele and Grumbles, 1979; Keon and Hargreaves, 1983; Jørgensen et al., 1998). Penetration is driven by enzymatic hydrolysis of cuticule and cell wall together with the formation of appressoria. After penetration of the epidermal cell, intracellular primary and subsequently secondary vesicles are developed in order to disrupt the invaded plant cell (Keon and Hargreaves, 1983). Intracellular hyphae are produced within the epidermal cell which finally enter mesophyll tissue, where intercellular growth of further hyphae occurs. During the infection process, chlorotic zones start to develop around infected cells, where chloroplasts are destroyed probably caused by toxins and effectors secreted by the fungus, or a host response by programmed cell death (Keon and Hargreaves, 1983). Intracellular vesicles are different between net type and spot type of *P. teres*. Whereas, *P. teres* f. *teres* behaves completely necrotrophic, with respect to infection and nutrition uptake, the intracellular vesicles of *P. teres* f. *maculata* are more haustorial-like and initially serve as feeding structures. Therefore, *P. teres* f. *teres* could referred to as complete necrotrophic, in contrast,

P. teres f. *maculata* could be seen as hemibiotroph (with a very short period of biotrophy) (Lightfoot and Able, 2010; Liu et al., 2011).

Both forms of *P. teres* produce different toxins in order to induce chlorotic and necrotic death of plant tissue. Necrotic lesions can appear 24 h after inoculation and sporulation can occur within one week. The chlorotic area surrounding a net blotch lesion was shown to be free of fungal hyphal growth (Smedegård-Petersen, 1977; Keon and Hargreaves, 1983). Three structurally similar phytotoxic compounds which induce necrosis and/or chlorosis, named toxin A, toxin B and toxin C, were purified from isolates of both *P. teres* types (Smedegård-Petersen, 1977; Bach et al., 1979). The determination of the chemical structure of the three toxins revealed that they are similar or identical (in case of toxin C) to aspergillomarasmine A, which is produced by *Aspergillus versicolor* and is present in indoor environments and on food products (Samson et al., 2004). In addition to low molecular weight compounds, proteinaceous metabolites extracted from culture filtrates of *P. teres* were able to induce necrotic lesions on barley cultivars (Sarpeleh et al., 2007, 2008).

1.1.1.4 Population diversity

Knowledge about the diversity and the genetic structure of a fungal population can be helpful in the management of fungal diseases, mainly for a successful development of resistant host varieties and the effective and long-lasting use of fungicides. *P. teres* was shown to have a high level of variability in the population compared to many other fungi even within a small-scale sampling area (Shipton et al., 1973; Campbell et al., 2002; Lehmensiek et al., 2010). Furthermore, populations separated by long distances exhibit a high genetic differentiation, which suggests limited gene flow (Jonsson et al., 2000; Lehmensiek et al., 2010). Phylogenetic studies revealed that the two mating-type genes of *P. teres* were found in a ratio of 1:1 in the population, suggesting that sexual reproduction is a driving force in the development of population structure (Rau et al., 2005).

1.1.2 Septoria tritici blotch in wheat caused by *Zymoseptoria tritici*

1.1.2.1 Importance

Septoria tritici blotch (STB) caused by the ascomycetous fungus *Zymoseptoria tritici* (Desm.) Quaedvlieg and Crous is a globally-distributed disease of wheat (Dean et al., 2012; Gurr and Fones, 2015; Torriani et al., 2015). Severe epidemics on STB-susceptible wheat cultivars can cause yield losses of up to 50% (Eyal et al., 1987). *Z. tritici* is thought to have emerged about 10,000 years ago from an ancestral population during the domestication of wheat in the Fertile Crescent (Stukenbrock et al., 2007). In the European Union, *Z. tritici* has become the most devastating foliar disease in wheat cultivation (Eyal et al., 1987; Shaw and Royle, 1989; Gurr and Fones, 2015). *Z. tritici* is favoured by humid climatic conditions that prevails in EPPO's "Maritime Zone" (Bouma, 2005) and includes the European regions Northern France, Germany, and the UK. Approximately 70% of annually used fungicides in European cereal cultivation are applied to control STB as the main disease (Ponomarenko et al., 2011).

1.1.2.2 Taxonomy

Z. tritici belongs to the class of Dothideomycetes in the order Capnodiales and the family Mycosphaerellaceae. *Mycosphaerella graminicola* (Fuckel) J. Schroeter in Cohn is the sexual stage of *Z. tritici* on wheat (Sanderson, 1976). Formerly, *Z. tritici* was referred to as *Septoria tritici* (Roberge in Desmaz.). In 2011, Quaedvlieg et al. introduced a novel genus *Zymoseptoria* to accommodate the Septoria-like species commonly infecting graminicolous hosts. All species of *Zymoseptoria* show a yeast-like growth in culture and up to three different types of conidia, namely pycnidial conidia, phragmospores on aerial hyphae and yeast-like proliferation via microcyclic conidiation (Quaedvlieg et al., 2011).

1.1.2.3 Life cycle

In winter wheat cultivation, *Z. tritici* survives the summer on residues of the previous wheat crop and starts infections in the autumn (Holmes and Colhoun, 1974; Brown et al., 1978; Serivastava and Tewari, 2002). *Z. tritici* is a specialized pathogen of wheat but is considered to survive in association with other alternative hosts belonging to the genera *Agropyron* spp., *Agrostis* spp., *Brachypodium* spp., *Bromus* spp., *Dactylis* spp. *Festuca* spp., *Hordeum* spp., *Glyceria* spp., *Poa* spp., *Secale cereale* and *Triticum* spp. (Sprague, 1950; Eyal, 1999). The exact role of alternative hosts in the epidemiology of the pathogen is not understood so far but it is thought that wild grass species can form a reservoir for the pathogen (Hoffmann and Schmutterer, 1999). This source of the fungus is probably important when wheat residues are absent. Primary infection starts with the germination of ascospores and pycnidiospores on the leaf surface (Hilu and Bever, 1957; Eyal et al., 1987;

Shipton et al., 1971; Suffert et al., 2011). Ascospores are dispersed by wind over long distances and are considered to be the main source of primary inoculum (Shaw and Royle, 1989; Suffert et al., 2011). Ascospores are produced in fruiting bodies of the sexual stage, which are called pseudothecia or ascocarps. *Z. tritici* has a heterothallic nature, therefore two different mating types (MAT1-1 and MAT1-2) are needed for sexual reproduction (Kema et al., 1996). As two lesions originating from both mating types need to coalesce, a high infection density is thought to lead to a higher number of pseudothecia than in an epidemic at lower infection densities (Cowger et al., 2002). Pseudothecia were also observed during growing season but appear long time after pycnidia (delay around 30-60 days) on infected leaves as a survival strategy in response to exhaustion of nutrition (Hunter et al., 1999; Eriksen and Munk, 2003). Pseudothecia that were produced during epidemics on green leaves stay viable on senescent leaves and plant debris as an ascospores source. However, ascospore release follows a seasonal pattern with a first peak in late autumn and a second peak at the end of the growing season (June or July) in the Northern Hemisphere in winter wheat cultivation (Hunter et al., 1999; Eriksen and Munk, 2003). Local secondary infections during the growing season are primarily driven by asexual conidia or pycnidiospores disseminated by rain splash or strong wind. STB is favoured by cool and wet weather with a temperature optimum between 16 to 21°C (Holmes and Colhoun, 1974). However, infections can also occur during winter at temperatures of at least 5°C. A successful infection requires a minimum of 6 h and up to four days of leaf wetness. Once the host is infected, the fungus exhibits a long latency phase of around 17 to 28 days before characteristic black fruiting bodies appear and a new generation of spores is produced. Spores produced in these fruiting bodies are exuded in sticky masses and are distributed mainly by rain splashes onto upper leaf layers and heads (De Wolf, 2008). Long, rainless periods reduce horizontal and upward vertical spread of spores to upper leaves, which are important in grain filling process (Eyal et al., 1987). Symptoms of *Z. tritici* on wheat leaves appear as irregular chlorotic areas, which later turn into necrotic lesions with typical black conidia (Eyal et al., 1987). On infected debris, pycnidiospores can be viable for several months (Hilu and Bever, 1957). In Figure 3, the dynamics of a STB disease epidemiology is shown with special regard to the importance of different inocula sources. The main source of primary infection was suggested to derive from wind-dispersed ascospores from far distant wheat debris, whereas secondary infection mainly was proposed to start from splash-dispersed pycnidiospores from neighouring wheat debris or from senescent basal leaves within wheat plants (Suffert et al., 2011).

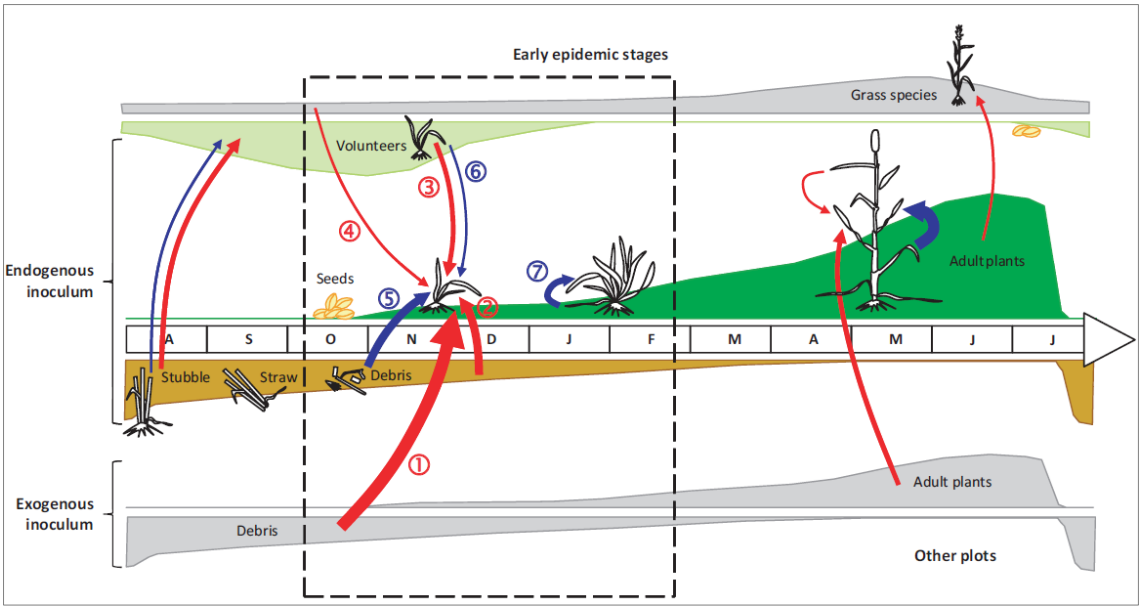


Figure 3: Dynamic of a Septoria tritici blotch epidemic. Figure is extracted from Suffert et al. (2011). Red arrows indicate infections caused by wind-dispersed ascospores and blue arrows indicate infections caused by splash-dispersed pycnidiospores of *Z. tritici*. The width of the arrows illustrates the suggested significance of each mechanism. 1: ascospores from distant infected wheat debris; 2: ascospores from neighbouring wheat debris; 3: ascospores from wheat volunteers; 4: ascospores from grass species (importance unclear); 5: pycnidiospores from neighbouring wheat debris; 6: pycnidiospores from wheat volunteers; 7: pycnidiospores from senescent basal leaves.

1.1.2.4 Infection process

Ascospores and conidia germinate on the leaf surface and penetrate the host plant through the stomata (Kema et al., 1996; Duncan and Howard, 2000). Most *Mycosphaerella* fungi, such as *Z. tritici*, show an extensive 'latent period' of symptomless fungal colonization (Leonard and Mundt, 1984). During this asymptomatic phase, hyphae of *Z. tritici* grow in the apoplastic space in close contact to plant cell walls (Kema et al.,1996; Marshall et al., 2011; Yang et al., 2013). As the fungus does not develop haustoria or other intracellular feeding structures, it remains questionable as to how the acquisition of nutrition is managed within that time. However, Kema et al. (1996) observed a movement of host chloroplasts towards the cell wall during infection which indicates an alteration of host cell physiology by the pathogen. In the latency phase, the growth of *Z. tritici* behaves more like an endophyte than a biotroph (Joosten et al., 1990; Solomon and Oliver, 2001, 2002; Thomma et al., 2005; Keon et al., 2007; Rudd et al., 2015; Sánchez-Vallet et al., 2015). Although the repertoire of plant cell wall degrading enzymes in *Z. tritici* is small compared to other phytopathogenic fungi, the expression profile gives evidence that they are important in plant colonization (Brunner et al., 2013). Some of the cell wall degrading enzymes are exclusively expressed during asymptomatic phase suggesting that release of nutrient from cell walls is used as nutrition but without visible damage to plant cells (Sánchez-Vallet et al., 2015). During colonization of their host plants, all endophytic and biotrophic fungi need to prevent host immune responses (Liu et al., 2003). Up to date, only two effectors of *Z. tritici* were shown to be essential for infection process (Marshall et al., 2011). *Mg*1LysM and *Mg*3LysM have

a signal peptide for secretion and a LsyM domain in common, which was demonstrated to prevent chitin-triggered immunity (Marshall et al., 2011; de Jonge et al., 2010; Sánchez-Vallet et al., 2013). After asymptomatic colonization of host tissue, a rapid switch to the necrotrophic, pathogenic phase coupled with sudden death of plant cells occurs. However, the reasons why *Z. tritici* first exhibits a long latent period before causing necrosis and the signals that trigger the switch to necrosis remain unknown (Sánchez-Vallet et al., 2015). The expression of cysteine rich effector proteins has been described in *Z. tritici*, but probably the fungus uses several other mechanisms to induce necrosis (Keon et al., 2007; Rudd et al., 2008, 2015; Brunner et al., 2013; Mirzadi Gohari et al., 2015).

1.1.2.5 Genetics and population diversity

The genome of *Z. tritici* was fully sequenced in 2011 and is used as a model for fundamental genetic studies of haploid plant-pathogenic fungi since then (Goodwin et al., 2011). Goodwin et al. (2011) discovered that the length of the genome is 39.7 Mb, which is comparable to other filamentous ascomycetes, but it has the highest number of chromosomes reported from ascomycetes so far, in total 21 (Goodwin et al., 2011). Another feature of the *Z. tritici* genome is the low number of genes encoding for cell wall degrading enzymes, which is more similar to endophytes than to other plant pathogenic fungi (Goodwin et al., 2011). Furthermore, genome studies revealed a high size range of chromosomes and that eight chromosomes could get lost during meiosis without visible effects on the fungus (Wittenberg et al., 2009; Goodwin et al., 2011). These so-called ‘dispensable chromosomes’ are proposed to originate from an ancient horizontal transfer from an unknown donor organism. The role of dispensable chromosomes remains elusive but it is thought that they might contribute to rapid adaptation to changing environmental conditions (Wittenberg et al., 2009). Studies on the genetic structure of populations indirectly reveals that sexual reproduction is very common in *Z. tritici* (Chen and McDonald, 1996; Linde et al., 2002). Moreover, it was reported that the fungus shows a very high effective population size which allows gene flow on a world-wide scale (Zhan et al., 2003). Zhan et al. (2003) further demonstrated that around 90% of the global genetic variation could be found within a single wheat field. This diversity of the fungus is a consequence of the sexual productive system leading to the formation of ascospores, which initiate epidemics every season (Chen and McDonald, 1996; Linde et al., 2002; Waalwijk et al., 2002).

1.2 Disease management in cereals

Diseases are a major threat in cereal production systems influencing both yield and grain quality (Oerke, 2006). Therefore, many control measures have been adopted over the last years in order to minimize disease severity. Integrated disease control relies on cultural

practices, breeding of pathogen resistant host varieties and the use of chemical control agents.

1.2.1 Cultural measures

Cultural practices, which include sanitation, tillage, crop rotation and adaption of the sowing date, are known to affect disease pressure in cereal production. The removal of infected crop residues from the field is one method to reduce inoculum and distribution of pathogens (Conway, 1996). Incorporation of residues into the soil and crop rotation are two further cultural methods in cereals to reduce pathogen inoculum. Minimum and no tillage have been shown to increase disease pressure of fungal pathogens especially of tan spot and Fusarium head blight (Jørgensen and Olsen, 2007). Inoculum of *P. teres* produced on straw was still able to initiate infection after nine months in the field (Piening, 1968). However, disease severity of STB and *P. teres* are less affected by crop rotation and reduced tillage compared to other pathogens. This is possibly due to the intensity of cereal production areas with ascospores being prevalent in all growing regions and are regarded as the main source of early season inoculum (Gladders et al., 2001; Brown and Hovmøller, 2002; Eriksen and Munk, 2003). Sowing date also shows a significant effect on disease development. Early autumn sowing of winter wheat can favour many diseases, such as eyespot and STB (Bødker et al., 1990; Bateman et al., 2007; Gladders et al., 2007; Jørgensen et al., 2014). On the other hand, late sowing can increase the risk of powdery mildew and yellow rust infections in spring (Jørgensen et al., 2014). Futhermore, a high input of nitrogen fertilizer can enhance the development of some foliar diseases, such as STB (Simon et al., 2003), yellow rust (Neumann et al., 2004) and powdery mildew (Olesen et al., 2003). Additionally, low or high crop densities can have an impact on the risk of disease epidemics. Many pathogens (e.g. powdery mildew) favour high seedling densities, whereas the splash-borne diseases caused by *Z. tritici* and *P. teres* were shown to have a higher risk at low seedling densities, where they profiting from crops more open to rain (Tompkins et al., 1992; Colbach and Saur, 1998). A major problem of cultural methods used to prevent diseases is the fact that a method reducing the risk of one disease, can enhance the risk of other diseases to occur (Jørgensen et al., 2014).

1.2.2 Resistance breeding

The growth of disease resistant cultivars represents a useful tool to combat plant pathogens (Loyce et al., 2008; Jørgensen et al., 2014). Plants respond to pathogen infections using transmembrane pattern recognition receptors (PRRs) to detect slowly evolving pathogen-associated molecular patterns (PAMPs) and polymorphic nucleotide-binding (NB) and leucine rich repeats (LRR) containing proteins to recognize pathogen specific effectors (Dangl and Jones, 2001). NB-LRR-mediated disease resistance was shown to be effective

against obligate biotrophic and hemibiotrophic pathogens, but not against necrotrophic pathogens (Glazebrook, 2005). After successful recognition of an infection, disease resistance is usually mediated by hypersensitive cell death response (HR) of the host plant (Jones and Dangl, 2006).

Over the last 25 years, breeding of resistant wheat cultivars has provided cultivars with disease resistance against yellow rust, brown rust, eyespot, powdery mildew and to some extent to STB (Loyce et al., 2008). Multi-resistant cultivars cover only a small proportion of cultivated wheat since they still show a lower yield in the absence of pathogens compared to cultivars with low disease resistance (Brown, 2002). Moreover, additional yield responses to fungicide treatment were often found even in resistant cultivars, which indicates that disease resistance does not cover all diseases and that fungicides may contribute to positive physiological effects on crops (Bartlett et al., 2002). However, field trials from Denmark, France, Sweden and the UK have indicated that resistant cultivars can reduce potential yield losses and the expected fungicide costs (Jørgensen et al., 2008). Key effectors of *Z. tritici* and *P. teres* are thought to be mainly proteinaceous (Sarpeleh et al., 2007, 2008; Rudd et al., 2010). In *P. teres*, single incompletely dominant resistance genes were identified in barley breeding lines (Schaller, 1955; Gray, 1966). As host resistance against *P. teres* obtained by single genes was overcome by pathotypes/biotypes of the pathogen, a durable approach was first accomplished using multiple resistance genes (Douiyssi et al., 1998).

1.2.3 Chemical control using fungicides

Since other disease control practices are often unsufficient to completely suppress plant diseases and positive yield response after chemical treatments can be observed, the use of chemical control agents is widely practiced. Chemical applications to control plant diseases target fungal diseases (fungicides), some bacterial diseases and on rare occasions phytoplasms (antibiotics), and by indirect control of vectors also viruses (insecticides) (Baldwin and Rathmell, 1988). Cereals in European cultivation systems are one of the most widely treated group of crops worldwide (Kuck and Gisi, 2006).

Fungicides are agents to protect plants against invasion by fungi and have been used for more than 200 years. At the beginning, fungicides were mainly used to protect cereal seeds and grapevines. After the Second World War, the number of crops treated with chemicals, the number of chemicals available, the area and frequency of applications, and the effectiveness of treatments have shown a huge increase (Brent and Hollomon, 2007). The use of fungicides has contributed to improve quality and quantity of agricultural products (Oerke et al., 1994). In cereals, the lack of disease resistant varieties against many pathogens has made the use of fungicides to be one of the most important tools to manage fungal diseases (Verreet et al., 2000).

Chalk, wood ash and sulphur are naturally occurring substances that have been used as fungicides in the early years but are toxic to many forms of life (Campbell, 1989). Since the 18th century, copper and lime sulphur became commonly used in cultivation of vegetables, fruits and ornamental plants and still are broadly used to manage crop diseases, especially on organic farms (Marzani, 2011). Later, further non-selective compounds, such as mercuric chlorides, and products of industrial processes, such as nitrophenols (Fent and Hunn, 1996), were used as fungicides; all of them showing toxicity to both users and non-target organisms. The modern era of synthetic fungicides began in the first half of the 20th century with inventions such as organomercury seed treatments, which were banned in the 1970s and 1980s due to their unfavourable toxicological profile (Oliver and Hewitt, 2014). Thereupon, efforts were initiated to develop new chemicals, which showed a more favourable toxicological and ecotoxicological profile, and a higher efficacy. In the late 1960s and 1970s, compounds such as benzimidazoles, 2-amino-pyrimidines, carboxanilides, phosphorothiolates, morpholines, dicarboximides, and sterol demethylation inhibitors (DMIs) were introduced (Brent and Hollomon, 2007). Particularly DMIs were subsequently improved over the next years leading to novel fungicides with a more potent action against plant pathogens (Anonymous, 2002). Over the last 20 years, numerous novel compounds belonging to chemical groups of phenylpyroles, anilinopyrimidines, benzamides, quinone outside inhibitors (QoIs, also called strobilurins) and succinate dehydrogenase inhibitors (SDHIs, earlier called carboxamides) were launched to the market.

Modern synthetic fungicides are mainly developed and sold by large, independent, multi-national companies. The development of a novel fungicide and the maintenance of existing products are very cost intensive and costs are continuously rising due to increased regulatory pressures. In contrast, progress in plant breeding is mainly driven by state agencies and universities (Oliver and Hewitt, 2014). Currently, the major companies are leading pesticide market worldwide: Syngenta (fungicide sales 2015: US$ 3,916 million, currently in merging process with ChemChina), Bayer CropScience (fungicide sales 2015: US$ 3,803 million, currently in merging process with Monsanto), BASF (fungicide sales 2015: US$ 2,917 million), DuPont and Dow Chemicals (fungicide sales 2015: US$ 418 and 750 million, respectively, both in merging process). Before developing a novel fungicide, the company needs to be convinced that the product will reach enough sales to justify high expenses for research and development.

Fungicides can be classified in different ways according to crop protection performance, their mode of action and their chemical structures. Currently, there are over 200 molecules that are marketed as fungicides in agriculture and which belong to several chemical classes. Most fungicides are complex organic molecules containing several functional groups often similar in different compounds within one mode of action (MOA) group (Oliver and Hewitt, 2014). Important differentiations are made between single- and multi-site modes of action and between protectant and eradicant (curative) effectiveness of molecules.

Protectant fungicides are applied before infection to prevent initial penetration of the pathogen into the host plant. Immobile protectant fungicides, also called contact fungicides, remain on the leaf surface forming a chemical blockade and are not distributed within the host plant. Non-systemic fungicides are often multi-site inhibitors, disrupting several biochemical processes by their ability to bind chemical groups, for example thiol moieties, which are commonly found in many enzymes (Oliver and Hewitt, 2014). Examples of protectant (contact) fungicides are inorganic copper salt (Bordeaux mixture), and sulphur, as well as dithiocarbamates (e.g. thiram, metiram and mancozeb) and pthalimides (e.g. captan, folpet) and some others (FRAC, 2016). Protectant fungicides have several restrictions in practical use as they need to be applied in advance of pathogen attack, they are impacted by degradation and erosion due to light and rain, and they need to remain in sufficient amounts on the leaf surface to stay active. Thus, an early warning of an infection risk is necessary to find the optimal timing of protective fungicides (Lucas, 1998). Factors such as rain duration and intensity, wind and temperature are related to the dissemination of ascospores in *Z. tritici* and are used in forecasting models of disease outbreaks (Royle, 1994; Parker et al., 1999; Eyal, 1999).

In contrast to that, systemic fungicides enter the plant and are translocated in the plant exhibiting apoplastic mobility and/or symplastic mobility. Systemic compounds can show protectant and curative activity and, therefore, can be used to kill already established infections, at least to some extent (Brent and Hollomon, 2007; Manners, 1993). Systemic fungicides act as single-site inhibitors, which means that they have a defined biochemical target-site in a pathogen (Taylor, 2001). Systemic fungicides are often specific in their toxicity, showing low toxicity to most non-target organisms (Narayanasamy, 2002). Compared with non-systemics, the popularity of systemic fungicides is increasing, particularly in cereals (Hewitt, 1998).

Intense research is performed to classify fungicides according to their biological and biochemical MOA. An up-to-date classification of fungicides can be found on the website of FRAC (FRAC, 2016). In total, agrochemical fungicides belong to 48 described MOAs and further unclear MOAs, whereof the most important classes are restricted to a limited number of MOAs.

Three main site-specific systemic classes of fungicides are currently in use to control cereal diseases. The C14-demethylation inhibitors (DMIs), which inhibit a step in the sterol biosynthesis of membranes, the quinone outside inhibitors (QoIs), which block the electron transfer in complex III of mitochondrial respiration, and the succinate dehydrogenase inhibitors (SDHIs), which lead to an inhibition of complex II and therefore are also involved in mitochondrial respiration.

The DMI class is a subgroup of sterol biosynthesis inhibitors (SBIs), which constitute the largest group of fungicides with respect to the number of compounds and current sales

(FRAC, 2016). DMIs interfere with the synthesis of ergosterol, which is the principal sterol in membranes of all fungi, except the oomycetes. Ergosterol acts as a functional component in the maintenance of membrane integrity and therefore, a reduction of ergosterol leads to membrane disruption and electrolyte leakage (Joseph-Horne et al., 1996). DMIs interact with sterol 14 α-demethylase (P450 monooxygenase) by inhibiting the removal of C14 methyl group from eburicol and, thereby, causing subsequent accumulation of precursor sterols and reduced production of ergosterol (Baldwin, 1983, 1990; Gadher et al., 1983; Baloch et al., 1984; Kelly et al., 1995). P450 monooxygenase is encoded by the *Cyp51* gene. DMIs presumably block the enzyme at its active site, thus hampering the access of the natural substrate (Kelly and Kelly, 2013). DMIs can be split into five chemical classes, whereof triazoles are commercially the most important and include epoxiconazole, prothioconazole, tebuconazole and propiconazole, but also a few pyrimidines and imidazoles (e.g. prochloraz) have reached market relevance (Oliver and Hewitt, 2014).

The first fungicide of the QoI class was first launched to the markets only two decades ago. Today, many different QoI fungicides are broadly used in crop protection with annual market sales approaching US$1 billion (Oliver and Hewitt, 2014). The first strobilurins (strobilurin A and B) were discovered in a wood-rotting basidiomycetous fungus, *Strobilurus tenacellus*, in 1977 (Anke et al., 1977). Studies confirmed that both strobilurin A and B were powerful antibiotics against several fungal species by inhibition of fungal energy production at the quinol oxidation in cytochrome bc1 complex (complex III) (Anke et al., 1979; Sauter et al., 1999). These natural products attracted agrochemical companies to produce synthetic compounds which showed similar or even more effective molecules. QoIs have several beneficial properties as they are active against a range of fungi including oomycetes, basidiomycetes and ascomycetes, they are non-toxic to non-target organisms and they are degraded rapidly in the environment (Oliver and Hewitt, 2014). Important QoI fungicides are for example azoxystrobin and pyraclostrobin.

The SDHI group of fungicides has a long history, however, SDHIs reached market relevance only several years ago as a consequence of the development of molecules with a broader spectrum of activity against many basidiomycete and ascomycete fungi. As SDHIs are investigated in the present study, a short summary of their history and the fungicidal activity are given in section 1.2.4.

1.2.4 Succinate dehydrogenase inhibitors (SDHIs)

In 1966, two oxathiin carboxamides (carboxin and oxycarboxin) were the first SDHIs described to show fungicidal activity (von Schmeling and Kulka, 1966). Both were launched to the market by Uniroyal in the years 1969 and 1975. They were mainly used as seed treatments to control a limited number of species within the basidiomycetous fungi, such as *Rhizoctonia* spp., *Ustilago* spp. and *Tilletia caries* in cereals, maize, cotton, oilseed rape

and legumes (reviewed Oliver and Hewitt, 2014). In 1974, two structural analogues, benodanil (BASF) and fenfuram (Shell), were introduced to the market, followed by a range of SDHIs such as mepronil and flutolanil mainly targeting rice diseases (Glättli et al., 2011). All these compounds have a limited spectrum in common, which is restricted to basidiomycetes, and a poor mobility in plants and are therefore referred to the first generation of SDHIs. Recently, new SDHI fungicides with a broader spectrum of activity, including basidiomycetous, ascomycetous and deuteromycetous plant pathogens on various crops, were developed. Boscalid (BASF) was the first of these SDHI fungicides with a broader spectrum and was launched in 2003 (Stammler et al., 2007; Stammler, 2008). In the last years, several other SDHIs, such as bixafen (Bayer CS), fluopyram (Bayer CS), fluxapyroxad (BASF SE), isopyrazam and benzovindiflupyr (Syngenta), and penthiopyrad (Mitsui) followed (Glättli et al., 2011; Stammler et al., 2015) and more are expected in the future e.g. pydiflumetofen (Syngenta). Today, SDHIs rank with QoI and DMIs in their importance in fungal disease control and their market-size (usage) has rapidly increased in many crops.

The target of SDHIs is the succinate dehydrogenase enzyme (SDH), also referred to as complex II or succinate-ubiquinone oxidoreductase, which is an essential enzyme of the tricarboxylic cycle and the mitochondrial electron transfer chain (Keon et al., 1991; Hägerhäll, 1997; Matsson and Hederstedt, 2001). The SDH enzyme is a mitochondrial heterotetramer composed of four nuclear-encoded subunits and is located in the inner mitochondrial membrane (Cecchini, 2003): SDH-A (also called Fp) is a hydrophilic flavoprotein, SDH-B (also called Ip) is an iron sulphur protein which contains three iron sulphur centres, and SDH-C (CybL) and SDH-D (CybS) are membrane anchoring subunits, which show a complexed prosthetic haem b group between both antiparallel helices (Sun et al., 2005; Ōmura and Shiomi, 2007; Ackrell, 2008). The SDH complex couples the oxidation of succinate to fumarate in the mitochondrial matrix with the reduction of ubiquinone (Q) to ubiquinol in the membrane during aerobic respiration (Horsefield et al., 2004, 2006). A schematic model of the SDH enzyme is given in Figure 4. Several studies have demonstrated that the amino acid residues involved in Q-binding are positioned near [3Fe-4S] clusters and the haem b group and are highly conserved residues between bacteria and eukaryotes (Yankovskaya et al., 2003; Sun et al., 2005; Horsefield et al., 2006).

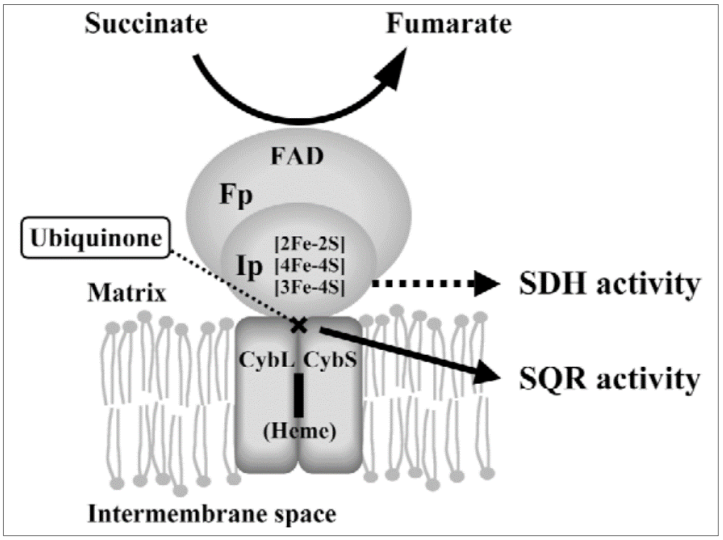


Figure 4: Schematic model of succinate dehydrogenase complex. Picture is taken from Ōmura and Shiomi (2007). The SDH complex is composed of subunits SDH-A (flavoprotein, Fp), SDH-B (protein containing iron-sulphur clusters, Ip), and SDH-C (CybL) and SDH-D (CypS) as membrane anchors with a complexed haem b group. The enzyme accomplishes succinate oxidation (SDH activity) with ubiquinone reduction (SQR activity).

SDHI fungicides bind at the Q-binding site (Q-site) of SDH complex and interrupt the reduction of ubiquinone to ubiquinol by competitive inhibition, finally impacting on fungal respiration (Keon et al., 1991; Matsson and Hederstedt, 2001; Horsefield et al., 2006; Huang et al., 2006). Modern SDHIs are thought to bind deeper into the Q-site than ubiquinone itself but show an overlapping binding to the natural substrate (Glättli et al., 2009, 2011; Fraaije et al., 2012; Sierotzki and Scalliet, 2013).

The structure of four SDHI fungicides and their structural alignments are shown in Figure 5. The overlay of SDHI molecules shows that they have several molecular features in common representing the fungicidal activity of these molecules. A first common feature is the central amide moiety, which forms hydrogen-bond interactions to amino acid residues of the Q-site. A second feature in common is the aromatic ring in the aniline part, which stabilizes the molecule in the Q-site by hydrophobic contacts or π-π-interactions. Moreover, most modern SDHIs additionally carry a nitrogen-containing heterocycle (pyrimidine or pyrazole), which further increases the binding affinity via hydrogen bounds (aromatic nitrogen) and π-π-interactions. The three-dimensional alignment demonstrates that the part of SDHI molecules which reaches deeper into the Q-site are structurally conserved, whereas the outer part of molecules (right hand side in Figure 5 B) appears to be more variable. The alignment of molecules further indicates that SDHIs all share a similar binding mode in the Q-site (Glättli et al., 2009, 2011; Stammler et al., 2015).

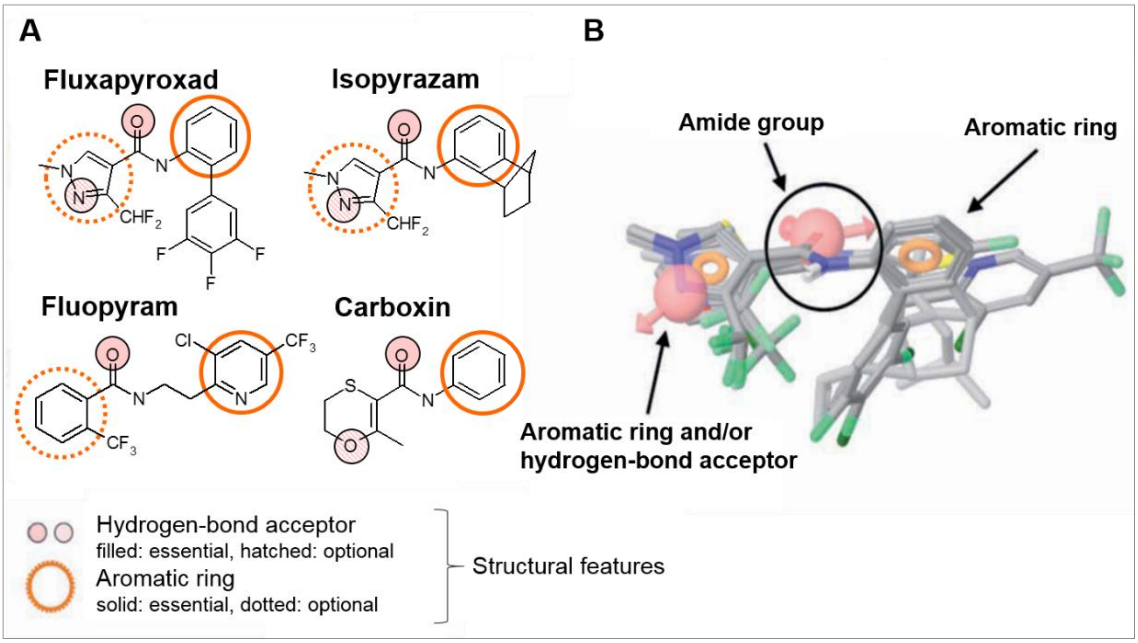


Figure 5: Structural properties of SDHI fungicides. Picture taken from Glättli et al. (2011) (modified). **A:** Chemical structure of four SDHIs with their common structural features to interact with amino acid residues of ubiquinone-binding site. **B:** Structural alignment of several SDHI molecules indicating interaction sites suggests similar binding mode at complex II.

1.2.5 Differences in fungicide use in European countries

The level of chemical input for disease control varies not only among countries but also within countries. Today, fungicides are used as a common practice to prevent severe disease epidemics. In European cereal production, the intensity of fungicide treatments varies from 0-4 times per season and has an average of two treatments (Jørgensen et al., 2014). Based on sold amounts of chemicals and national surveys from the years 2006 and 2007 in Germany, France, the United Kingdom and Denmark, it was observed that the usage in Denmark was much lower than in other countries. In France, regional differences could be found which showed higher use intensities in Northern France than in southern regions. The highest total use of fungicides was applied in the UK. These differences can be explained by different pest and disease pressures, different climatic conditions, different operating policy action plans to reduce pesticide usage, different organisation of advice to farmers, and different prices of pesticides (Jørgensen et al., 2014).

Across Europe in winter wheat and winter barley, two applications of fungicides (excluding seed treatments) are generally applied per season although this can be higher in intensive cropping areas. In winter wheat, the first application is applied during stem extension, at growth stage 30/31, to target stem base and early foliar pathogens, such as eyespot, *B. graminis* f. sp. *tritici* and *Z. tritici*. The second application is applied to protect crops against foliar diseases during flag leaf emergence, at growth stage 37, such as *B. graminis* f. sp. *tritici*, *Puccinia* spp. and *Z. tritici* (reviewed Oliver and Hewitt, 2014). In winter barley, the major pathogens are *B. graminis* f. sp. *hordei*, *Rhynchosporium secalis*, *P. teres* and

Puccinia hordei. In contrast to that, in spring barley, usually only a single fungicide application is sufficient to control pathogens such as *B. graminis* f. sp. *hordei* and *R. secalis* (reviewed Oliver and Hewitt, 2014).

1.3 Evolution of fungicide resistance

Huge achievements have been made in modern agriculture. However, certain cultural practices, such as monoculture, using susceptible cultivars and nitrogenous fertilizer that could enhance disease susceptibility, have contributed to increase the destructive potential of plant diseases. Therefore, the control of plant diseases now is often dependent on the use of fungicides (Schwinn, 1992). The spread of new damaging diseases in a changing climatic environment, the evolution of pathogens that break resistance of host cultivars and the emergence of pathogens showing a loss of sensitivity to fungicides are the major challenges of modern plant protection (Hollomon and Brent, 2009). Charles Darwin declared all organisms to be survivors of natural selection competing with others in their specific environment (Darwin, 1859). Fungicides are a part of the environment of phytopathogenic fungi in agricultural ecosystems and show a direct effect on survival of fungi. Therefore, it is obvious that pathogens evolve mechanisms to resist those lethal effects. Fungal genomes are very plastic and show many thousands of polymorphisms (Cuomo et al., 2007) and produce a high number of progeny, under favourable conditions, in many propagation cycles each season (FRAC, 2016).

Fungicide resistance is defined as a heritable and stable genetic modification of a fungus to overcome the effects of a fungicide (Delp und Dekker, 1985; Steffens et al., 1996). There are four different resistance mechanisms that are described in fungicide resistant fungi (reviewed FRAC, 2016):

1) Alteration of the target-site of fungicides
2) Exclusion of fungicides from the cell
3) Overexpression of the target enzyme of fungicides
4) Detoxification of the fungicide

The most important mechanism in phytopathogenic fungi relies on point mutations in genes encoding for the target enzymes of fungicides (Brent and Hollomon, 2007). These mutations cause alteration of the target proteins of fungicides, which leads to a reduced or inhibited binding efficiency of fungicides. The effect on the fungicide sensitivity caused by different target-site mutations can lead to a range of diverse efficacy losses (depending on the mutation). Single-site inhibiting fungicides mainly target one specific enzyme in a biochemical process. A point mutation causing one amino acid exchange can rapidly and effectively block fungicide interaction within such target-sites (Brent and Hollomon, 2007).

In contrast, multi-site inhibitors interfere with several biochemical steps. Here, the evolution of resistance is more slowly because the fungus requires a combination of many mutations to overcome effects of fungicides (Brent and Hollomon, 2007). The emergence of resistance is described in the literature as 'qualitative' resistance (also called 'single-step' or 'disruptive') and 'quantitative' resistance (also called 'multi-step', 'continuous', and 'directional') (Brent and Hollomon, 2007). The qualitative resistance is often associated with a failure of disease control and is characterised by a sudden loss of efficacy of fungicides with a clearly separated resistant subpopulation. This form of resistance is often derived from target-site mutations in specific genes (monogenic) (Brent and Hollomon, 2007; De Miccolis Angelini et al., 2015). G143A, which causes high resistance levels to QoI fungicide, is a well-studied example of a 'single-step' evolution of fungicide resistance (Heaney et al., 2000; Gisi et al., 2002; Fraaije et al., 2002, 2005; Lucas and Fraaije, 2008; Torriani et al., 2009; Lesniak et al., 2011). In contrast, the quantitative resistance is characterised by a slow, continuous 'shift' of the sensitive population towards a population with a reduced fungicide sensitivity. This form of resistance is often caused by several mutations in different genes (polygenic), whereof individual mutations can have minor effects leading to additive effects when combined (Brent and Hollomon, 2007; De Miccolis Angelini et al., 2015). The slow adaptation of the *Z. tritici* population against DMI fungicides is an example of a continuous resistance evolution, however, it is mainly expressed by several mutations within *Cyp51* gene or overexpression of the same gene (Cools and Fraaije, 2008, 2013; Cools et al., 2013).

Spontaneous mutations in genomes continually occur in all organisms. It is thought that mutations which can cause fungicide resistance randomly occur in the population of pathogens, however, under non-selective conditions they do not provide advantage and can disappear again (Brent and Hollomon, 2007). Under fungicide treatment, such resistant individuals have a higher competitiveness compared to the sensitive population and propagation within the population occurs. Effective resistance mechanisms that are observed in resistant individuals do not necessarily mean that practical disease control failure will evolve (Brent and Hollomon, 2007). Target-site alterations were often connected to a decreased enzyme efficiency (Fisher et al., 2004; Scalliet et al., 2012). This could lead to a reduced fitness of isolates in untreated conditions, consequently hampering the further increase of resistance alleles in the population (Brent and Hollomon, 2007). A common feature of fungicide resistance (due to alterations in the target protein) is that products, of the same MOA group, are generally considered to be cross-resistant, but not to other fungicide groups (FRAC, 2016).

Active exclusion of fungicides from the fungal cell can contribute to resistance and was reported in some phytopathogenic species such as *B. cinerea* (Kretschmer et al., 2009; Kretschmer, 2012) and *Z. tritici* (Leroux and Walker, 2011; Omrane et al., 2015). Multi-drug-resistance (MDR, as it is called) is known to have relevance in pathogens and cells,

including bacteria but also human cancer cells (Perez-Tomas, 2006; Morschhäuser, 2010; Bassetti et al., 2013). Thereby, overexpression of efflux pumps of the ATP-binding cassette (ABC) transporter or major facilitator superfamilies (MFS) are involved. The overexpression of such efflux transporters can confer resistance to a broad range of structurally unrelated toxic molecules, including fungicides. However, resistance levels in the investigated fungal pathogens were low (~20-fold tolerance) (Kretschmer, 2012). Therefore, MDR has minor relevance compared to target-site resistance in practice but could further contribute to resistance 'strength' in addition to target-site resistance. Research on MDR is still at the beginning in plant pathogenic fungi and was described in single field isolates in only few species (Chapeland et al., 1999; Kretschmer et al., 2009; Leroux and Walker, 2011; Omrane et al., 2015).

A third resistance mechanism is the overexpression of target genes. Thereby target-site enzymes of fungicides are expressed in a higher number, which increases the likelihood of fungal natural substrates to interact with enzymes that are not blocked by fungicides (FRAC, 2016). On this account the fungicide efficacy can be reduced. An example is the overexpression of CYP51 in *Z. tritici*, which resulted in a 10-40-fold transcript level leading to a 7-16-fold reduction of DMI sensitivity *in vitro* (Cools and Fraaije, 2013).

The last reported resistance mechanism is based on the detoxification of fungicides by an increased metabolism or enzymatic detoxification by hydrolases, glutathione S-transferases or cytochrome P450 monooxygenases. Examples for metabolic adaptations involved in fungicide resistance are fenhexamid resistance in *B. cinerea* (Leroux et al., 2002) and benzimidazole resistance in *F. graminearum* (Sevastos et al., 2016). Detoxification of fungicides in fungal cells is not well understood so far, but seems to have minor relevance compared to other resistance mechanisms. In contrast, herbicide resistance in weeds is often archived by an increased or altered metabolism (Bryant, 2004; HRAC, 2016).

The risk for the development of fungicide resistance is dependent on different chemical features of the fungicides, the pathogen and the agricultural system (Kuck and Russell, 2006; Brent and Hollomon, 2007). A model to calculate the risk for fungicide resistance development was recently designed by Grimmer et al. (2015). 61 European cases of resistance against single-site-acting fungicides were compared with respect to the number of years from product introduction to the first detection of resistance (FDR time). Different traits that significantly affect the FDR time were identified. These observations showed that the number of latency phases of a pathogen per year, the number of host varieties, effects of the agricultural system (e.g. glasshouse or field) as well as the molecular complexity of fungicides are key factors affecting the time of resistance development (Grimmer et al., 2015). In research, the term resistance is often used when a fungus tolerates higher concentrations of a given fungicide, independent of the level of resistance. However, if

resistance levels are low, or the level of a resistance allele stays low in a population, resistance does not necessarily lead to a control failure of fungicides in the field. To separate these two events, the term 'field resistance' can be useful to describe a scenario, in which the pathogen population has evolved high frequencies of resistance that cause a control failure of fungicides in the field (Brent and Hollomon, 2007). As a consequence of a wider use of systemic single-site acting fungicides, the resistance development started to raise as a practical problem in agriculture. As a result, strategies were discussed to solve this phenomenon (Schwinn, 1982). In 1981, the Fungicide Resistance Action Committee (FRAC) was founded to address these problems. Since then, FRAC has a leading role in determining fungicide resistance management strategies (Highwood, 1990; Marzani, 2011). Guidelines are reviewed in annual FRAC meetings and can be followed on the FRAC webpage (FRAC, 2016). Current recommendations for resistance management in cereals include tatics such as a limited number of applications, the alternation and use of mixtures of different MOA groups, and an optimal timing, in a rather preventative than curative application, of fungicide sprays (van den Bosch et al., 2011, 2014; van den Berg et al., 2013).

1.3.1 Resistance to succinate dehydrogenase inhibitors

First cases of resistance towards SDHIs were described 5-7 years after the market launch of carboxin and other first-generation SDHIs in diseases such as corn smut and chrysanthemum rust (Ben-Yephet et al.,1975; Georgopoulos et al., 1975; Abiko et al., 1977; Leroux and Berthier, 1988). Several studies revealed that resistance is based on single-site mutations in genes encoding the target enzyme of SDHIs (Keon et al., 1991; Skinner et al., 1998; Matsson et al., 1998; Matsson and Hederstedt, 2001; Ito et al., 2004). After the introduction of broad-spectrum SDHIs, SDHI resistance was observed in field isolates of some other plant pathogens, such as *B. cinerea* on various crops (Stammler et al., 2007; Veloukas et al., 2011; Yin et al., 2011), *Corynespora cassiicola* (Miyamoto et al., 2008, 2010), *Sclerotinia sclerotiorum* (Glättli et al., 2009) and *Alternaria alternata* (Avenot and Michailides, 2007; Avenot et al., 2008, 2009). Genetic analysis of the *Sdh* genes in these isolates revealed that mutations which lead to SDHI resistance were found in genes for subunits SDH-B, SDH-C and SDH-D of SDH enzyme. SDH enzymes in different fungal species show different lengths of amino acid sequences. Due to that, homologous (orthologous) amino acids in different species have different numbers within the protein. A prominent position of an amino acid alteration is positioned in the highly conserved subunit SDH-B. Histidine at orthologous positions 277, 272 and 267 in *A. alternata* (Avenot and Michailides, 2007), *B. cinerea* (Stammler et al., 2007; Veloukas et al., 2011) and in laboratory mutants of *Z. tritici* (Skinner et al., 1998; Glättli et al., 2011; Fraaije et al., 2012; Scalliet et al., 2012) was substituted in SDHI resistant isolates. Those positions correspond to histidines at position 257 in *Ustilago maydis* (Keon et al., 1991) and 229 in *Xanthomonas*

campestris pv. *citri* (Li et al., 2006) at which amino acid substitutions influencing SDHIs sensitivity, have been detected in former years. In most cases, substituted amino acids were directly positioned in direct proximity to the Q-site. In some plant pathogens, such as *A. alternata*, substituted amino acids distant to the Q-site have been detected, e.g. C-H133R and D-H134R (Avenot et al., 2009). Histidines at these positions are highly conserved amongst species and confer haem b stabilization between SDH-C and SDH-D subunit (Cecchini, 2003). An up-to-date overview of SDHI resistance in plant pathogens can be found on the FRAC-webpage (FRAC, 2016) and is reviewed in Stammler et al. (2015).

1.3.2 Fungicide resistance in *P. teres* and *Z. tritici*

P. teres is classified as a 'medium-risk' pathogen for the evolvement of fungicide resistance (FRAC, 2016). In recent years, F129L was detected in the European population of *P. teres*, which was shown to mediate 'moderate' levels of QoI resistance (Semar et al., 2007; Sierotzki et al., 2007). In some phytopathogenic species, G143A, which causes high levels of QoI resistance, has not yet been detected, although QoIs have been used significantly against these pathogens in the field. It was shown that different *Puccinia* species (Grasso et al., 2006a), *Alternaria solani* (FRAC, 2016), different *Monilinia* species (Miessner and Stammler, 2010; Luo et al., 2010), *P. teres* (Sierotzki et al., 2007) and others (Stammler, 2012; Stammler et al., 2012) had an intron after the triplet that encodes glycine at position 143. It was proposed that G143A could be lethal in these species, because the substitution could have strong effects on the splicing process leading to a deficient CYTB (Grasso et al., 2006b). As a second mechanism of QoI resistance, G137R was reported in *P. teres* and *A. solani* (Sierotzki et al., 2007; FRAC, 2016). However, F129L and G137R were generally shown to cause lower resistance factors than G143A, and show limited impact on the field efficacy of QoIs even when higher frequencies are observed in the population (Semar et al., 2007). Frequencies of F129L in European populations of *P. teres* were found to be highest in the UK, however, never reaching 100% in the population. Lower frequencies of F129L were observed in countries such as Germany and France (FRAC, 2016).

Most fungicide treatments in Europe target STB as the main foliar disease of wheat. Today, *Z. tritici* is mainly controlled by fungicides of classes SDHIs, DMIs and multi-site fungicides, such as chlorothalonil (Oliver and Hewitt, 2014). *Z. tritici* has a high adaptive potential to environmental changes (Stukenbrock et al., 2011). Formerly, *Z. tritici* was mainly controlled by QoI fungicides, which are classified as high risk fungicides for the development of resistance. The efficacy of QoIs was drastically reduced due to resistance development in *Z. tritici* populations in North-Western Europe and other regions of the world (FRAC, 2016). In *Z. tritici*, QoI resistance is mainly mediated by the amino acid substitution G143A in CYTB (Fraaije et al., 2003, 2005; Torriani et al., 2009). In *Z. tritici*, F129L in CYTB plays a minor role but was detected in a few isolates from Ireland (Lucas and Fraaije, 2008; Kildea et al., 2010). Moreover, fungicides of the DMI class are under threat, mainly in intensive wheat

growing areas, especially where STB control mainly relies on DMIs (Stammler and Semar, 2011; Cools et al., 2013). Populations of *Z. tritici* in these regions showed a gradual 'shift' towards more and more insensitive populations to DMI fungicides. It was shown that the accumulation of successive mutations in the *Cyp51* gene of *Z. tritici* led to these advanced adapted isolates (Leroux et al., 2007; Cools and Fraaije, 2008, 2013).

SDHIs are not yet faced with real practical problems of resistance in these two pathogens (Dubos et al., 2013). In 2012, first SDHI-resistant field isolates were reported in *P. teres* and *Z. tritici* (FRAC, 2016). The emergence of SDHI resistant individuals in the European population of *P. teres* on barley and *Z. tritici* on wheat are topic of the present work. Detected target-site mutations that have been identified were communicated to FRAC after each growing season. The results presented in this work have been partially published (Rehfus et al., 2016, 2017, accepted 2017).

2 Objectives

Fungicide resistance and its management is an important concern in crop protection. Without resistance management, single resistant isolates of pathogens could rapidly propagate under selective conditions and could cause severe efficacy losses in the field within a few years. This happened in many phytopathogenic fungi with methyl benzimidazole carbamates and dicarboximides in the 1980s and the QoI fungicide class in the last years. To implement effective resistance management strategies, the mechanisms underlying resistance and their impact on the control of pathogens is of great importance. The aims of this study were to detect and characterise resistance mechanisms towards SDHI fungicides in two cereal pathogens, namely *Pyrenophora teres* and *Zymoseptoria tritici*, in Europe. The study focused on the following main areas:

1. Detection of SDHI resistance mechanisms in *P. teres* and *Z. tritici*
2. Development of reliable quantification tools to determine resistance frequencies in Europe
3. *In vitro* and *in planta* sensitivity evaluation of resistant isolates
4. Determination of fitness penalties associated with mutations in both pathogens
5. Evaluation of occurrence of multiple resistance in the pathogens
6. Estimation of field performance of SDHIs in the future

3 Material and Methods

3.1 Technical devices

Technical devises used in the present study are listed in Table 1. Technical equipment commonly used in laboratories, such as pipettes and centrifuges, are not listed.

Table 1: Technical devises used.

Technical devise	Origin
Airbrush (nozzle size 0.3 / 0.5 mm), SATAminijet® 3000 B HVLP	SATA GmbH & Co. KG, Kornwestheim, Germany
Analytical balance, MC 410S	Sartorius AG, Göttingen, Germany
Application chamber, SPK011 (applying 400 L ha^{-1})	BASF SE, Ludwigshafen, Germany
Blue light transillumination, UVT-28 L	Herolab GmbH Laborgeräte, Wiesloch, Germany
Cell counting chamber, Thoma bright line	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany
Gel documentation system, EasyDoc plus	Herolab GmbH Laborgeräte, Wiesloch, Germany
Gel electrophoresis, Sub-Cell GT Basic System	Bio-Rad Laboratories Inc., Hercules, US
Homogenisation, Mixer Mill MM200	Retsch GmbH, Haan, Germany
Homogenisation, Grindomix GM200	Retsch GmbH, Haan, Germany
Inoculation station	BASF SE, Ludwigshafen, Germany
KNF Vacuum / pressure pump	KNF Neuberger GmbH, Freiburg, Germany
Microscope, Olympus IX70	Olympus Deutschland GmbH, Hamburg, Germany
Thermal cycler, DNA Engine DYAD	Bio-Rad Laboratories Inc., Hercules, US
Thermal cycler, Mastercycler gradient	Eppendorf AG, Hamburg, Germany
Pyrosequencing preparation, PyroMark Q96 work station	Qiagen, Hilden, Germany
Pyrosequencer, PSQ 96MA	Qiagen, Hilden, Germany
Real-time PCR cycler, Rotor-Gene Q 2-Plex	Qiagen, Hilden, Germany
Spectrophotometer, NanoDrop 2000	Thermo Fisher Scientific Inc., Waltham, US
Sunrise™ absorbance reader	TECAN Group AG, Männedorf, Switzerland
UV light transillumination, UVT-28 ME-HC	Herolab GmbH Laborgeräte, Wiesloch, Germany
Water purification, Q-POD® MilliQ	Merck KGaA, Darmstadt, Germany
XC10 Colour Camera	Olympus Deutschland GmbH, Hamburg, Germany

3.2 Chemicals and consumables

Chemicals and consumables used in the present work are shown Table 2. Standard reaction tubes and pipette tips are not listed.

Table 2: Consumables used.

Consumables	Manufacterer
10,000 x GelGreen nucleic acid stain	Biotium Inc., Hayward, US
6x Orange DNA loading dye	Fermentas GmbH, St. Leon-Rot, Germany
8 cm plant pots	Pöppelmann GmbH und Co. KG, Lohe, Germany
96well microtiter plate	VWR International GmbH, Darmstadt, Germany
96well PCR plate	4titude Ltd., Wotton, UK
Acetone	Bernd Kraft GmbH, Duisburg, Germany
Adhesive PCR seal	4tidude Ltd., Wotton, UK
Ampicillin, sodium salt	AppliChem GmbH, Darmstadt, Germany
Bacto™ Peptone	Becton, Dickinson and Company, Franklin Lakes, US
Combitips plus	Eppendorf AG, Hamburg, Germany
D-(+)-glucose	Sigma Aldrich, St. Louis, US
DEPC-water	Ambion Inc., Austin, US
Difco™ Agar	Becton, Dickinson and Company, Franklin Lakes, US
Difco™ ISP medium 2	Becton, Dickinson and Company, Franklin Lakes, US
Difco™ LB Broth, Miller	Becton, Dickinson and Company, Franklin Lakes, US
Difco™ Peptone	Becton, Dickinson and Company, Franklin Lakes, US
EDTA	Calbiochem, Merck KGaA, Darmstadt, Germany
Ethanol	Sigma Aldrich, St. Louis, US
Gauze	Lohmann & Rauscher GmbH & Co. KG, Neuwied, Germany
Gelatine	VWR International GmbH, Darmstadt, Germany
Glacial acetic acid	Riedel-de Haën, Seelze, Germany
Glycerol	VWR International GmbH, Darmstadt, Germany
Yeast extract	Merck KGaA, Darmstadt, Germany
HPLC water (Chromasolv Plus)	Sigma Aldrich, St. Louis, US
Magnesium chloride	Merck KGaA, Darmstadt, Germany
Magnesium sulphate	Merck KGaA, Darmstadt, Germany
Malt extract	VWR International GmbH, Darmstadt, Germany
Oat flakes (Kölln Schmelzflocken)	Peter Kölln GmbH & Co. KGaA, Elmshorn, Germany
Petri dish (ø 92mm)	Greiner Bio-One International GmbH, Frickenhausen, Germany
Potassium chloride	Merck KGaA, Darmstadt, Germany
Potassium sulphate	Merck KGaA, Darmstadt, Germany
Sodium acetate	Sigma Aldrich, St. Louis, US

Consumables	Manufacterer
Sodium chloride	Sigma Aldrich, St. Louis, US
Sodium hydroxide	Merck KGaA, Darmstadt, Germany
O´GeneRuler, 1 kb DNA ladder	Fermentas GmbH, St. Leon-Rot, Germany
6x Orange DNA loading dye	Fermentas GmbH, St. Leon-Rot, Germany
PyroMark annealing buffer	Qiagen, Hilden, Germany
PyroMark binding buffer	Qiagen, Hilden, Germany
PyroMark Gold Q96 reagents	Qiagen, Hilden, Germany
RNase Away	Molecular Bio-Products Inc., San Diego, US
Streptavidin sepharose high performance	GE Healthcare, Buckinghamshire, UK
Streptomycin sulphate	Sigma Aldrich, St. Louis, US
Tris Base, Molecular biology grade	Merck KGaA, Darmstadt, Germany
Tryptone	Formedium Ltd., Hunstanton, UK
Tween20	Sigma Aldrich, St. Louis, US
Ultra Clear Cap strips	Thermo Fischer Scientific Inc., Waltham, US
Biozym LE agarose	Biozym Biotech Trading GmbH, Wien, Austria

3.3 Enzymes, kits and bacterial strain

Table 3 lists enzymes, kits and bacterial strain used in the present study.

Table 3: Enzymes, kits and bacterial strain used.

Name	Manufacturer
*Bgl*II, FastDigest	Fermentas GmbH, St. Leon-Rot, Germany
CloneJET PCR Cloning Kit	Fermentas GmbH, St. Leon-Rot, Germany
Maxima Hot Start PCR Master Mix (2x)	Fermentas GmbH, St. Leon-Rot, Germany
NucleoSpin DNA Plant 8 II Kit	Macherey-Nagel GmbH & Co. KG, Düren, Germany
NucleoSpin Gel and PCR Clean-up Kit	Macherey-Nagel GmbH & Co. KG, Düren, Germany
NucleoSpin Plasmid Kit	Macherey-Nagel GmbH & Co. KG, Düren, Germany
Phusion Hot Start, High-Fidelity DNA Polymerase Mastermix	Finnzymes OY, Espoo, Finland
Fast Blue qPCR Mastermix (2x)	Eurogentec, Seraing, Belgium
XL-1 Blue Competent Cells	Agilent Technologies, Santa Clara, US

3.4 Buffer und solutions

The buffers and solutions indicated in Table 4 and Table 5 were used in the present work.

Table 4: Solutions and reaction buffers used.

Name	Composition	Notes
10% acetone	10% [v/v] acetone	
TAE buffer (50x stock solution)	2 M TRIS-base 1 M acetic acid 5 mM EDTA	dilution of stock solution to 1x working solution
Tris-HCl buffer (10x stock solution)	0.5 M TRIS-base	pH 8 (with HCl), autoclaved, dilution of stock solution to 1x working solution with sterile water

Table 5: Buffers used for pyrosequencing.

Name	Composition	Notes
70% ethanol	70% [v/v] ethanol	HPLC water used
2 M sodium hydroxide (stock solution)	2 M NaOH	HPLC water used, dilution of stock solution to 0.2 M working solution with HPLC water
10x washing buffer (stock solution)	100 mM TRIS	HPLC water used, pH 7.6 (with acetic acid), dilution of stock solution to 1x working solution with HPLC water

3.5 Growth media

Liquid and agar media used for the cultivation of fungi and bacteria are listed in Table 6.

Table 6: Growth media used.

Media	Composition	Notes
Inoculation medium - *P. teres*	0.25% malt extract (w/v) 0.25% gelatine (w/v)	gelatine dissolved in hot water
Inoculation medium - *Z. tritici*	0.02% Tween20 (v/v)	
ISP2-(strep) agar	3.8% [w/v] ISP medium 2 2% [w/v] agar (0.003 % [w/v] streptomycin sulphate)	autoclaved, pH 7.2, cooled to 60°C before addition of streptomycin (100 mg mL^{-1} stock solution [sterile filtered] to 100 mg L^{-1})
LB-(amp) medium (agar)	2.5% [w/v] LB-broth powder (2% agar) (0.01% [w/v] ampicillin sodium salt)	autoclaved, cooled to 60°C before addition of ampicillin (100 mg mL^{-1} stock solution [sterile filtered] to 100 mg L^{-1})
Malt-(strep) medium (agar)	2% [w/v] malt extract (2% [w/v] agar) (0.003% [w/v] streptomycin sulphate)	autoclaved, cooled to 60°C before addition of streptomycin (100 mg mL^{-1} stock solution [sterile filtered] to 100 mg L^{-1})
Malt medium amended with gycerol	2% [w/v] malt extract 15% [v/v] glycerol	autoclaved
POA agar*	5% [w/v] peanut leaf extract (50 g peanut leaflets in 500 mL of water) 1.5% [w/v] oat flakes 2% [w/v] agar	autoclaved, poured thin (15 mL agar per petri dish with ø 92 mm)
SOC medium	2 % [w/v] tryptone 0.5 % [w/v] yeast extract 10 mM [w/v] NaCl 2.5 mM [w/v] KCl 10 mM [w/v] $MgCl_2$ 10 mM [w/v] $MgSO_4$ 20 mM [w/v] glucose	autoclaved, pH 7.0
YBA medium d.c.	2% (w/v) Bacto™ Peptone 2% (w/v) yeast extract 4% (w/v) sodium acetate	autoclaved
YBG medium d.c.	2% (w/v) Bacto™ Peptone 2% (w/v) yeast extract 4% (w/v) glycerol	autoclaved, pH 6.8

* recipe extracted from Speakman and Pommer (1986)

3.6 Oligonucleotides

The oligonucleotides listed in Table 7 served as primers for standard PCR and Sanger sequencing in this work. Primers and probes, which were used for SNP detection, are indicated in Table 8. Biotinylated primers and probes were obtained from Eurogentec (Seraing, Belgium). All other oligonucleotides were synthesized by an internal DNA laboratory of BASF SE.

Table 7: Oligonucleotides used for standard PCR. Sequences are shown in 5'→3' orientation. Fungal species, target sequences, amplicon sizes and annealing temperatures for Phusion polymerase (T_m) are given. Oligonucleotides for *Cyp51* gene were available from previous work in our laboratory.

Species	Name	Sequence (5'→3')	Target	Size [kb]	T_m [°C]
P. teres	KES 1825 (fw)	CATAACCGAGGAAGCTTGAGTG	*SdhB*	1.2	66
	KES 1837 (rv)	CAAACACAACTCGCAATTAACGC			
	KES 1827 (fw)	ATCACCCAACACCACCATCG	*SdhC*	0.85	69
	KES 1828 (rv)	ATGTTGCAAACTTCAATCGTACCC			
	KES 1833 (fw)	CGATCCTTCAACCCACCTCCGA	*SdhD*	0.75	71
	KES 1834 (rv)	ACCCGCTTATGCATGCCACAG			
Z. tritici	KES 304 (fw)	ATGGCTCTTCGACTCGCG	*SdhB*	1.001	64
	KES 3 (rv)	GTGAAAGCCATGCTCTTCTTG			
	KES 584 (fw)	ATGTTGGCACAGAAGCTCAC	*SdhC*	0.839	69
	KES 550 (rv)	TTACGATTCCATACTTCAGAAAGGC			
	KES 583 (fw)	ATGGCCTCCACCGCCCT	*SdhD*	0.697	62
	KES 2124 (rv)*	CCATCTACAACTTCTGCTCAATC			
	KES 540 (fw)	ATGGGTCTCCTCCAGGAAGTC	*Cyp51*	1.623	66
	KES 541 (rv)	TCAGTTCTTCTCCTCCTTCTCCTC			

* sequence of oligonucleotide KES 2124 is extracted from Fraaije et al. (2012) (Mgsdhdr1)

Table 8: Oligonucleotides and probes used for pyrosequencing and qPCR. Sequences are shown in 5'→3' orientation. Fungal species, target sequence and additional information (notes) is given. Note that two assays are available for C-H152R detection. Assays for detection of G143A in *Z. tritici*, F129L and G137R in *P. teres* were already established in previous studies in our laboratory. Probes used for qPCR had 5' fluorescein (5' FAM) reporter dye and 3' black-hole-quencher (3' BHQ-1).

Species	Name	Sequence (5'→3')	Target	Notes
***P. teres* - SNP detection by pyrosequencing**	KES 1845 (fw)	ACAGGACGCCCTCAACAACAG	partial *SdhB*	
	KES 1846 (rv)	ACTCTCCCTATTTGCCACGTGAT		5' biotinylated
	KES 1847 (fw)	GAGCTTGTACCGATGC	B-H277Y/L/R	sequencing primer
	KES 1848 (fw)	ATCTACAGGCCGCAAATCAC	partial *SdhC*	
	KES 1849 (rv)	CCAAATGCCTCAATCCGTTAAG		5' biotinylated
	KES 1851 (fw)	CGTTCCCCTTCTTCTTT	C-H134R, C-S135R	sequencing primer
	KES 1956 (fw)	TGGCCTCATCGCTCA	C-N75S	sequencing primer
	KES 2025 (fw)	CTTAGACTTGCAGCAACTG	partial *SdhC* (C-G79R)	5' biotinylated
	KES 2026 (rv)	ATACCGAAGAGGTAGAGAGAACCG		
	KES 2027 (rv)	CGGAGAGAACGATAC	C-G79R	sequencing primer
	KES 2020 (fw)	TTTCCGCTGGTCTCATTCC	partial *SdhD*	
	KES 2021 (rv)	AGAGTGCAAGACCGAGAACAAC		5' biotinylated
	KES 2028 (fw)	CTGAACCCTGTAACC	D-D124N/E	sequencing primer
	KES 2023 (fw)	CGCTCTTCTGGTAGTC	D-H134R	sequencing primer
	KES 2024 (fw)	AGATCATGCATCGTCG	D-D145G	sequencing primer
	KES 2080 (fw)	AACAACCCCCAGATCATGC	partial *SdhD* (D-E178K)	5' biotinylated
	KES 2081 (rv)	CCTAGCAACAGCCTCGGTAATAC		
	KES 2082 (rv)	ATACCAACATCATTCGT	D-E178K	sequencing primer
	KES 432 (fw)	TCCTAACTTAAAAGGTTACACAAGGCTT	partial Cyt *b* (F129L)	
	KES 433 (rv)	AACCATTTTGGGCTATGTTGGTA		5' biotinylated
	KES 434 (fw)	CGGAACTTAGACAGCC	F129L	sequencing primer

Species	Name	Sequence (5'→3')	Target	Notes
	KES 630 (fw)	GGCTGAAATGCTGCTTAATGT	partial Cyt *b* (G137R)	5' biotinylated
	KES 631 (rv)	AATTTTCACCTCAAAGGCTCATT		
	KES 632 (rv)	CAAAGGCTCATTTGC	G137R	sequencing primer
***Z. tritici* - SNP detection by pyrosequencing**	KES 602 (fw)	AGTACCTCGGACCAGCTGTCCT	partial *SdhB*	
	KES 603 (rv)	CCGCTTTCCAATCATCTCGTTC		5' biotinylated
	KES 604 (fw)	GAGCTTGTACCGATGC	B-H267Y/R/L, B-T268I, B-I269V	sequencing primer
	KES 655 (fw)	ACCGCAAATAACCTGGTACCTCT	partial *SdhC*	5' biotinylated
	KES 656 (rv)	AATCATACTCGCCGTATCCCAAA		
	KES 657 (rv)	CCGTATCCCAAACCA	C-H152R	sequencing primer
	KES 770 (rv)	CTCCCGAGGCGGCGAC	C-N86K/S, C-G90R	sequencing primer
	KES 2138 (fw)	CCGGTGACGTTTCATTCGTT	partial *SdhC* (C-H152R)	
	KES 2139 (rv)	ACTCGCAACACTCAACCCCACAA		5' biotinylated
	KES 2140 (fw)	GTTGAATGGAGTGAGG	C-H152R	sequencing primer
***Z. tritici* – SNP detection by qPCR**	KES 1841 (rv)	AATCGTCTTGGTCAAGACCTGCAA	partial Cyt *b* (C-T79N)	
	KES 1839 (fw)	CACCTCGCAATCTACAAACCGCAAATATC		MAMA primer (T79)
	KES 1840 (fw)	CACCTCGCAATCTACAAACCGCAAATAGA		MAMA primer (N79)
	St-sdhc-79	AGCCTTCTACGCCTTCGG		probe (5' FAM and 3' BHQ-1)
	KES 64 (rv)	CCCTAGAACATTAACATGAACAATCG	partial Cyt *b* (G143A)	
	KES 132 (fw)	CAATAAGTTAGTTATAACTGTTGCCC		MAMA primer (G143)
	KES 73 (fw)	GCACTCAATAAGTTAGTTATAACTGTTGCAG		MAMA primer (A143)
	St-cytb-143	CCCTAAGAATGCGGTTGCCATCATCA		probe (5' FAM and 3' BHQ-1)

3.7 Software

The software used in this work is given in Table 9.

Table 9: Software used.

Name	Provider
BLAST	BASF SE, Ludwigshafen, Germany
FastPCR	PrimerDigital Ltd, Helsinki, Finland
LaserGene™ 12	DNASTAR Inc., Madison, US
Magellan™	TECAN Group AG, Männedorf, Switzerland
MegAlign™ Pro	DNASTAR Inc., Madison, US
Pyrosequencing Assay Design (version 1.0.6)	Qiagen, Hilden, Germany
RESLAB	BASF SE, Ludwigshafen, Germany
Rotor-Gene Q Series (version 2.0.2)	Qiagen, Hilden, Germany
R studio (version 0.98.1091)	RStudio, Inc.
SeqMan™ Pro	DNASTAR Inc., Madison, US
The PyMOL Molecular Graphics System (version 1.8)	Schrödinger, LLC.

3.8 Cultivation of living organisms

3.8.1 Cultivation of wheat and barley plants

Fungicide sensitivity tests and growth competition studies of fungal isolates were carried out in a glasshouse. Barley cultivar ‘Astrid’ served as host plant for experiments performed with *P. teres*. Wheat cultivar ‘Riband’ was used as host for glasshouse trials with *Z. tritici*. Both cereal cultivars (around 10 plants per pot) were grown in the glasshouse on Universal perlite soil (BASF SE) with 16 h light and at 20°C until growth stage BBCH 11 before inoculation.

3.8.2 Cultivation of fungal isolates

P. teres was cultivated on 2% malt-strep agar for mycelial growth and on thin poured POA agar for sporulation. Isolates were cultivated for 10 days at 22°C in the dark to allow mycelial growth and for spore production with a 12 h dark and 12 h light cycle (soft-white light tubes supplemented with near ultra-violet light). It is important to note that POA plates should not be sealed with parafilm. *Z. tritici* was cultivated on ISP2-strep agar for 7 days at 18°C and with a 12 h dark and 12 h light cycle. Isolates were directly used for sensitivity assays, growth competition tests, DNA preparation and further downstream procedures, or were stored at -80°C in 2% (w/v) malt medium with 15% (v/v) glycerol.

3.9 Fungal isolates and leaf samples

Fungal isolates and leaf samples from all over Europe were either obtained from 'Random Monitoring' performed by the company EpiLogic (Freising-Weihenstephan, Germany) or by internal monitoring programmes on commercial or trial sites of BASF SE and other institutions.

3.9.1 'Random monitoring' and 'Trial site monitoring'

'Random Monitoring' in European countries was carried out by the company EpiLogic. Samples were taken in all major barley and wheat growing areas in Europe in order to get an overview of the pathogen population in European countries.

Air-borne *P. teres* spores were collected by a spore trap mounted on the roof of a car (Figure 6). Most isolates of *P. teres* used in this study were obtained from BASF European pathogen monitoring programmes in the years 2012-2015. In total, 175 isolates were collected in 2012, 248 isolates in 2013, 245 isolates in 2014 and 253 isolates in 2015. Collection of isolates was done in June and July of each year. Thus, collection was started in southern countries (e.g. Italy) and was ended in northern countries (e.g. Denmark, Sweden) at the end of July. One to 15 single-spore isolates of *P. teres* were obtained from each route and were pre-analysed for SDHI resistance in an *ex vivo* test, based on detached leaves which had been treated with discriminating test concentrations of 0, 0.64 and 2.5 mg L^{-1} fluxapyroxad (technical a.i.). Isolates which showed ≥40% of necrotic leaf area at 0.64 mg L^{-1} fluxapyroxad in these detached leaf tests were further analysed in the present study. Analysed isolates are listed in Supplementary Table 37.

'Random monitoring' in Europe was additionally performed for the pathogen *Z. tritici*, however, with a different isolate collection method. During the growing season, pycnidiospores act as the main source of inoculum of new wheat plants. Pycnidiospores of *Z. tritici* are rarely detected in the air. Therefore, sampling of *Z. tritici* isolates was realised by collecting STB-infected leaves from all major wheat growing regions. Sampling was organised by BASF including shipment to EpiLogic. Subsequently, isolates were generated and tested for their sensitivity to fluxapyroxad in a microtiter test. In total, 484 isolates were collected in 2012, 456 isolates in 2013, 690 isolates in 2014, 630 isolates in 2015 and 504 isolates in 2016. Isolates that showed EC_{50} values of >0.3 mg fluxapyroxad L^{-1} in this test were further analysed in the present study. Origin and sampling date of *Z. tritici* isolates are given in Supplementary Table 38.

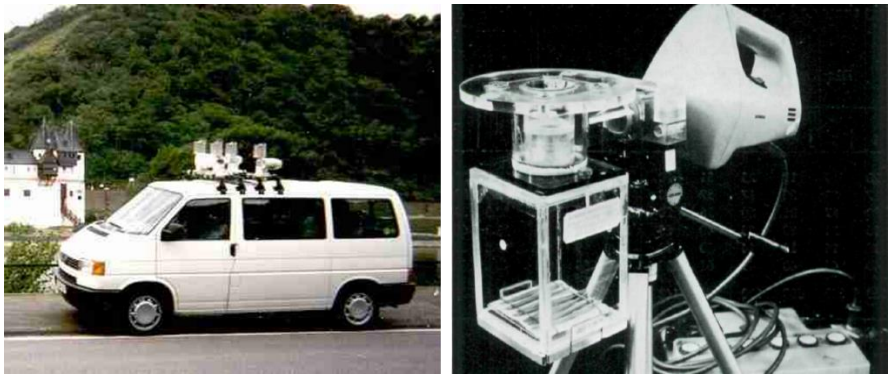


Figure 6: Spore jet trap mounted on a car to sample air-borne spores of *P. teres*. Barley leaves in the collection chamber were used to trap spores (http://www.epilogic.de/).

In addition to 'Random monitoring', isolates collected from trial sites were included in the present study. 'Trial site monitoring' programmes comprised 337 isolates of *P. teres* from 2013 collected in France and Germany. In the case of *Z. tritici*, isolates were obtained from different trial sites in Europe. These included 100 isolates from 2012 collected in France, 487 isolates from 2013 collected in Germany, and 25 isolates from 2015 collected in Ireland.

Experiments that were conducted on multiple resistance of both pathogens, QoI 'Random monitoring' (*P. teres*) and DMI 'Random monitoring' (*Z. tritici*) made by EpiLogic, were additionally considered.

3.9.2 'Field monitoring'

Net blotch- or STB-infected leaves, that were sampled by BASF SE field technicians, farmers or governmental institutions and universities, were additionally studied. Collectors sent 20-30 dried barley or wheat leaves per sample (field). These had been randomly collected from the fields, which were either used as a trial site or are commercial fields. Samples, which were taken from trial sites, were mainly taken from untreated plots. Most samples were collected in April and May. The number, origin and sampling date of these leaf samples are given in Supplementary Table 39 and Table 40.

The leaf samples were mainly taken to extract DNA to quantify allele frequency, which are leading to SDHI resistant phenotypes, by pyrosequencing or qPCR (see section 3.10.3). Therefore, in the case of net-blotch infected barley, 20 small pieces of lesions (~5 mm^2) were excised from different leaves obtained from one sample. Scissors and pinzettes were placed in 100% EtOH and were flamed after each sample. Gloves were removed, whenever contact with infected leaf material could not be avoided. These pieces of lesions were pooled in an Eppi for each sample and DNA extraction followed.

In the case of *Z. tritici*, all wheat leaves were grinded together in a homogeniser (Grindomix GM200) for 3 min, 10,000 rpm. The equipment, that was used for the homogenisation, was thoroughly cleaned after each sample. The remaining sample material was removed by washing in water with soap. The surface was additionally cleaned by an incubation of 5 min

in RNase Away, which also removes DNA contaminations. Afterwards, equipment was again cleaned in water and was dried. This method is more time-consuming than cutting lesions but is useful in that it considers all lesions from all leaves that were taken from one site. However, when lower infection densities on leaves (<10%) were observed, green leaf areas were first removed (roughly) to obtain higher fungal DNA ratio in the extract.

3.9.2.1 Generation of *P. teres* and *Z. tritici* from infected leaves

Some infected leaf samples were additionally used to generate isolates of *P. teres* and *Z. tritici*. Isolation protocols were already established in our laboratory. In case of *P. teres*, this was mainly done to obtain spot-type isolates. For this, two filter papers were placed in a petri dish. The upper filter paper was prepared before by using a scalpel to cut small openings in it (in two parallel rows). Here, the ends of dried leaf segments with typical net-blotch lesions (3-5 segments per petri dish) were inserted. The filter papers were moistened with sterile water. It is important to note that leaf segments should not have direct contact to water. The petri dishes were then incubated for 3-4 days at 18-22°C with a 12 h dark and 12 h light cycle (soft-white light tubes supplemented with near ultra-violet light). After that, single spores of typical *P. teres* conidiophores were transferred under the binocular to 2% malt-strep agar plates. These were incubated for a further 5-10 days at 24°C in the dark.

Z. tritici strains were isolated from infected leaf samples from Ireland and the United Kingdom in 2016. The dried leaves were surface sterilised in 2.5% sodium hypochlorite solution for 60 s and washed twice in sterile water. After leaves had dried, lesions were excised and transferred to 2% malt-strep agar. Petri dishes were incubated for 1-2 days at 22°C until pycnidia start to release spores. Spores produced by a single pycnidium were regarded as an isolate and were transferred to ISP2-strep plates. Petri dishes were incubated for 7 days at 18°C and 12 h of light.

3.10 Molecular biological methods

3.10.1 Standard molecular techniques

Standard molecular techniques, such as DNA extraction, PCR and gel electrophoresis, were performed according to manufacturers' or established protocols.

The extraction of genomic DNA was done using Nucleo Spin® Plant kits (single column isolation or 48-well scale vacuum processing). Homogenisation of bacterial or fungal material was performed by freezing 15 to 30 mg cells, conidia or mycelia on dry ice and consequent grinding for 1 min at 20 Hz (Retsch) and the addition of a metal bead. For DNA isolation, the manufacturers' protocol using PL1 lysis buffer for the extraction from plant material was used. DNA was stored at 4°C for short time storage and at -20°C for long time

storage. Concentration and purity of DNA was determined using photometric measurement by Nanodrop2000. PCR was performed to amplify target DNA sequences. Pathogen specific oligonucleotides are listed in Table 7. Reactions were preapared as shown in Table 10. To avoid contaminations, all reactions were prepared in a clean bench. A NTC with DEPC-water instead of template DNA served as control.

Table 10: Preparation of PCR reactions.

Component	Volume [µL] in a final volume of 25 µL
2 x Mastermix (Maxima/Phusion Flash)	12.50
DEPC-H_2O	7.50
Primer fw (10 pmol μL^{-1})	1.25
Primer rv (10 pmol μL^{-1})	1.25
Template DNA	2.5

Phusion® High-Fidelity DNA polymerase (Thermo Fisher Scientific) was used for the cloning and sequencing of target genes. Phusion DNA polymerase possesses 5'→3' polymerase activity, 3'→5' exonuclease activity (proofreading) and generates blunt-ends. Appropriate annealing temperatures were determined by gradient PCR and can be found in Table 7. To reduce costs for large-scale pyrosequencing procedure, Maxima Hot Start *Taq* Polymerase (Thermo Fisher Scientific) was taken to amplify target DNA sequences. Another advantage of Maxima DNA polymerase is the inactivity at room temperature, which helps to avoid extension of unspecific annealed primers or primer dimers, and allows preparation of large PCR setups at RT. Temperature programmes used for PCR reactions are given in Table 11.

Table 11: PCR programme to amplify target DNA sequences. Maxima Hot Start *Taq* polymerase (Phusion High Fidelity Polymerase) were used for amplification. Annealing temperature is dependent on primer pair and polymerase used.

Function	Temperature [°C]	Time [min]	Cycles
Initial denaturation	95 (98)	4:00 (0:30)	1
Denaturation	95 (98)	0:15 (0:10)	35-39
Annealing	45-72	0:30 (0:05)	
Elongation	72	1:00 per kb (0:15-0:30 per kb)	
Final elongation	72	5:00	1
Cooling	4	∞	

In a next step, amplified PCR fragments were either used for sequencing of genes, SNP detection by pyrosequencing (see section 3.10.3) or studies on promotor insertions of *Mg*MFS1 (see section 3.13). DNA fragments were separated in a 1% TAE agarose gel either using ethidium bromide and UV-light or GelGreen and a blue light table (430-490 nm) to visualise DNA fragments. Ethidium bromide was preferred whenever the exact size of DNA fragments was important for example in the process of assay development. The result

of gel electrophoresis was documented with EasyDoc plus gel documentation system. For further Sanger sequencing, appropriate PCR products were excised from the gel and cleaned-up using NucleoSpin Gel and PCR Clean-up kit. Sequencing was performed with oligonucleotides used for the corresponding PCR reaction. In a few cases, the amplification led to unspecific byproducts during PCR e.g. *Cyp51* gene of some isolates. To obtain specific sequencing, the PCR product of appropriate size was excised from the gel and was cleaned-up and cloned prior to sequencing. For blunt-end cloning, CloneJET PCR Cloning Kit was used. PCR products were ligated into pJet1.2/blunt cloning vector and vectors were transformed in *Escherichia coli* XL1-Blue competent cells. SOC media was used for the recovery of cells after transformation and LB-amp agar for selection. Clones were picked and transferred to LB-amp media for further propagation. Plasmide DNA was isolated using NucleoSpin Plasmid Kit. For the verification of correct insert length, plasmids were digested using *Bgl*II restriction enzyme (Table 12) and separated by gel electrophoresis. Sequencing of the plasmids which contained the sequence of interest, was performed with oligonucleotides pJet1.2 fw and pJet1.2 rv.

Table 12: Preparation of *Bgl*II restriction reactions.

Component	Volume [µL] in a final volume of 20 µL
Plasmid DNA	3
FastDigest® buffer	2
*Bgl*II FastDigest®	1
DEPC water	14

3.10.2 Sequencing

Sequencing of PCR products and plasmids were done internally by BASF SE DNA laboratory. Resulting sequences were analysed using DNASTAR lasergene programmes (DNASTAR, Madison, USA). SeqMan programme was used to obtain contigs of two reads (fw and rv primer), whereas MegAlign programme was used to compare nucleotide and protein sequences between samples or organisms. Sequences were additionally checked using BLAST (internal BLAST of BASF SE) to verify correctness of origin.

3.10.3 SNP detection systems

In the present study, genetic polymorphisms causing a reduction of fungicide efficacy were either detected by quantitative PCR (qPCR) or by pyrosequencing. Most mutations were detected using pyrosequencing, as it allows a high through-put but also the detection of more than one mutation in one assay. In some cases, no matching pyrosequencing primer could be designed, e.g. due to repetitive nucleotides at positions before or after mutations. Here, qPCR assays based on TaqMan probes were developed.

3.10.3.1 Quantitative real-time PCR (qPCR)

Quantitative PCR enables the real-time measurements of DNA through PCR amplification. Detection and quantification of mutated DNA during this work was based on TaqMAMA genotyping assays (Glaab and Skopek, 1999). TaqMAMA combines quantitative PCR using a TaqMan probe with modified allele-specific PCR primer (MAMA primer). TaqMan® probes, originally designed by Roche Molecular Systems Inc. (Pleasanton, US), are dual labeled hydrolysis probes with a reporter fluorophore at the 5' end and a quencher fluorophore at the 3' end. The fluorogenic probe (~20 bp) is complementary to the target sequence and anneals specifically between the two PCR primers. TaqMan probe principle utilizes the 5'→3' exonuclease activity of *Taq* polymerase, which leads to hydrolysation of the probe while elongation. Thereby the reporter dye is released from the close vicinity of the quencher dye which leads to an increase of fluorescence. The increase of fluorescence is proportional to amplified PCR product.

To guarantee discrimination of SNP allelic polymorphisms, MAMA primer were used in combination to TaqMan assay in the present study. A MAMA primer is either specific for the investigated SNP or the wild type sequence. Besides the nucleotide exchange that is investigated, a second nucleotide exchange in the MAMA primer is leading to a mismatch in both sequences, which can significantly improve discrimination between alleles (Cha et al., 1992). MAMA primer nucleotide exchanges were selected according to Li et al. (2004) to obtain greatest allelic discrimination and these are shown in Table 8. The relative amounts of allelic SNP variants in a sample can be quantified by equal aliquots of the pooled DNA measured in two separate PCR reactions, and which contain a specific primer pair to one or the other allelic SNP variant. If efficiency of both PCR reactions is similar, a 50% mixture of both alleles should reach a detectable level of fluorescence at the same cycle for the two amplifications. For mixtures of unequal ratios of the two alleles, the difference in cycle number between both reactions can serve for the calculation of relative allele amounts (Germer et al., 2000). In the present study, allele-specific discrimination was performed for the molecular detection and quantification of G143A in CYTB and C-T79N in SDH-C of *Z. tritici*. Components and preparation of qPCR reactions are described in Table 13. As TaqMan probes are sensitive to light, light sources were avoided as far as possible for the preparation of qPCR reactions.

Table 13: Preparation of qPCR reaction (TaqMAMA).

Component	Volume [µL] in a final volume of 25 µL
Fast Blue qPCR Mastermix (2x)	12.5
DEPC-water	7.25
Primer fw (10 pmol μL^{-1})	1.25
Primer rv (10 pmol μL^{-1})	1.25
TaqMan probe (10 pmol μL^{-1})	0.25
Template DNA	2.5

The two-step temperature programme used for qPCR is shown in Table 14. All qPCR reactions were run on Rotor-Gene-Q maschine.

Table 14: Programme used for qPCR reactions.

Function	Temperatur [°C]	Time [min]	Cycle
Initial denaturation	95	5:00	1
Denaturation	95	0:10	40
Annealing & Elongation	60	0:45	

The measurement of the fluorescence was done at the end of elongation after each cycle. Cq (cycle of quantification) values were obtained by Rotor-Gene Q Series Software. The cq value defines the cycle at which measured fluorescence significantly exceeds background fluorescence and is used for the calculation of allele frequencies (sensitive and resistant). The frequency of sensitive to resistant allele was calculated according to Germer et al. (2000):

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

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

For validation of assays, DNA of a wild type isolate and DNA of a mutated isolate, mixtures of them (95/5, 90/10, 70/30, 50/50, 30/70, 10/90 and 5/95%) and 1:10 dilution series of both DNA samples were tested. Quantification of non-mutated/mutated DNA was reliable above a value of 2-3% and accuracy of the assays was 1%, which means values can vary ± 1%. The specificity of primer pairs was tested by performing qPCR reactions on non-target DNA isolated from pathogens ubiquitious in nature e.g. *B. cinerea* and *A. alternata* but also from cereal pathogens infecting same hosts e.g. *Pyrenophora tritici-repentis*, *R. secalis*, several rust species and *B. graminis*. In each run, a NTC, a DNA sample containing 100% sensitive allele and a DNA sample containing 100% resistant allele and a 50% mixture of both DNA samples served as controls.

3.10.3.2 Pyrosequencing

Pyrosequencing is a DNA sequencing methodology based on the principle of sequencing-by-synthesis. It allows real-time sequencing of 20 to 30 basepairs and thereby can be used for mutation detection and quantification. The technique uses a cascade of enzymatic reactions that starts with nucleotide incorporation and ends in a detectable light signal (bioluminescence).

Four enzymes are needed in a pyrosequencing reaction. The Klenow fragment of DNA polymerase I, ATP sulfurylase, luciferase and apyrase (Figure 7). The reaction mixture also contains the enzyme substrates adenosine phosphosulfate (APS) and D-luciferin and the sequencing template with an annealed primer. The four nucleotides are added one after each other to the reaction mixture. If the added nucleotide is incorporated by DNA

polymerase, pyrophosphate (PPi) is released which itself is substrate for ATP sulfurylase producing ATP. ATP is converted by luciferase mediated turnover from D-luciferin to oxyluciferin and thus to light. A CCD camera then detects light emission. A fourth enzyme, apyrase, removes unincorporated nucleotides and ATP before addition of the next base (Ahmadian et al., 2000, 2006).

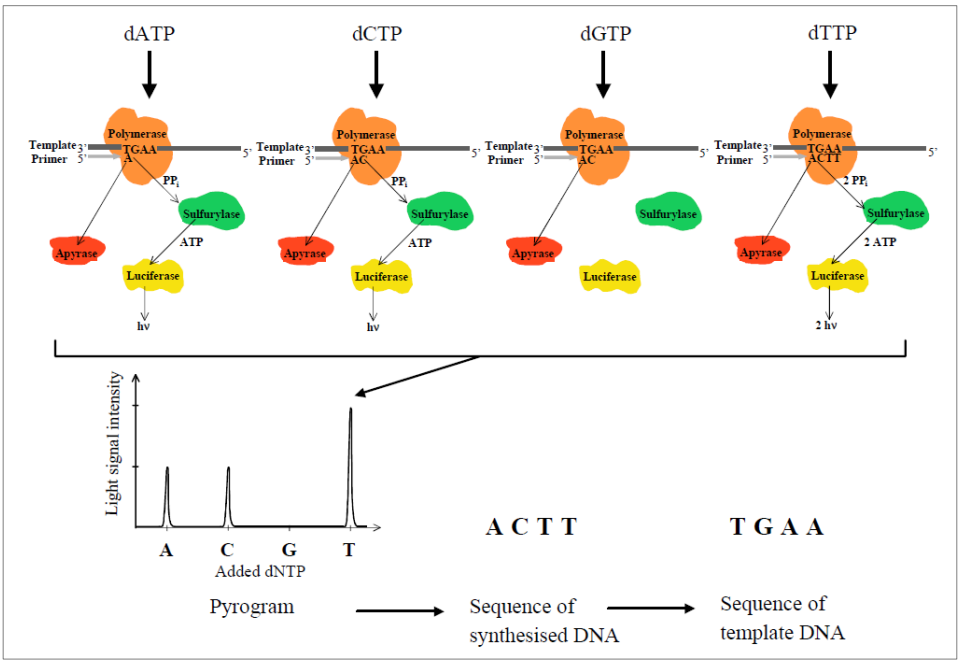


Figure 7: Schematic procedure of pyrosequencing. The figure is extracted from Ahmadian et al. (2006). If the added dNTP is complementary to the template and is therefore incorporated by Klenow Polymerase into the DNA strand, a pyrophosphate (PPi) is released. PPi is converted by ATP Sulfurylase into ATP. ATP serves as substrate for Luciferase enzyme emitting detectable light.

In the present work, pyrosequencing was not only performed to detect point mutations in *P. teres* and *Z. tritici* but was also used for quantification of mutations in a DNA pool. To allow quantitative point mutations analysis, pyrosequencing assays were developed using the Pyrosequencing Assay Design Software. Standard PCR, using Maxima Hot Start PCR Master Mix and the primer pairs with biotinylated oligonucleotides (listed in Table 8), were employed for amplification of all gene fragments (~200 bp). The following conditions were applied for all pyrosequencing assays: initial heating at 95°C for 15 s, 40 cycles at 94°C for 15 s, 55°C for 30 s and 72°C for 20 s, followed by a final elongation step at 72°C for 5 min. Every template was applied in duplicate.

Table 15: Temperature programme used for PCR reaction before pyrosequencing.

Function	Temperatur [°C]	Time [min]	Cycle
Initial denaturation	95	1:00	1
Denaturation	94	0:15	40
Annealing	55	0:30	
Elongation	72	0:20	
Final elongation	72	5:00	1

After the immobilisation of PCR products to streptavidin sepharose beads implementing the Vacuum Prep Worktable and a clean-up step with ethanol (70%), single strand preparation was performed using sodium hydroxide (0.2 M) followed by a washing step in tris-acetate (10 mM). Single stranded samples were transferred to annealing buffer amended with the according sequencing primer (listed in Table 8) and were heated at 80°C for 2 min in an incubator. After cooling, the samples were then pyrosequenced using PyroMark Gold Q96 Reagents on a PSQ 96MA machine, as described by the manufacturers.

Table 16: Preparation of DNA template for pyrosequencing reaction. A: Immobilisation of template DNA to streptavidin sepharose beads; **B:** Reaction solution for pyrosequencing.

	Component	Volume [µL]	Notes
A	PCR reaction (amplified template DNA)	25	incubation for 15-20 min at RT (1200 rpm)
	Binding buffer	37	
	Streptavidin Sepharose	3	
B	Annealing buffer	38.75	addition of bound template DNA
	Sequencing primer	1.25	

For validation of assays, DNA of a wild type isolate and DNA of a mutated isolate as well as mixtures of them (95/5, 90/10, 70/30, 50/50, 30/70, 10/90 and 5/95%) were tested. Quantification of non-mutated/mutated DNA was reliable above a value of 10% and accuracy of all assays was 5%, which means values can vary ± 5%. In addition, specificity of primer was checked by performing pyrosequencing on non-target DNA isolated from pathogens ubiquitious in nature e.g. *B. cinerea* and *A. alternata* but also from cereal pathogens infecting same hosts e.g. *P. tritici-repentis*, *R. secalis*, several rust species and *B. graminis*.

3.11 Fungicide sensitivity tests

The effect of different mutations in the *Sdh* genes on various SDHIs was studied in the present study. *In vitro* sensitivity tests were performed in a microtiter scale to test wild type and *Sdh* mutated isolates in high sample numbers. In addition, *in planta* studies were performed on barley and wheat seedlings in the glasshouse.

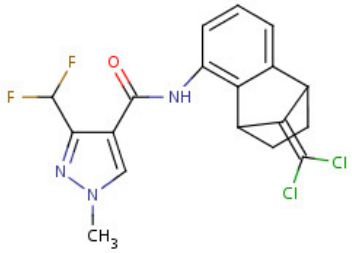
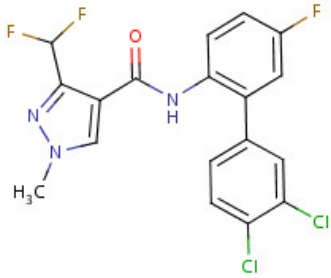
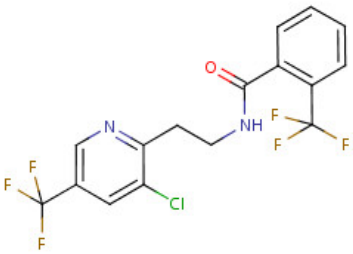
3.11.1 Fungicides used

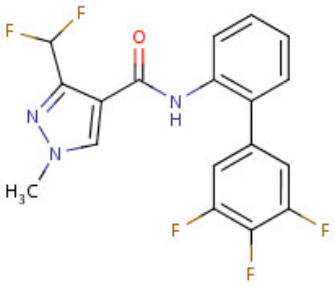
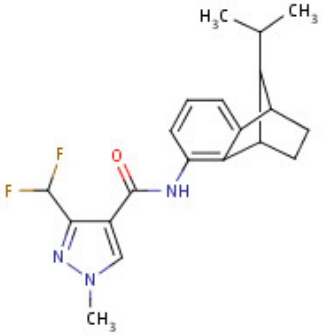
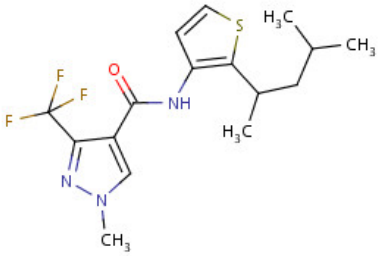
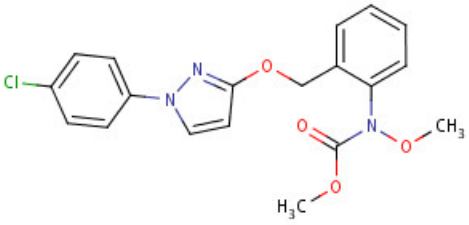
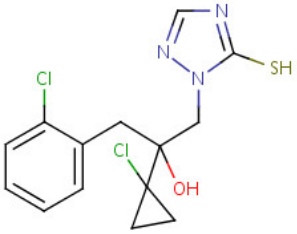
The tested compounds (Table 17) are foliar applied SDHI fungicides that are already or will soon be available on the European market for the control of *P. teres* in barley and *Z. tritici* in wheat. In *in vitro* tests, fungicides were mainly used as technical active ingredients obtained from Sigma Aldrich, an exception was benzovindiflupyr, which was not available and thus synthesized by BASF SE. In glasshouse tests, either ready-formulated products

(solo-SDHI compounds) were used (when commercially available) or technical active ingredients were formulated and then applied to plants (see section 3.11.3.2).

SDHIs have a low water solubility (0.49 mg L^{-1} bixafen, 16 mg L^{-1} fluopyram, 3.44 mg L^{-1} fluxapyroxad, 0.55 mg L^{-1} isopyrazam and 1.38 mg L^{-1} penthiopyrad at 20°C, http://sitem.herts.ac.uk/aeru/ppdb/en/). Therefore, inappropriate handling during preparation of fungicide concentrations can lead to unintentional precipitation of SDHIs. Stock solutions of SDHIs (10,000 mg L^{-1}) were prepared in dimethyl sulphoxide (DMSO). Further dilutions were made in (sterile) water. 100 mg L^{-1} solutions of most SDHIs (such as bixafen, fluxapyroxad, fluopyram and penthiopyrad) are vulnerable to precipitation. Therefore, further dilutions should be prepared rapidly (some SDHIs precipitate after a while) but mixing should occur carefully without shaking or vortexing.

Table 17: Fungicides used.

Fungicide class	**Technical a.i. (example for trade mark)**	**Company (launch date in Europe)**	**Structural formula**
SDHI	Benzovindiflupyr (no solo product available)	Syngenta Agro GmbH (expected 2017)	
SDHI	Bixafen (no solo product available)	Bayer Crop Science (2013)	
SDHI	Fluopyram (Luna Privilege®)	Bayer Crop Science (2016)	

Fungicide class	Technical a.i. (example for trade mark)	Company (launch date in Europe)	Structural formula
SDHI	Fluxapyroxad (Imbrex®)	BASF SE (2013)	
SDHI	Isopyrazam (Zulu®)	Syngenta Agro GmbH (2013)	
SDHI	Penthiopyrad (Fontelis®/Intellis®)	DuPont (2013)	
QoI	Pyraclostrobin (Comet®)	BASF SE (2002)	
DMI	Prothioconazole (Proline®)	Bayer Crop Science (2004)	

3.11.2 Microtiter tests

In vitro tests with fungicides were performed in 96-well microtiter plates according to monitoring methods described on the web page of FRAC (FRAC, 2016) with some minor changes. Pure technical grades of bixafen, fluopyram, fluxapyroxad, isopyrazam, penthiopyrad and benzovindiflupyr were dissolved in DMSO to prepare 10,000 mg L^{-1} stock solutions. In addition to SDHIs, pyraclostrobin (as the formulated product Comet®), prothioconazole and tolnaftate (both as technical a.i. obtained from Sigma Aldrich) were measured in some microtiter tests. Dilution of fungicides was conducted in sterile deionized water immediately before mixing with spore suspensions. Fungicide dilutions were prepared double-concentrated in 48-deep-well plates and were mixed in a ratio of 1:1 with spore suspensions in the 96-well plates. The following final concentrations of fungicide were used in microtiter tests: 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 and 30 mg a.i. L^{-1}. Seven appropriate fungicide dilutions and a water control were chosen for each fungicide used and experiment.

Table 18: Preparation of fungicide dilution series.

Dilution (concentration in fungicide dilution [mg L^{-1}])	**Final concentration in microtiter plate [mg L^{-1}]**
9.8 mL water + 0.2 ml 10,000 mg L^{-1} solution (200)	(100)
7.0 mL water + 3.0 ml 200 mg L^{-1} solution (60)	30
9.0 mL water + 1.0 ml 200 mg L^{-1} solution (20)	10
9.0 mL water + 1.0 ml 60 mg L^{-1} solution (6)	3
9.0 mL water + 1.0 ml 20 mg L^{-1} solution (2)	1
9.0 mL water + 1.0 ml 6 mg L^{-1} solution (0.6)	0.3
9.0 mL water + 1.0 ml 2 mg L^{-1} solution (0.2)	0.1
9.0 mL water + 1.0 ml 0.6 mg L^{-1} solution (0.06)	0.03
9.0 mL water + 1.0 ml 0.2 mg L^{-1} solution (0.02)	0.01
9.0 mL water + 1.0 ml 0.6 mg L^{-1} solution (0.006)	0.003
10.0 mL water	0

P. teres isolates were grown for 10 days and *Z. tritici* isolates for 7 days prior to microtiter test. Spore suspensions of *P. teres* were obtained by harvesting spores from POA plates using a Drigalski spatula and 4 mL of YBA d.c. medium. Compared to many other fungi, *P. teres* generally produces a relatively low number of spores on artificial media. 1-3 POA plates of each isolate (dependent on the spore production of the isolate) were grown to obtain enough spores for microtiter test with many different fungicides. The obtained spore suspension was filtered through sterile 4-fold gauze to remove mycelium. In contrast, *Z. tritici* produces a high number of spores on artificial media. Spores were harvested by a sterile cotton swab from ISP2-strep plates and were transferred to 2-4 mL of YBG d.c.

medium. Spores were counted microscopically using a haemocytometer (Thoma cell chamber) and adjusted to 4E+03 cells mL^{-1} for *P. teres* and 1.6E+04 cells mL^{-1} for *Z. tritici* with the appropriate media. Different treatments with spores and without spores (blank) were tested in four replicates in 96-well microtiter plate by mixing 50 µL of fungicide dilutions with 50 µL spore suspension or media without spores. The set-up of a microtiter plate can be seen in Figure 8. To allow growth of *P. teres* and *Z. tritici* isolates in microtiter plates, these were incubated at 18°C in darkness. The growth was measured in a photometer (96-well reader) at 405 nm 5 days after set-up in the case of *P. teres* and 7 days after set-up in the case of *Z. tritici*. The values were corrected by comparison with the blanks. The EC_{50}-values (concentration with a fungal growth inhibition of 50% relative to the untreated control) were determined by probit-analysis and mean value of technical replicates was calculated using log-values. Reslab-Software (BASF SE software using SAS calculation) was used to calculate EC_{50} values.

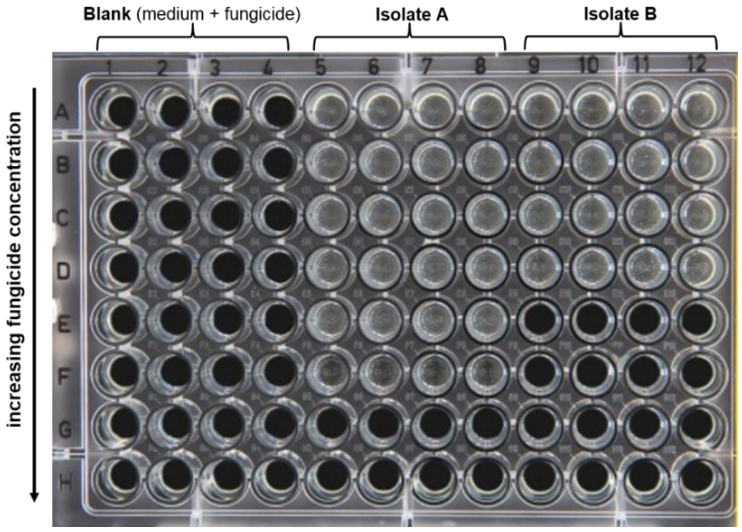


Figure 8: Experimental set-up of a microtiter plate assay. No fungicide is applied in row A. Fungicide concentration is increasing in rows B-H. Columns 1-4 are not inoculated with fungal spores. Columns 5-12 are inoculated with the same number of spores each well.

3.11.3 Sensitivity tests in the glasshouse

The impact of SDH alterations on SDHIs was investigated in glasshouse studies. The sensitivity of SDHIs was determined on barley cv. 'Astrid' or wheat cv. 'Riband', both of which were inoculated as seedlings in growth stage BBCH 11. Both cereal varieties that were used in trials are highly susceptible to either net blotch disease or STB. Fungicides were applied one-day preventative or four-days curative.

3.11.3.1 Inoculation

Spore suspensions of *P. teres* were obtained from 10-days-old cultures, which were grown on POA petri dishes (see section 3.8.2). Spores were harvested from 2-3 petri dishes per isolate (low sporulating isolates were transferred to up to 10 POA plates to obtain enough

spores). Spores were removed from plates by the addition of 4 mL of 0.25% malt + 0.25% gelatine medium and the use of a Drigalski spatula. The suspension was filtered through two layers of gauze to separate spores from mycelium. Finally, the spore density was counted in a Thoma cell chamber and adjusted to 1E+04 spores mL^{-1} in malt-gelatine medium. Spore suspensions of *Z. tritici* were prepared by using a sterile cotton swab to transfer spores to 4 mL of 0.02% Tween20 in water. The suspension was filtered through two layers of gauze and spores were counted. A spore density of 2.5E+06 cells mL^{-1} was adjusted in 0.02% Tween20-water. Spore suspensions were prepared in sufficient volumes, calculating 1-2 mL suspension for one pot with 10 seedlings.

Plants that had been inoculated with one isolate, were separated (one isolate per glasshouse trolley) and placed on a wet fleece, which was additionally covered by a semipermeable foil. The plants were inoculated with an airbrush (nozzle size: 0.5 mm for *Z. tritici*, 0.8 mm for *P. teres*) until plants were covered but no run-off was observed. After inoculation, seedlings on trolleys were covered with a light permeable plastic box and were transferred to a glasshouse chamber at 20°C and 80% humidity and 15 h light period. The plastic surroundings were removed after two days in the case of *P. teres* and after four days in the case of *Z. tritici*.

Note that the glasshouse management optimised the lights in 2016 in glasshouse chambers, which were used for the cultivation of inoculated barley and wheat plants. These optimised light conditions (old lights: Philips Master HPI-T Plus 400W/645, new lights: DH Licht CHD AGRO 400W 230V) allowed higher infection pressures of *Z. tritici*, particularly in glasshouse trials conducted in the winter months.

3.11.3.2 Fungicide application

Fungicides were applied in a spray chamber with flat fan nozzles, which uses water amounts equal to 400 L ha^{-1}. A maximum of 16 plant pots were treated at a time with 100 mL of fungicide solution.

Sensitivity tests with *P. teres* were carried out using two different concentrations of each fungicide. Three SDHIs were compared in glasshouse studies on *P. teres* (fluxapyroxad, fluopyram and penthiopyrad). These three SDHIs are available as solo-formulated products, Imbrex® (BASF SE), Luna Privilege® (Bayer CS) and Fontelis® (DuPont). Fungicides were applied in full registered rate doses (125 g a.i. ha^{-1}) or at a third of the registered rate (41.5 g a.i. ha^{-1}). Dilutions of formulated products were conducted in water. Three replicates per treatment and isolate were laid out in a fully randomised experimental design.

Glasshouse trials with *Z. tritici* were conducted at full registered field rate doses of SDHIs and included all technical a.i.s of SDHIs that were tested in microtiter tests. Registered rates are 75 g a.i. ha^{-1} benzovindiflupyr, 100 g a.i. ha^{-1} bixafen, 100 g a.i. ha^{-1} fluopyram, 125 g a.i. ha^{-1} fluxapyroxad and isopyrazam and 300 g a.i. ha^{-1} penthiopyrad. It was observed that

precipitation of SDHIs can occur in fungicide dilutions using the standard formulation (5% acetone and 0.05% wettol) designated in the established protocol. Therefore, Agnique®AMD10 (BASF SE) was used as alternative formulation substance to avoid precipitation of SDHIs. SDHIs were first dissolved in DMSO (10,000 mg L^{-1} to 20,000 mg L^{-1}) and were then diluted in 0.15% (v/v) Agnique AMD10 in water. The blank formulation (0.15% Agnique AMD10, 2% (v/v) DMSO) was additionally applicated in the tests to see whether these adjuvants show an effect on the growth of *Z. tritici*. In addition to technical a.i.s, the solo-product Imbrex® was applied in these tests. Four replicates per treatment and isolate were laid out in a fully randomised experimental design.

3.11.3.3 Rating

Infection success of both cereal pathogens was visually rated in diseased leaf area (%). Thereby, all leaves that were present at the time of application and inoculation were rated. Leaves that have evolved after the inoculation of the pathogen were not included. Diseased leaf area was assessed 10 dpi in the case of *P. teres* and 21 dpi in the case of *Z. tritici*. The efficacy (inhibition) of fungicides was calculated according to Abbott (1925):

% efficacy= (% disease untreated - % disease treated) x 100% (% disease untreated)$^{-1}$

3.12 Competition studies of SDHI resistant isolates

3.12.1 Competition studies *in planta*

Infection behaviour of SDHI resistant isolates was studied *in planta* on barley seedlings cv. ‘Astrid’ in the case of *P. teres* and wheat seedlings cv. ‘Riband’ in the case of *Z. tritici* (seedling age BBCH 11). Competition studies, which included several sensitive field isolates and resistant isolates, were performed. Mixtures that contain one resistant and one sensitive isolate in each mixture (1:1), were propagated together over several infection cycles without use of any fungicides. Spore suspensions were prepared as described in section 3.11.3.1. Suspensions were adjusted to 2.5E+04 spores mL^{-1} of *P. teres* and 2.0E+06 spores mL^{-1} of *Z. tritici*. These were applied alone or were mixed in a ratio of 1:1 with another isolate. Each pot, which had ~10 cereal seedlings, was inoculated with 2 mL of the spore suspension. Each isolate or mixture was inoculated to six pots. *P. teres* infected leaves were rated and harvested 10-21 days later. *Z. tritici* was harvested 28 days after inoculation. All infected leaves of each mixture/isolate were washed in 15 mL of either 0.25% malt and 0.25% gelatine medium (*P. teres*) or 0.02% Tween-water (*Z. tritici*). Subsequently, spore suspensions were filtered through 2-fold gauze and were used to inoculate the next round of fresh plants.

An aliquot of 2 mL of each spore suspension (at starting point and at each propagation cycle) was taken to quantify alleles leading to resistance by pyrosequencing or qPCR (see section 3.10.3).

In total, five competition experiments were performed. Two experiments were carried out on *P. teres* isolates, whereas the other three experiments were done on SDHI resistant mutants of *Z. tritici*. The list of isolates that were mixed in 'fitness' tests in the glasshouse can be found in Supplementary Table 42 and Table 44. In the set-up of competition studies, uninoculated plants were placed between different isolates/mixtures to see if there is any unintended spreading of isolates. All competition studies were carried out in a separate glasshouse chamber to avoid having fungicide treated plants from other trials inside the same chamber.

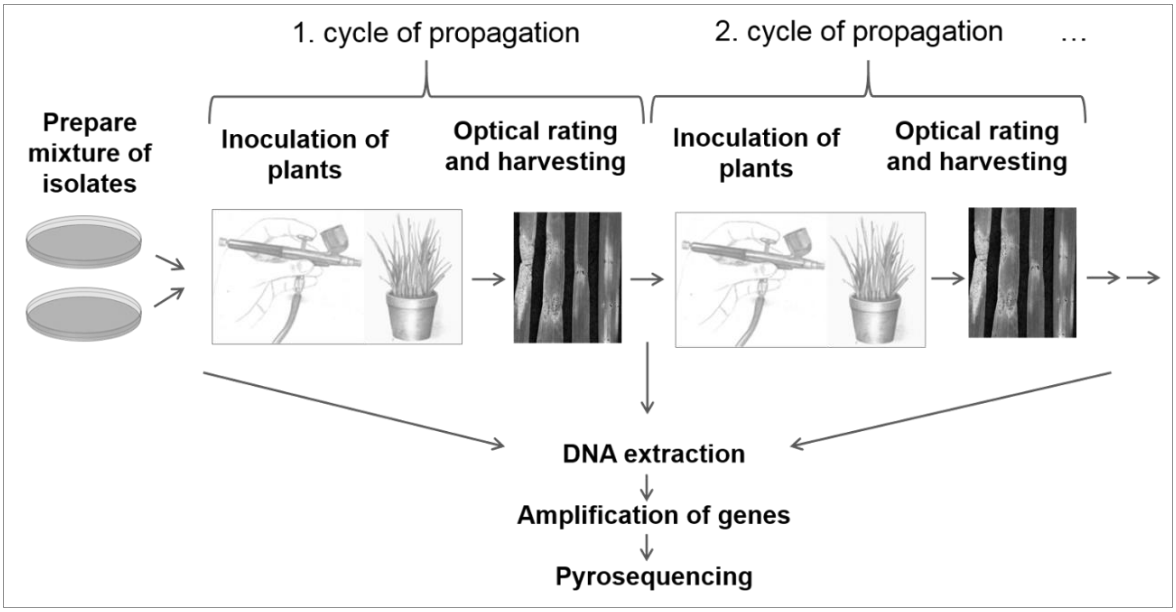


Figure 9: Schematic procedure of competition studies *in planta* with different *P. teres* and *Z. tritici* isolates. Isolates used in mixtures are given in the results and in Supplementary Table 42 and Table 44.

3.12.2 Generation of SDHI-resistant mutants of *Z. tritici*

SDHI resistant mutants were created on YBG agar amended with fluxapyroxad (10 and 1 mg L^{-1}). The generation of fungicide resistant mutants of *Z. tritici* by selection on agar is facilitated by the high number of spores that are produced on artificial media compared to most other fungi. Five ISP2-strep plates of each parental isolate was freshly transferred 7 days prior to the experiments. All spores, pooled from the plates, were transferred to 4 mL of sterile water. Total cell numbers were calculated by counting spores in a dilution (1:10,000 dilution) and two independent measurements. Around 1.0E+08 spores were plated out onto YBG agar amended with fluxapyroxad (as the formulated product, Imbrex®). Different SDHI sensitive field isolates and the reference isolate IPO323 served as parental isolates and are listed in Table 19. Putative SDHI-resistant mutants were picked from

growing colonies after 10-14 days of incubation at 21°C and 12 h of artificial light. Mutants were grown for another generation on selective media before the detection of resistance mechanisms was started.

Table 19: SDHI sensitive *Z. tritici* isolates that were used as parental isolates for generation of SDHI resistant mutants.

Name	Origin	Year	QoI resistance
IPO323	Netherlands (reference isolate)	1981	no
3718	Germany	2004	no
2847	Ireland	2002	G143A
3573	Ireland	2003	G143A
3955	Germany	2005	G143A
5821	United Kingdom	2015	F129L

The exposure to UV-light was avoided to reduce likelihood for other random mutation to occur in the genome. Fungal material was directly transferred to PCR reactions (Mastermix used for standard PCR reaction with addition of 2.5 µL of water) by using sterile pipet tips. Pyrosequencing and qPCR assays, described in section 3.10.3, were used to screen known SNPs which can lead to SDHI resistance. In addition, some mutants were analysed by sequencing of the genes *SdhB*, *SdhC* and *SdhD*, as described in section 3.10.1.

3.13 Studies to detect enhanced efflux of *Z. tritici*

Overexpression of MDR transporters is known in *Z. tritici* isolates from the field (Leroux and Walker, 2011; Omrane et al., 2015). The *Z. tritici* isolates studied were additionally tested for such an enhanced efflux of unspecific toxic compounds. Two different methods were used. On the one hand, microtiter tests with tolnaftate were performed. Tolnaftate is a thiocarbamate antimycotic, which is used in humane medicine but not in agricultural systems and has a different mode of action (inhibition of squalene epoxidase in sterole biosynthesis) (Ryder et al., 1986) compared to the fungicide classes studied. An enhanced tolerance to tolnaftate, therefore, could give indication of a higher efflux of toxic compounds by the isolates (Leroux and Walker, 2011). The tested concentrations of tolnaftate in microtiter tests were 0, 0.03, 0.1, 0.3, 1, 3, 10 ,30 mg a.i. L^{-1}.

Z. tritici isolates were further analysed for the presence of an insertion in the promotor of *Mg*MFS1 transporter. This PCR-based method was extracted from Omrane et al. (2015). The promotor of *Mg*MFS1 of several SDHI resistant field isolates was amplified using KES 2143 and KES 2144. Sizes of amplicons were analysed in a gel (1% TAE) using ethidium bromide.

Table 20: Oligonucleotides used for *Mg*MFS1 promotor studies in *Z. tritici*. Primer sequences were extracted from Omrane et al. (2015).

Name	Sequence (5' → 3')	Amplicon size	T_m
KES 2143	ACATGATCCCTGATCCGTTC	700 bp (without insert), 1200 bp (with insert)	57
KES 2144	CGGCGACTTCTTGCTGAA		

3.14 Homology modelling

The homology models of *P. teres* and *Z. tritici* SDH protein subunits SDH-B, SDH-C and SDH-D were performed by Dr. Janosch Achenbach (BASF SE) and Dr. Antje Wolf (BASF SE). The standard settings of the modelling tool in MOE was used to construct homology models (Molecular Operating Environment, Version 2010.1, Chemical Computing Group Inc., Montreal, Canada). As a structural template for this model, the available SDH X-ray structure of SDH-carboxin complex from *Gallus gallus* (PDB 2WQY) with a resolution of 2.1 Å was chosen. The overall sequence identity to *G. gallus* for subunits B, C and D are 63.95%, 35.5% and 35.9% for *P. teres* and 67.76%, 30.71% and 28.16% for *Z. tritici*. The amino acid substitutions leading to SDHI resistance were manually implemented into the three-dimensional structure of the reference strain protein. Alignments of the amino acid sequence were performed using BLOSUM62 substitution matrix.

3.15 Bioinformatic analyses

Oligonucleotides used for standard PCR and qPCR were designed with the programme FastPCR. Oligonucleotides used for pyrosequencing assays were designed using Pyrosequencing Assay Design Software. Blasts and alignments were made on BASF internal Bioinformatics site (includes NCBI data base). Colouring and virtual mutagenesis of homology models of *P. teres* and *Z. tritici* SDH enzymes were applied in PyMOL software. Reslab software was used to determine EC_{50} values in microtiter tests and inhibition values in glasshouse studies. Statistical evaluation was done with statistic software R. Glasshouse trials were analysed by Lagrange-Multiplier-Test (P=0.05), individually for each fungicide and dose rate, using logit transformation to normalise the data. In competition studies, the increase or decrease of mutants was tested by applying linear regressions. Slope of regressions (lm test) was tested (P=0.001, P=0.01, P=0.05) to evaluate significance of increase or decrease. Significant differences of infection rates and spore production of isolates were tested in an unpaired t-test (P=0.05) in comparison to sensitive isolates.

4 Results

The emergence of SDHI resistance in the two cereal pathogens *P. teres* and *Z. tritici* was observed. Resistant isolates of both pathogens were obtained from intensive monitoring programmes ('Random monitoring' and 'Trial site monitoring') which were carried out in the whole of Europe. Resistance mechanisms were detected and characterised for their impact on various SDHIs. Alignments of protein sequences and homology modelling were used to visualise localisation of alterations in the target enzyme of SDHIs. Quantitative detection systems were established to obtain further information about frequencies of resistant alleles in the whole Europe. In addition, the competitiveness of resistant isolates compared to wild types was examined and an analysis of multiple resistance mechanisms towards SDHIs and other fungicide classes (mainly QoI and DMIs) was carried out. In the following, both pathogens are depicted separately starting with *P. teres*.

4.1 Emergence of SDHI resistance in *Pyrenophora teres* in Europe

4.1.1 Detection and analysis of resistant isolates

Isolates of *P. teres* were collected as air-borne conidia from different regions of Europe. Their sensitivity towards SDHI-fungicides has been analysed by EpiLogic since 2007. The sensitivity was tested in an *ex vivo* bioassay with detached barley leaves. This test used three discriminating fluxapyroxad concentrations to differentiate sensitive (-), moderately (+/++) and advanced (+++) resistant isolates.

Before 2012, isolates were found to have sensitivities within the regular baseline range and were classified as sensitive (-). In the season 2012, two *P. teres* isolates from Northern Germany were shown to have a higher tolerance towards fluxapyroxad and were categorized as moderately resistant. In the following years, more isolates which exhibited moderate resistance towards fluxapyroxad were detected in Germany and other European countries. Isolates which showed an advanced resistance phenotype were first found in 2013 in France and Germany. All 'suspicious' isolates sampled between 2012 and 2015, and some selected wild type isolates were analysed in the present study and are listed in Supplementary Table 37.

An overview of the different amino acid substitutions, that were detected in SDHI resistant *P. teres* isolates obtained from 'Random monitoring', is given in Table 21.

Table 21: Overview of amino acid substitutions in *P. teres* isolates from European countries. Isolates were obtained from 'Random monitoring' in Europe from 2012 to 2015.

SDH-variant	Years of detection	Codon WT >Codon *Sdh* mutant	Number of detected isolates
B-H277Y	2012 to 2015	CAC>TAC	27
C-S73P	2015	TCG>CCG	1
C-N75S*	2014 to 2015	AAC>AGC	18
C-G79R	2013 to 2015	GGT>CGT	134
C-H134R	2013 to 2015	CAC>CGC	29
C-S135R	2013 to 2015	AGC>AGA(G)	9
D-D124N	2013	GAC>AAC	1
D-D124E	2013 to 2015	GAC>GAA	6
D-H134R	2013 to 2015	CAC>CGC	8
D-G138V	2015	GGT>GTT	2
D-D145G	2013 to 2015	GAT>GGT	12
D-E178K	2014	GAG>AAG	3

* C-N75S was first detected in an additional 'Trial site monitoring' of BASF SE in 2013; in 'Random monitoring' it was first found in 2014

Amplification of the target genes (*SdhB*, *SdhC* and *SdhD*) and sequencing using specific *P. teres* oligonucleotides of *SdhB* (KES 1825 and KES 1837), *SdhC* (KES 1827 and KES 1828) and *SdhD* (KES 1833 and 1834) genes were performed. All sequencing reactions were carried out using both primers, forward and reverse, to obtain two reads of each sequence. DNA sequences were virtually spliced by alignments with cDNA sequences available on genome database of *P. teres*. In total, 12 SDH-variants were detected in resistant isolates of *P. teres* in European countries. Sequenced *Sdh* genes of isolates showed only one mutation in each isolate, never two or more *Sdh* mutations in combination. Not all isolates were used to sequence whole *SdhB*, *SdhC* and *SdhD* genes. Isolates from 2015 were mainly analysed by pyrosequencing to detect SDHI resistance mechanisms.

4.1.2 Impact of mutations leading to SDHI resistance in *P. teres* isolates

The sensitivity of SDHI sensitive and SDHI resistant *P. teres* isolates was measured in microtiter tests and glasshouse trials to analyse the effect of different mutations on several SDH-inhibiting fungicides.

4.1.2.1 Sensitivity of *P. teres* isolates *in vitro*

The sensitivities of *P. teres* isolates, which showed a mutation in the *Sdh* genes, were determined in microtiter tests against a range of commercially available SDHI fungicides. The compounds bixafen, fluxapyroxad, isopyrazam, penthiopyrad, benzovindiflupyr and fluopyram are foliar-applied SDHI fungicides that are available on the European market for the control of net blotch in barley. The pooled results from three individual experiments are illustrated in Figure 10. The EC_{50} values of all tested isolates (20 wild type isolates and 57 *Sdh* mutant isolates with 10 different genotypes) ranged from 0.003 to 1.649 mg bixafen

L^{-1}, 0.003 to 0.820 mg fluxapyroxad L^{-1}, 0.003 to 0.971 mg benzovindiflupyr L^{-1}, 0.011 to 4.133 mg isopyrazam L^{-1}, 0.010 to 9.297 mg penthiopyrad L^{-1} and 0.006 to 0.454 mg fluopyram L^{-1}. The sensitive isolates (SDH wild type enzyme), including isolates from 1998 to 2014 collected in France, Germany, the United Kingdom, Poland, Hungary and Belgium, showed a relatively low range of EC_{50} values varying from 0.003 to 0.009 mg fluxapyroxad L^{-1}, 0.003 to 0.007 mg bixafen L^{-1}, 0.003 to 0.019 mg benzovindiflupyr L^{-1}, 0.011 to 0.056 mg isopyrazam L^{-1}, 0.010 to 0.061 mg penthiopyrad L^{-1} and 0.006 to 0.055 mg fluopyram L^{-1}. The highest EC_{50} values measured for all SDHIs were obtained for isolates carrying the amino acid substitutions to arginine in SDH-C and SDH-D subunit, C-H134R, C-G79R, C-S135R and D-H134R. The substitution with the greatest impact on the sensitivity of SDHIs, with exception of penthiopyrad, was C-H134R, showing mean EC_{50} values of 0.441 mg fluxapyroxad L^{-1}, 0.460 mg bixafen L^{-1}, 0.751 mg benzovindiflupyr L^{-1}, 2.500 mg isopyrazam L^{-1}, 5.755 mg penthiopyrad L^{-1} and 0.417 mg fluopyram L^{-1}. The substitution causing the second largest impact on the sensitivity of fluxapyroxad and bixafen, and the largest on penthiopyrad was C-G79R with EC_{50} values of 0.440, 0.396 and 6.603 mg a.i. L^{-1}. In comparison, the isolates carrying C-S135R exhibited the second highest impact on benzovindiflupyr and isopyrazam sensitivities, with mean EC_{50} values of 0.612 and 1.478 mg a.i. L^{-1}, respectively. The substitutions, D-E178K, B-H277Y and D-D124N/E showed the lowest impact on all SDHIs tested.

At the time these experiments were performed, only one isolate of D-D124N and D-D124E have been available. In Figure 10, these two substitutions were taken together for the calculation of mean EC_{50} value. However, D-D124E in comparison to D-D124N showed a slightly higher impact on the sensitivity to most SDHIs. EC_{50} values of the D-D124E mutant were 0.054 mg fluxapyroxad L^{-1}, 0.045 mg bixafen L^{-1}, 0.073 mg benzovindiflupyr L^{-1}, 0.159 mg isopyrazam L^{-1}, 0.299 mg penthiopyrad L^{-1} and 0.138 mg fluopyram L^{-1} and EC_{50} values of D-D124N mutants were shown to be 0.025 mg fluxapyroxad L^{-1}, 0.037 mg bixafen L^{-1}, 0.092 mg benzovindiflupyr L^{-1}, 0.125 mg isopyrazam L^{-1}, 0.279 mg penthiopyrad L^{-1} and 0.108 mg fluopyram L^{-1}. Some substitutions, such as C-N75S and D-D145G, were shown to cause an intermediate increase of EC_{50} values compared to wild types.

Different SDH amino acid substitutions confer different levels of resistance to SDHIs. However, regarding each substitution separately, all SDHIs tested in microtiter tests are affected in a similar manner. In microtiter tests, the highest EC_{50} values were obtained for isolates carrying SDH-variants, C-G79R, C-H134R and C-S135R, followed by D-H134R. Medium levels of resistance were observed for isolates carrying C-N75S, D-D124E and low levels of resistance for isolates having B-H277Y, D-D145G and D-D124N. It was demonstrated that all SDHI fungicides show a similar response to different SDH alterations which implies cross-resistance of resistant isolates to all SDHIs tested.

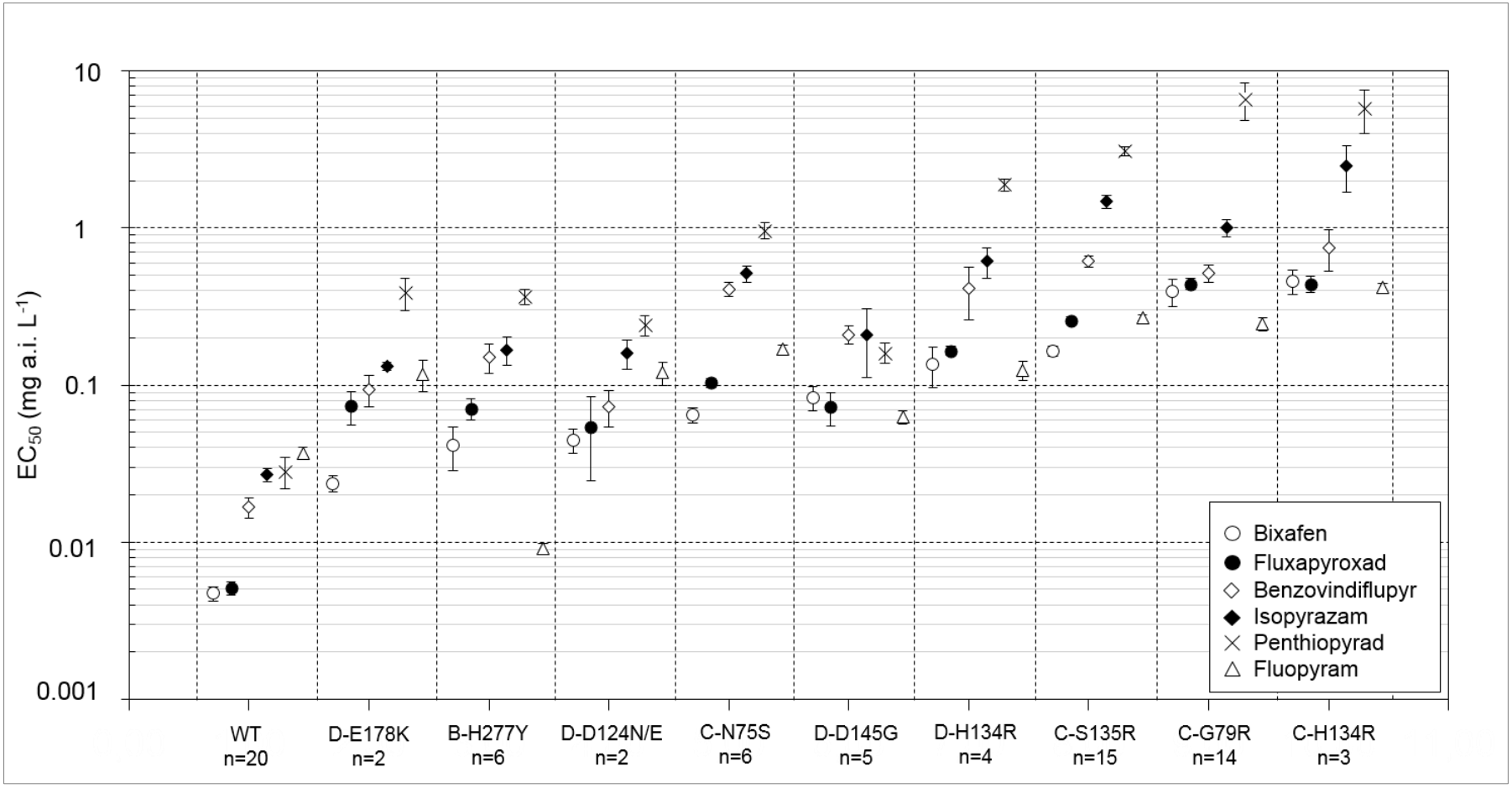


Figure 10: SDHI sensitivity of *P. teres* isolates carrying different mutations in the *Sdh* genes. Photometric measurements (OD_{405}) detecting mycelial growth of *P. teres* in microtiter plates were used to determine EC_{50} values (probit calculation) of SDHI fungicides. Water control and seven concentrations (up to 10 mg a.i. L^{-1}) of bixafen, fluxapyroxad, benzovindiflupyr, isopyrazam, penthiopyrad and fluopyram applied in four replicates were mixed with spore suspensions of *Sdh* wild type isolates and several *Sdh* mutated isolates. SDH amino acid substitution, the number of isolates and standard errors (some standard errors are smaller than the symbol itself) calculated from three individual experiments, are given. These results have been partially published recently (Rehfus et al., 2016, Figure 3).

The RF values and the cross-resistance pattern are given in Table 22. RF values ranged from 0.25 to 204, dependent on the SDH-variant and the SDHI that was observed. Resistance factors were calculated as ratios of EC_{50} of resistant to EC_{50} of sensitive isolates and are dependent on wild type activity of compounds and the set of wild types that are used. Fungicides (in Figure 10 and Table 22) are sorted due to their intrinsic activity towards SDHI sensitive isolates. Bixafen and fluxapyroxad had highest activity on wild type isolates of *P. teres*, followed by benzovindiflupyr, isopyrazam, penthiopyrad and fluopyram. Although, RF values of fluopyram were lower in comparison to other SDHIs, the classification of mutants was similar. Fluopyram in generell was shown to behave cross-resistant to other SDHIs. Low RF values of fluopyram were mainly driven by low activities of this substance towards wild type isolates of *P. teres* compared to other SDHIs tested. One exception of cross-resistance was observed in *P. teres* in the case of B-H277Y mutants, which showed a more sensitive phenotype towards fluopyram than wild type isolates. B-H277Y mutants, however, only showed low resistance factors in case of other SDHIs tested.

Table 22: Resistance factors of *P. teres* isolates showing different SDH-variants towards SDHIs. Resistance factors were calculated as ratios of mean EC_{50} of resistant isolates / EC_{50} of sensitive isolates shown in Figure 10. Bixafen (bixa), fluxapyroxad (fluxa), benzovindiflupyr (benzo), isopyrazam (isopyra), penthiopyrad (penthio) and fluopyram (fluo) are sorted due to their intrinsic activity on wild type isolates (left to the right). Colours indicate the strength of resistance with RF values < 0 white (no resistance), ≤ 20 bright yellow, 21 to 50 dark yellow, 51 to 100 orange, and >100 red.

	RF values					
SDH-variants	Bixa	Fluxa	Benzo	Isopyra	Penthio	Fluo
D-E178K	5	14	6	5	14	3
B-H277Y	9	14	9	6	13	0.25
D-D124N/E	9	11	4	6	9	3
C-N75S	14	20	24	19	34	5
D-D145G	18	14	12	8	6	2
D-H134R	29	32	24	23	67	3
C-S135R	35	51	36	55	109	7
C-G79R	84	86	31	37	234	7
C-H134R	97	86	45	93	204	11

4.1.2.2 Sensitivity of *P. teres* isolates *in planta*

In microtiter tests, it could be confirmed that different SDH-variants of *P. teres* have an influence on the SDHI efficacy and cause a range of diverse sensitivity losses. Spores in a microtiter plate are directly exposed to high concentrations of fungicides without considering the substance behaviour in interaction with plants. The efficacy of three solo SDHI compounds on *Sdh* mutated isolates was tested under controlled conditions in the glasshouse (Figure 11). Fontelis® (tech a.i. penthiopyrad), Imbrex® (tech a.i. fluxapyroxad) and Luna Privilege® (tech a.i. fluopyram) are commercially available SDHI solo fungicides either used in speciality crops (fluopyram and penthiopyrad) and/or cereal crops (fluxapyroxad and penthiopyrad). In Figure 11 A, inhibition levels of compounds are shown at full doses of the registered field rates (125 g a.i. ha^{-1}). SDHIs solo compounds controlled wild type isolates of *P. teres* with a mean inhibition of 92% fluopyram, 98% fluxapyroxad

and 95% penthiopyrad when the fungicides were applied one-day preventative. The isolates carrying an SDH amino acid exchange showed a broad range of inhibition levels, which depended on the SDH-variant, the product that was used, and the applied dose of fungicides.

At full rates, significant efficacy losses of all three compounds were observed for SDH-variants showing C-G79R, C-H134R, D-D124E and D-H134R. No significant or weak reduction of inhibition levels was observed for isolates having B-H277Y, D-D124N and D-D145G. However, isolates carrying C-N75S and C-S135R exchange were fully controlled by fluxapyroxad at full doses. Fluopyram and penthiopyrad showed significant efficacy losses regarding C-N75S and C-S135R mutants even at full doses. Inhibition levels at a dose equal to a third of the full rate of the three SDHIs are given in Figure 11 B. Here, the impact of SDH-variants on SDHI efficacy was more pronounced but SDH-variants showed the same ranking as with the full doses.

Regarding the impact of different SDH-variants, results from microtiter tests were confirmed in glasshouse studies. Isolates which carry amino acid substitutions C-G79R and C-H134R had the highest impact on the SDHI efficacy, whereas B-H277Y, D-D145G and D-D124N had no significant or only a low impact on SDHI efficacies. The observation from microtiter tests that fluopyram shows a weaker activity against wild type isolates compared to other SDHIs was also visible in glasshouse tests. At a third rate, wild type isolates were inhibited by fluopyram to 81%, by fluxapyroxad to 97% and by penthiopyrad to 85%. Fluopyram showed a slightly better activity on B-H277Y mutants (85 % inhibition) compared to wild types, which had been also seen in microtiter tests. Fluxapyroxad, which showed an RF of 14 in the case of B-H277Y in microtiter, was also not significantly affected by this exchange, even at a third of the full rate of the fungicide. Therefore, RF values evaluated in microtiter tests do not necessarily allow conclusions on the efficacy *in planta*. However, the ranking of SDH-variants with respect to their impact on SDHI efficacies were shown to be similar in both glasshouse and microtiter tests.

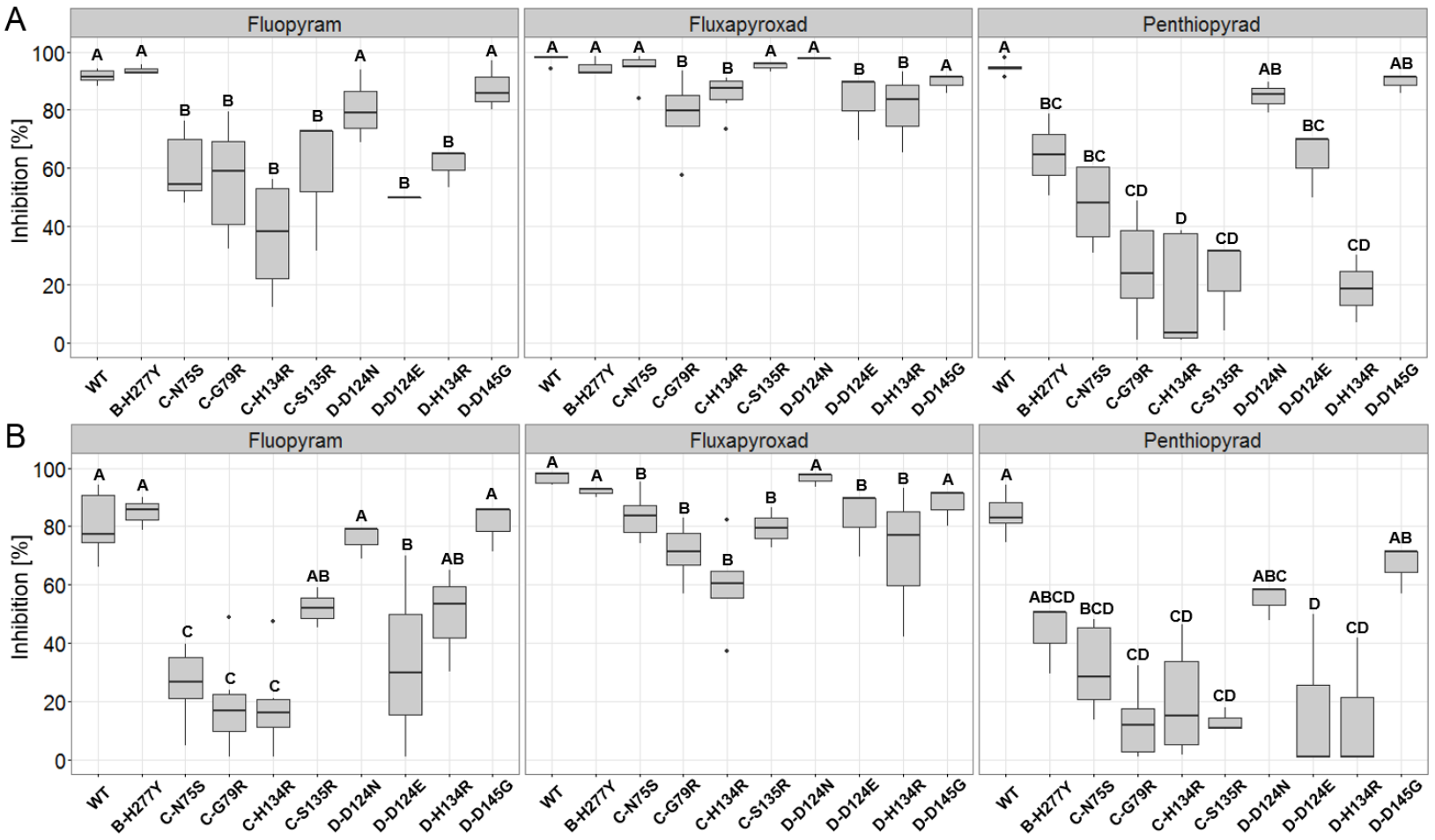


Figure 11: Efficacy of fluopyram (Luna Privilege®), fluxapyroxad (Imbrex®) and penthiopyrad (Fontelis®) against SDHI sensitive and resistant isolates of *P. teres* in glasshouse experiments. Water control and different treatments were applied in three replicates one day before inoculation of barley cv. Astrid (BBCH 11) with *Sdh* wild type (n=2) and *Sdh* mutated isolates including SDH-variants: B-H277Y, C-N75S, C-G79R, C-H134R, C-S135R (each n=2) and D-D124N, D-D124E, D-H134R, D-D145G (each n=1). **A:** Box-and-Whiskers of inhibition levels [%] of SDHIs when applied at full doses of registered field rates (125.0 g a.i. ha-^{1}). **B:** Box-and-Whiskers of inhibition levels [%] of SDHIs when applied at a third of the registered field rate (41.7 g a.i. ha-^{1}). Within a fungicide, same letters on top of Box-and-Whiskers mean that they do not differ significantly according to Lagrange multiplier to range test (P=0.05).

4.1.3 Localisation of amino acid exchanges in the SDH enzyme

4.1.3.1 Alignments of *P. teres* SDH amino acid sequences to other phytopathogenic fungi

SDH-B, SDH-C and SDH-D amino acid sequences of *P. teres*, SDH wild type and SDHI resistant isolates, were aligned to sequences of other phytopathogenic fungi. The fungi included are important plant pathogens that cause serious damage in the production of speciality (e.g. fruit, vine, vegetables) or arable (e.g. soy beans, potatoes, cereals) crops. *P. teres* sequences were obtained in the present study, sequences of other plant pathogens were extracted from NCBI or BASF internal sequence database. The alignment of SDH-B sequences revealed that the SDH-B subunit shows a high conservation of amino acids. The sequence alignment in Figure 12 shows that histidine at position 277, which can be exchanged to tyrosine in resistant isolates of *P. teres*, is highly conserved amongst other phytopathogenic fungi. Sequence alignments including bacteria, mammals and plants releaved that this histidine is highly conserved within all kingdoms of living organisms (Cecchini, 2003).

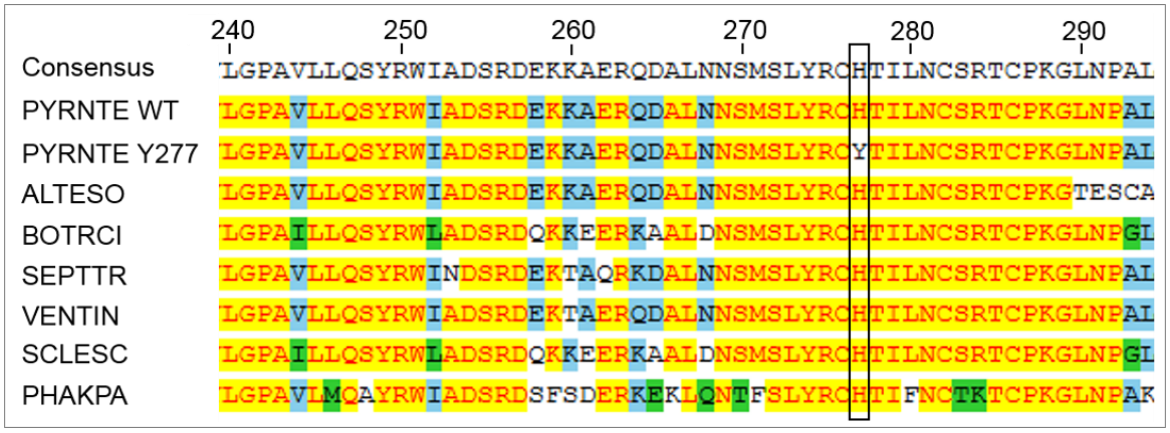


Figure 12: Alignment of partial SDH-B amino acid sequence of *P. teres* and other phytopathogenic fungi. Sequences of ascomycetous and basidiomycetous fungi are named by species EPPO code (PYRNTE: *Pyrenophora teres*, ALTESO: *Alternaria solani*, BOTRCI: *Botrytis cinerea*, SEPTTR: *Zymoseptoria tritici*, VENTIN: *Venturia inaequalis*, SCLESC: *Sclerotinia sclerotiorum*, PHAKPA: *Phakospora pachyrhizi*). In the case of *P. teres*, a wild type sequence and the sequence of an SDH-B H277Y mutant is included. The numbers of amino acids that are given in the figure are based on the sequence length of *P. teres*.
Colour code: > 80% conserved, ≥ 50% conserved, ≥ 50% similar, not conserved. Sequence alignments were made by the multiple alignment tool on bioinformatics webpage of BASF SE, which uses muscle-calculation.

In Figure 13, the alignment of partial SDH-C amino acid sequences of sensitive and resistant isolates of *P. teres* and other phytopathogenic fungi is shown. Amino acids G79 and H134 of *P. teres* are conserved amongst other fungi. Other positions, such as N75 and S135, which are altered in some resistant isolates of *P. teres*, are conserved when compared to most fungi but show exceptions, as for example *S. sclerotiorum* (F75) and *P. pachyrhizi* (T135). Position 73, where S73P exchange can occur in *P. teres*, shows no conservation in different fungal species.

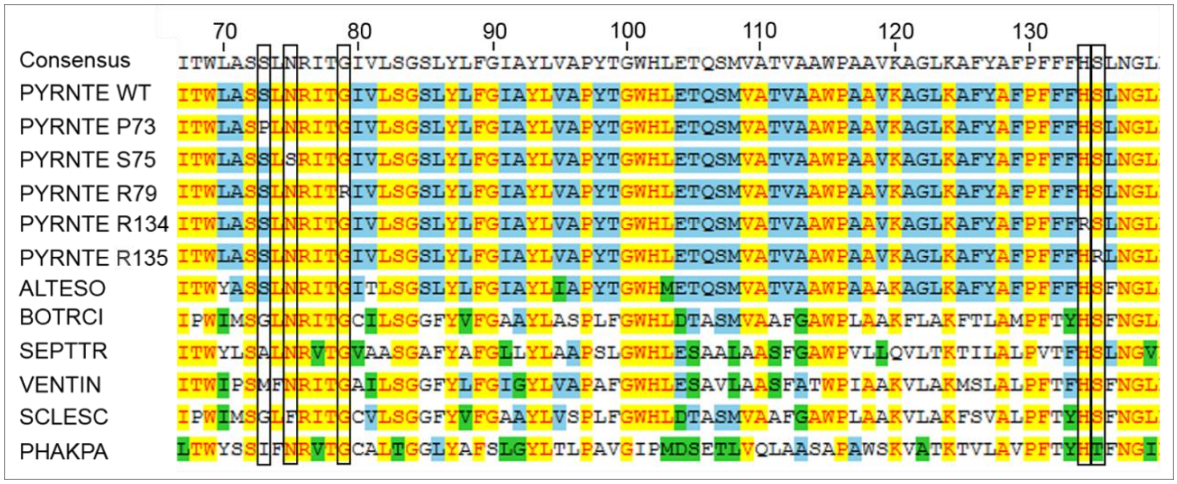


Figure 13: Alignment of partial SDH-C amino acid sequence of *P. teres* and other phytopathogenic fungi. Sequences of ascomycetous and basidiomycetous fungi are named by species EPPO code. In case of *P. teres*, a wild type sequence and the sequences of SDH-C S73P, N75S, G79R, H134R and S135R mutants are included. The numbers of amino acids that are given in the figure are based on the sequence length of *P. teres*. Colour code: > 80% conserved, ≥ 50% conserved, ≥ 50% similar, not conserved. Sequence alignments were made by the multiple alignment tool on bioinformatics webpage of BASF SE, which uses muscle-calculation.

The alignment of SDH-D amino acid sequences of mutants of *P. teres* and other fungi is given in Figure 14. Here, most exchanges found in SDHI resistant isolates of *P. teres* are located at a position which is completely conserved over all fungi investigated. There is only one exception, D-E178K, a position at which *P. pachyrhizi* carries an asparagine instead of glutamic acid.

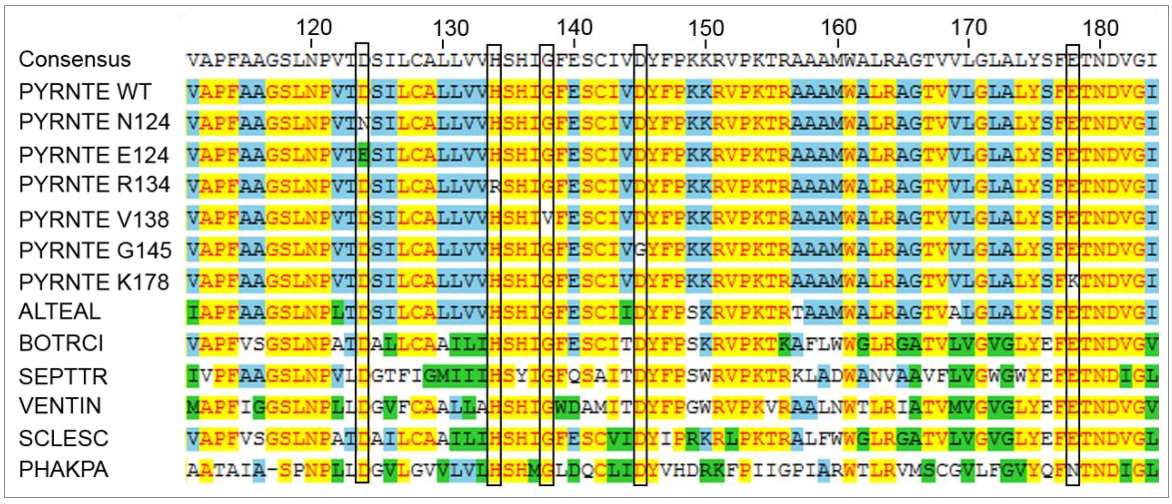


Figure 14: Alignment of partial SDH-D amino acid sequence of *P. teres* and other phytopathogenic fungi. Sequences of ascomycetous and basidiomycetous fungi are named by species EPPO code (instead of ALTESO as in Figure 12 and Figure 13, ALTEAL (*A. alternata*) sequence is shown). In case of *P. teres*, a wild type sequence and the sequences of SDH-D D124N/E, H134R, G138V, D145G and E178K mutants are included. The numbers of amino acids that are given in the figure are based on the sequence length of *P. teres* SDH. Colour code: >80% conserved, ≥ 50% conserved, ≥ 50% similar, not conserved. Sequence alignments were made by the multiple alignment tool on bioinformatics webpage of BASF SE, which uses muscle-calculation.

4.1.3.2 Homology modelling of the SDH enzyme of *P. teres*

Amino acid substitutions that were detected in SDHI resistant *P. teres* isolates were analysed as to their localisation within the SDH enzyme. Identification of locations of SDH-variants B-H277Y, C-S73P, C-N75S, C-G79R, C-H134R, C-S135R, D-D124N/E, D-H134R, D-G138V, D-D145G and D-E178K were carried out using a homology model of the *P. teres* SDH enzyme (Figure 15). SDH modelling of the wild type enzyme of *P. teres* was performed by Dr. Janosch Achenbach (BASF SE), whereas mutagenesis and illustration of the SDH model was part of the present work. Modelling was based on the X-ray structure of *Gallus gallus* (PDB 2WQY) because an X-ray structure of the *P. teres* SDH was not available. In the SDH homology model, carboxin is indicated at the binding site of SDHIs and haem b group is depicted, which is naturally complexed by highly conserved histidine residues of SDH-C and SDH-D chains (Cecchini, 2003; Horsefield et al., 2004).

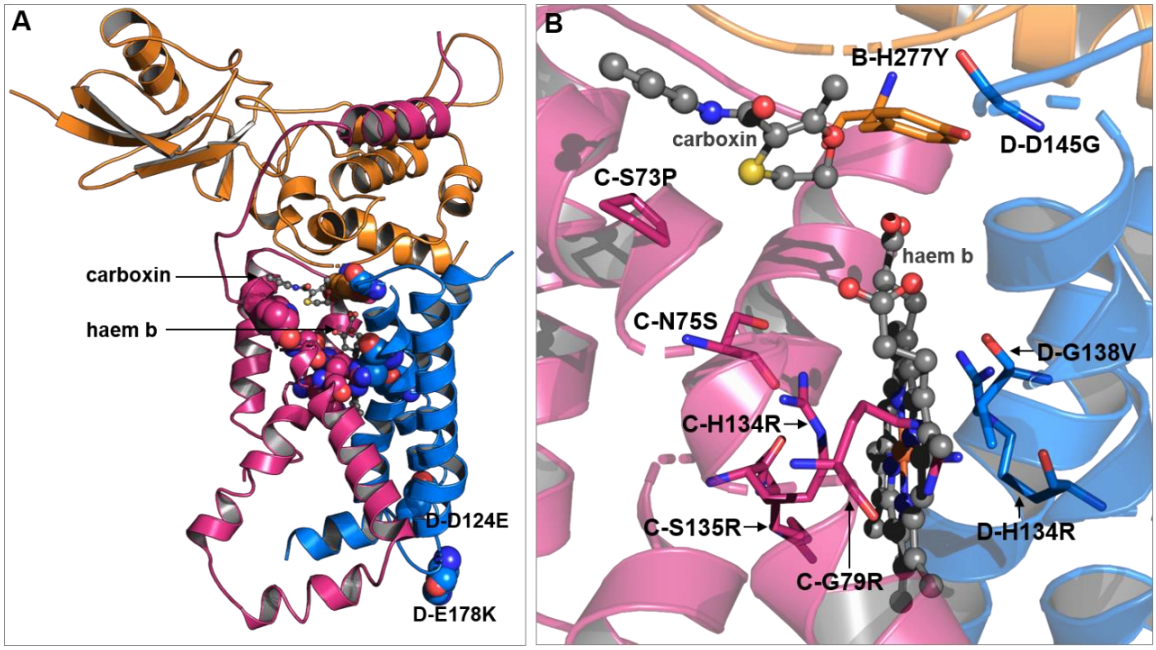


Figure 15: Localisation of amino acid substitutions leading to SDHI resistance in homology model of *P. teres* succinate dehydrogenase enzyme. Homology model of SDH subunits SDH-B, SDH-C and SDH-D is based on X-ray from *G. gallus* (PDB 2WQY) with the docked pose of carboxin in ubiquinone-binding pocket and complexed haem b group between SDH-C and SDH-D. SDH-B (orange), SDH-C (lilac) and SDH-D (blue) subunits are shown in cartoon style (helices denote α-helical secondary structures and ribbons denote β-sheets). Carboxin and haem b are depicted in ball-and-stick models showing CPK colouring (carbon=grey, nitrogen=blue, oxygen=red, sulfur=yellow, iron=dark orange). Substituted amino acid residues of B-H277Y, C-S73P, C-N75S, C-G79R, C-H134R, C-S135R, D-D124E, D-H134R, D-G138V, D-D145G and D-E178K are depicted in sphere/stick models showing CPK colouring except for carbon atoms, which are shown in the colour of corresponding subunit. Hydrogen atoms are not shown in any of the molecules. **A:** Overview of positions of amino acid substitutions in SDH-B, SDH-C and SDH-D subunits of SDH enzyme. Alterations are found in direct neighbourhood of the Q-site such as B-H277Y, C-S73P and D-D145G, in close neighbourhood to haem b group in case of C-N75S, C-G79R, C-H134R, C-S135R, D-H134R and D-G138V or in far distance to both sites such as D-D124N/E (D-D124E shown) and D-E178K. **B:** Closer view to alterations located near Q-site and haem b group.

With respect to their localisation within the SDH enzyme, substitutions can be found at different positions in SDH-B, SDH-C and SDH-D subunits. Amino acids at positions 277 in SDH-B subunit, 73 in SDH-C subunit and 145 of SDH-D subunit are placed in the direct vicinity of the Q-site (SDHI binding site). Amino acids in subunit SDH-C at positions 75, 79,

134 and 135, and in subunit SDH-D at positions 134 and 138 are situated near the haem b group within SDH complex. Two further substitutions in far distance to both sides (Q-site and haem b group) in subunit D, at positions 124 and 178, were detected.

The distance of the substituted amino acids to carboxin and haem b group is given in Table 23. Since the *P. teres* SDH model was aligned to the chicken SDH model, length specifications are just estimations to illustrate positions within the enzyme. Amino acid arginine in the case of C-G79R mutants is much bulkier compared to glycine and makes the original position of haem b unlikely (0 Å of arginine to haem b implies a 'clash' of arginine with haem b). Interestingly, the histidines that coordinate the central iron atom of haem b group can be substituted to arginine in SDHI resistant isolates of *P. teres* (C-H134R and D-H134R). Arginine is not known to coordinate haem groups, which would mean the loss of one coordination partner for haem b in case of these mutants.

Table 23: Distance of amino acid substitutions in *P. teres* to carboxin binding site and haem b group. Nearest atoms of both carboxin and haem b group to altered amino acid residue were calculated in the measurement tool of PyMol. Length measurements can be found in the Supplementary Material, Figure 48.

SDH-variant	Distance to carboxin [Å]	Distance to haem b group [Å]
B-H277Y	1.3	3.4
C-S73P	3.1	6.6
C-N75S	3.9	4.3
C-G79R	6.9	0
C-H134R	6.9	2.1
C-S135R	13.9	2.4
D-D124E	26.9	12.3
D-H134R	9.6	2.0
D-D145G	4.5	7.5
D-E178K	36.5	21.8

4.1.4 Frequency of SDHI resistant isolates of *P. teres* in Europe

SDHI resistant isolates of *P. teres* were sampled in Europe over a period of four years starting in 2012 in 'Random monitoring'. Sequencing of the *Sdh* genes of resistant isolates showed a range of different mutations which caused amino acid variations in the SDH enzyme. In addition to 'Random monitoring', quantitative pyrosequencing assays were established to detect the frequency of genotypes leading to SDHI resistance directly from DNA pools of net blotch infected leaf samples. These leaf samples were collected mainly from trial sites but also from commercial fields all over Europe and were sampled by field technicians of BASF SE, farmers or governmental institutions and universities.

4.1.4.1 'Random monitoring'

The 'Random monitoring' included 175 isolates from 2012, 248 isolates from 2013, 245 isolates from 2014 and 253 isolates from 2015. During this work, two isolates from 2012, 72 isolates from 2013, 118 isolates from 2014 and 138 isolates from 2015 were analysed.

Thereof, the two isolates in 2012, 62 isolates in 2013, 74 isolates in 2014 and 113 isolates in 2015 showed target-site mutations in the *Sdh* genes.

In 2012 in a region between Rostock and Lübeck in Germany, the first two SDHI resistant isolates were detected carrying B-H277Y exchange (Figure 16). Isolates collected in other European countries were shown to be fully sensitive towards SDHIs.

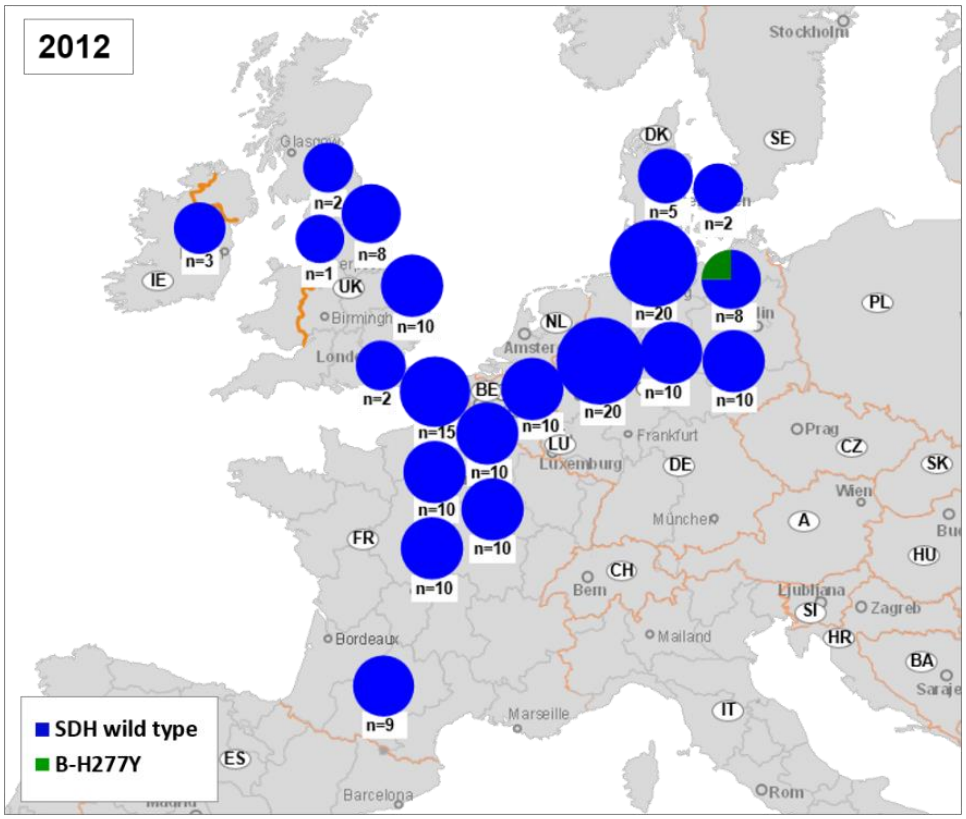


Figure 16: Occurence of SDH-variants leading to SDHI resistance in isolates of *P. teres* collected in 'Random monitoring' from various European countries in 2012. Colour code of SDH-variants and the number of isolates are given in the figure. The size of the disc represents the number of isolates and the location of the disc indicates the origin of collected isolates. Isolates were sampled in Ireland (IE) (n=3), the United Kingdom (UK) (n=23), in France (FR) (n=64), Belgium (BE) (n=10), Germany (DE) (n=68) and Denmark (DK) (n=7).

A strong increase in SDHI resistance in *P. teres* to 25% of the sampled isolates was observed in 2013 (Figure 17). SDHI-resistant isolates were found in France, Germany, the United Kingdom, Belgium and Denmark. In France, 14% and in Germany 44% of all sampled isolates showed target-site mutations in the *Sdh* genes. Within insensitive isolates in 2013, a range of different mutations in *Sdh* genes was found leading to substitutions such as B-H277Y, C-G79R, C-H134R, C-S135R, D-D124N and D-D124E, D-H134R and D-D145G. The most frequent amino acid exchange detected was found to be C-G79R, whereas other SDH changes occurred at lower frequencies. Isolates collected from Sweden, Poland, Czech Republic, Hungary and Italy were shown to be SDHI sensitive.

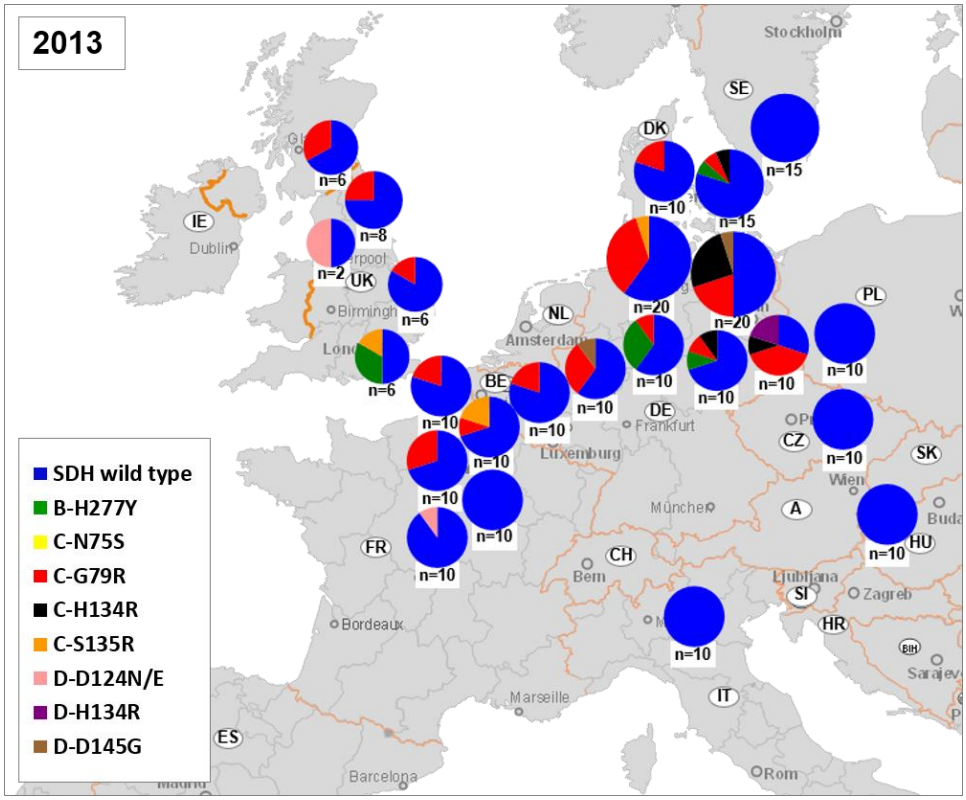


Figure 17: Occurence of SDH-variants leading to SDHI resistance in isolates of *P. teres* collected in 'Random monitoring' from various European countries in 2013. Colour code of SDH-variants and the number of isolates are given in the figure. The size of the disc represents the number of isolates and the location of the disc indicates the origin of collected isolates. Isolates were sampled in the United Kingdom (UK) (n=28), in France (FR) (n=50), Belgium (BE) (n=10), Germany (DE) (n=80), Denmark (DK) (n=25), Sweden (SE) (n=15), Poland (PL) (n=10), Czech Republic (CZ) (n=10), Hungary (HU) (n=10) and Italy (IT) (n=10).

The distribution and frequency of insensitive isolates of *P. teres* in Europe for the year 2014 is given in Figure 18. A further increase in SDHI-resistant *P. teres* isolates from 25% in 2013 to 30% in 2014 was observed in Europe (see Figure 20). In contrast to 2013, isolates sampled in 2014 from the United Kingdom and Denmark were sensitive towards SDHIs. In most northern and eastern countries, such as Denmark, Poland and Croatia, no SDHI resistant isolates of *P. teres* were detected in 2014. In Sweden, one isolate carrying C-G79R exchange was observed. Highest proportion of SDHI-resistant isolates were collected in France, Germany and Belgium. Large regional differences were found with the highest frequencies of resistant isolates in northern parts of France and Germany. In Germany, only a slight increase of resistant isolates from 44% in 2013 to 47% in 2014 was observed, whereas in France a significant increase in SDHI-resistant isolates from 14% in 2013 to 70% in 2014 was found. In 2014, there was an increase in the frequency of other SDH-variants compared to C-G79R. In Germany, C-G79R was found in a lower number of isolates compared to 2013, and the frequency of amino acid exchange D-D145G increased to 12% in isolates sampled. In France, the frequency of isolates carrying C-G79R increased in 2014 to 41% compared to the year 2013. Besides C-G79R, C-N75S, with a frequency of 16% in France, gained importance in 2014. A new mutation which caused D-E178K was observed in a region from central France.

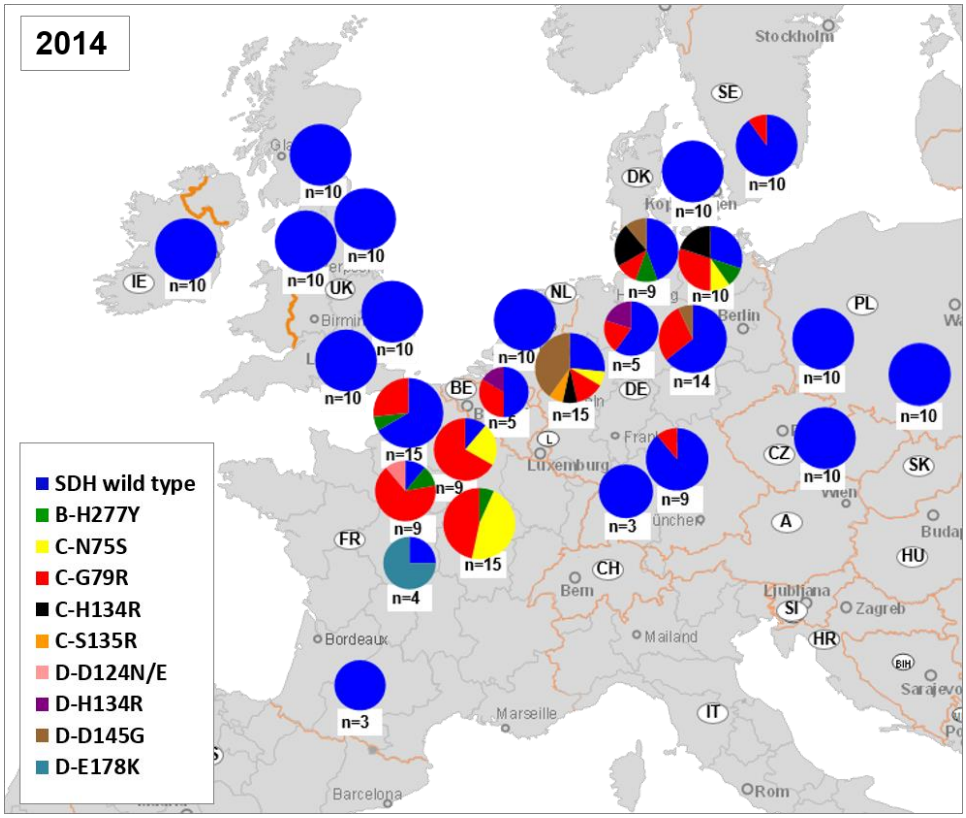


Figure 18: Occurence of SDH-variants leading to SDHI resistance in isolates of *P. teres* collected in 'Random monitoring' from various European countries in 2014. Colour code of SDH-variants and the number of isolates are given in the figure. The size of the disc represents the number of isolates and the location of the disc indicates the origin of collected isolates. Isolates were sampled in Ireland (IE) (n=10), the United Kingdom (UK) (n=50), in France (FR) (n=55), Belgium (BE) (n=5), the Netherlands (NL) (n=10), Germany (DE) (n=65), Denmark (DK) (n=10), Sweden (SE) (n=10), Poland (PL) (n=20) and Czech Republic (CZ) (n=10). These results were published recently (Rehfus et al., 2016, Figure 8).

SDHI resistant isolates of *P. teres* and their frequency in Europe in the year 2015 are given in Figure 19. In France, the most frequent amino acid substitution was shown to be C-G79R. In contrast, isolates collected from Northern-Germany showed a higher diversity of SDH-variants, with C-H134R, C-G79R, D-D124E and B-H277Y being the most frequent. In addition, a new SDH-C variant, C-S73P, was found. In the UK, only eight isolates were collected in 2015 whereof five isolates showed SDHI resistance. These isolates carried mutations leading to C-N75S, C-G79R, D-H134R and a new variant, namely D-G138V. Isolates collected in countries such as Poland, Italy and the Netherlands were shown to be fully sensitive towards SDHIs. A low frequency of C-G79R mutants were detected in Denmark and Sweden in 2015.

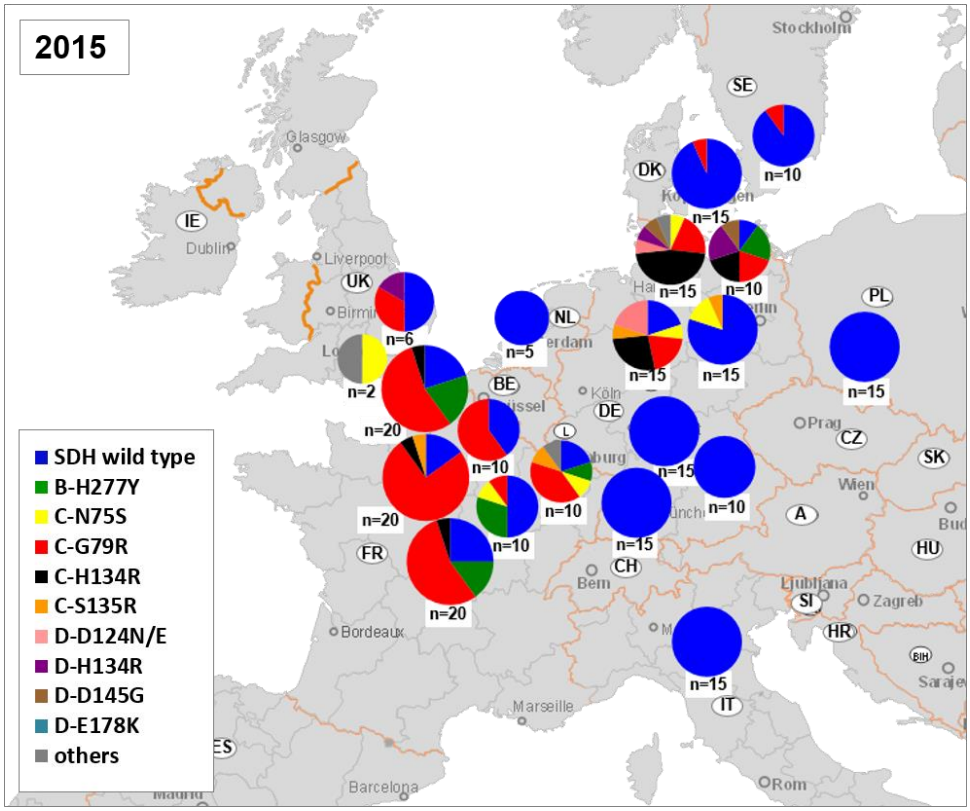


Figure 19: Occurence of SDH-variants leading to SDHI resistance in isolates of *P. teres* collected in 'Random monitoring' from various European countries in 2015. Colour code of SDH-variants and the number of isolates are given in the figure. The size of the disc represents the number of isolates and the location of the disc indicates the origin of collected isolates. Isolates were sampled in the United Kingdom (UK) (n=8), in France (FR) (n=80), the Netherlands (NL) (n=5), Germany (DE) (n=95) and Denmark (DK) (n=15), Sweden (SE) (n=10), Poland (n=15) and Italy (n=15). Other SDH-variants than that listed in the figure were C-S73P (n=1) collected in Germany and D-G138V (n=2) collected in UK and France.

In Figure 20, an overview of the frequency of SDHI resistant isolates over all the years in Europe is given. In 'Random monitoring', an increase of SDHI resistant isolates has been observed since 2012. In 2013, 25% of all collected isolates showed an alteration in the *Sdh* genes. In 2014, this proportion raised to 30% and to 45% in the year 2015 (Figure 20 A). In the years 2013 to 2015, the most frequent amino acid substitution was C-G79R, present in >50% of all resistant isolates. However, in 2014 and 2015, significant proportions showed other alterations, such as C-N75S, C-H134R and D-D145G.

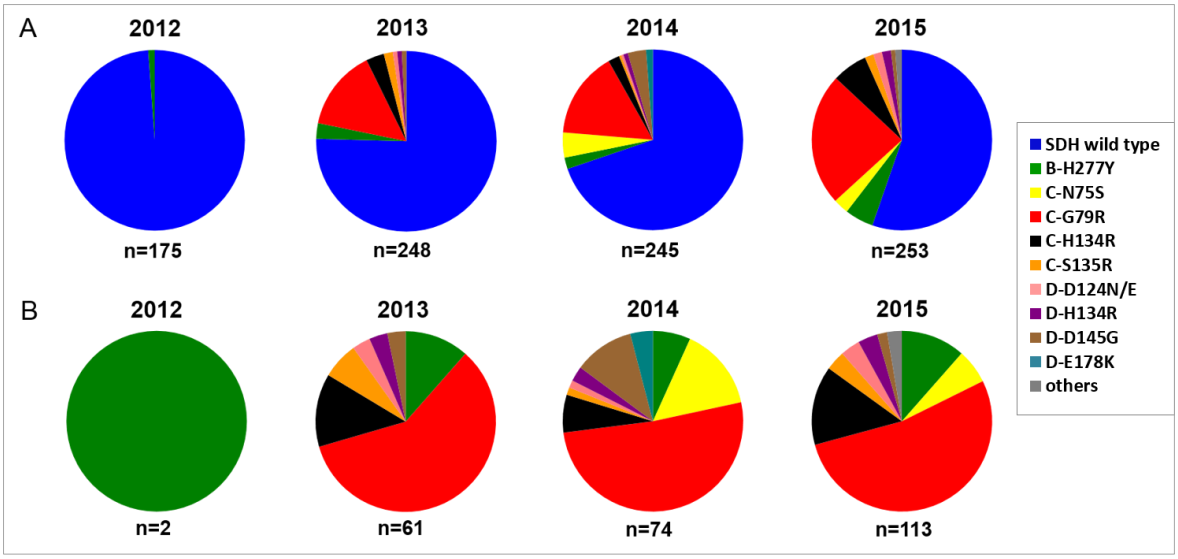


Figure 20: Frequency of SDH amino acid substitutions leading to SDHI resistance in *P. teres* in Europe from 2012 to 2015. Colour code of SDH-variants and the number of isolates are given in the figure. **A:** Increase of SDHI resistant isolates and corresponding SDH-variants collected in 'Random Monitoring' in many European countries from 2012 to 2015. **B:** Frequency of SDH-variants within SDHI resistant isolates collected in 'Random Monitoring' in countries such as France, the United Kingdom, Germany, Belgium, Denmark and Sweden.

4.1.4.2 Field samples

Sequence information of sensitive and resistant isolates of *P. teres* were used to establish quantitative pyrosequencing assays. Pyrosequencing assays for the detection of B-H277Y, C-N75S, C-G79R, C-H134R, C-S135R, D-D134N/E, D-H134R, D-D145G and D-E178K variants were then used to determine the frequency of alleles leading to SDHI resistance in infected leaf samples. Assays to detect C-N75S and C-G79R are sequenced in 5'→3' orientation, whereas all other assays are designed forward in a 3'→5' sequencing orientation. One sample included 20 to 30 net-blotch infected leaves, which had been randomly collected within a field at each site. DNA was extracted from 20 net blotch lesions of these leaves at each site and was analysed.

The frequency of sensitive alleles ('SDH wild type' as it is referred to in the figures) was calculated by adding the frequency of different resistance alleles and substraction from 100%. Studied SNPs should represent the main proportion of alleles leading to SDHI resistance. However, alterations C-S73P and D-G138V (first detected in 2015 in a low number of isolates) are not covered by pyrosequencing assays.

The frequencies of alleles leading to SDHI resistant phenotypes are shown in Figure 21 in an overview for the whole of Europe in the year 2014. In 2014, 243 samples were taken from untreated plots from trial sites or commercial fields of 17 different countries. SNPs, which are known to cause SDHI resistance, were detected in France, Germany, Belgium and Denmark. However, frequencies were highest in Northern-France and the middle and northern parts of Germany. In one sample from Denmark, low frequencies of the allele, which causes C-G79R variant, and in another sample, low frequencies of D-D124E, were

observed. In all other countries, pyrogrammes showed 100% wild type sequence in all analysed samples.

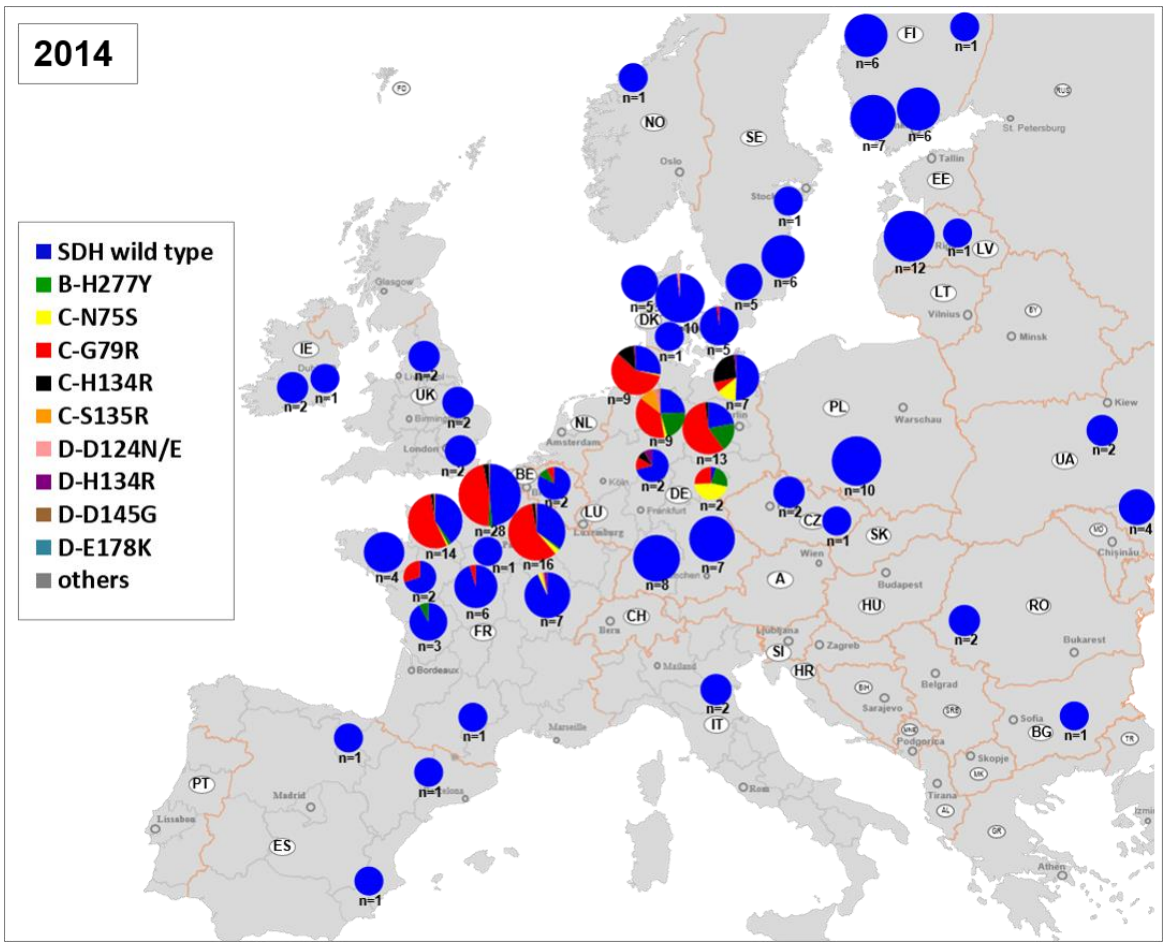


Figure 21: Frequency of genotypes causing SDHI resistance in *P. teres* collected in 'Field monitoring' from various European countries in 2014. Net blotch infected leaf samples (one sample includes a pool of 20 infected leaves randomly collected from one site) were taken from commercial sites and from untreated plots of trial sites. Frequency of alleles leading to SDHI resistance was measured using quantitative pyrosequencing. Colour code of SDH-variants and the number of samples are given in the figure. The size of the disc represents the number of analysed samples and the location of the disc indicates the origin of collected leaf samples. Samples are taken from Ireland (IE) (n=3), the United Kingdom (UK) (n=6), France (FR) (n=82), Spain (ES) (n=3), Belgium (BE) (n=2), Germany (DE) (n=57), Denmark (DK) (n=21), Sweden (SE) (n=12), Finland (FI) (n=20), Norway (NO) (n=1), Latvia (LV) (n=13), Poland (n=10), Czech Republic (CZ) (n=3), Italy (n=2), Ukraine (UA) (n=6), Romania (RO) (n=2) and Bulgaria (BG) (n=1).

Frequencies of resistance alleles in samples from 2015 are given in Figure 22. In 2015, 261 samples from 18 different countries were analysed. The situation in 2015 was comparable to that in 2014. The highest proportion of SDHI resistant genotypes were found in France and Germany. However, altered alleles were also observed in low frequencies in southern regions of both countries. In addition, the frequency of resistant genotypes in Belgium increased in 2015 to 50% of resistant alleles. Low frequencies were also observed in the United Kingdom for the first time in 'Field monitoring'. In one Italian sample, a low frequency of C-G79R was shown. All other countries, including Denmark, were shown to have sensitive populations.

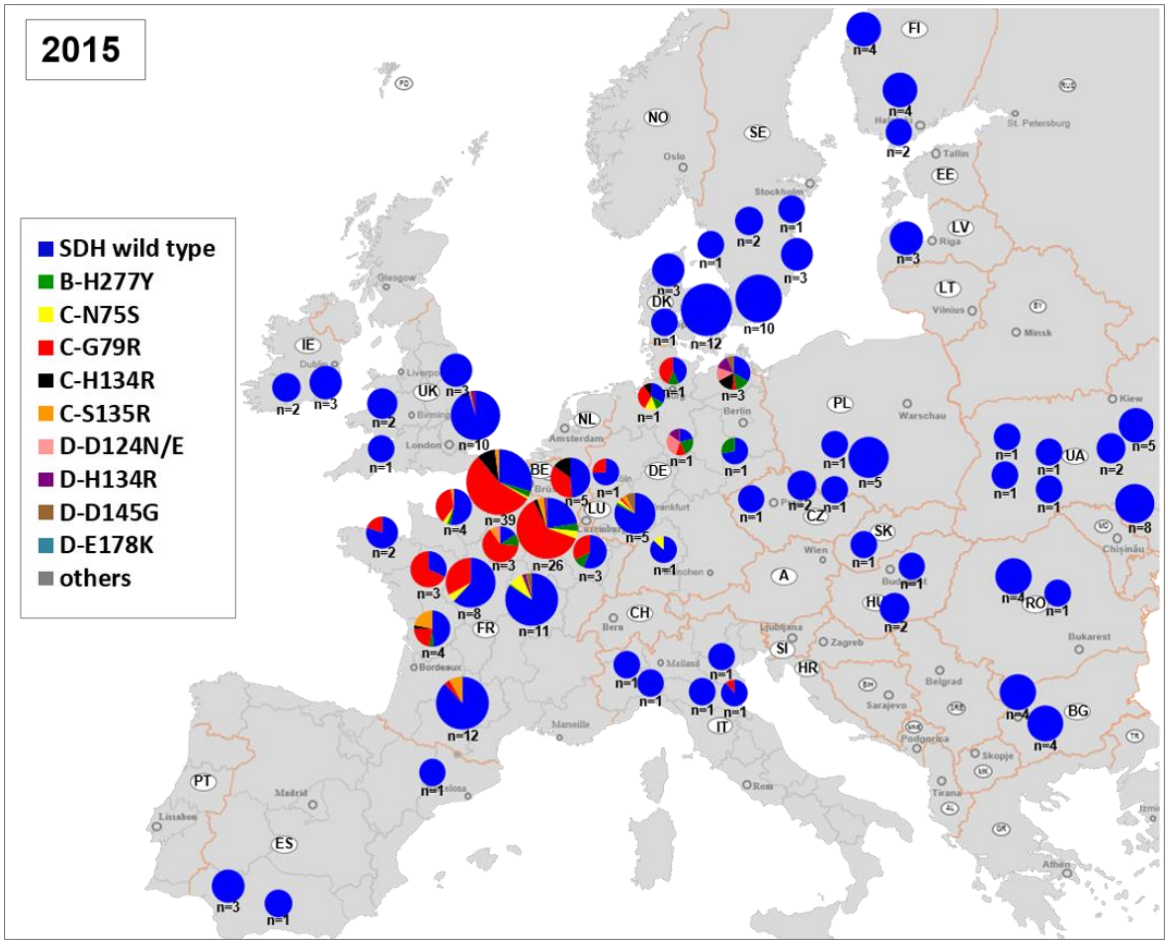


Figure 22: Frequency of genotypes causing SDHI resistance in *P. teres* collected in 'Field monitoring' from various European countries in 2015. Net blotch infected leaf samples (one sample includes a pool of 20 infected leaves randomly collected from one site) were taken from commercial sites and from untreated plots of trial sites. Frequency of alleles leading to SDHI resistance was measured using quantitative pyrosequencing. Colour code of SDH-variants and the number of samples are given in the figure. The size of the disc represents the number of analysed samples and the location of the disc indicates the origin of collected leaf samples. Samples are taken from Ireland (IE) (n=5), the United Kingdom (UK) (n=16), France (FR) (n=115), Spain (ES) (n=5), Belgium (BE) (n=5), Germany (DE) (n=14), Denmark (DK) (n=16), Sweden (SE) (n=17), Finland (FI) (n=10), Latvia (LV) (n=3), Poland (n=6), Czech Republic (CZ) (n=4), Italy (n=5), Hungary (HU) (n=3), Slovakia (SK) (n=1), Ukraine (UA) (n=19), Romania (RO) (n=5) and Bulgaria (BG) (n=8). These results were published recently (Rehfus et al., 2017, Figure 1).

In 2016, 183 samples from 19 different countries were analysed. The overview of the different genotypes found in infected leaf samples from European countries in 2016 is given in Figure 23. Countries which showed high levels of mutated alleles in the 2014 and 2015, such as France, Germany and Belgium, were also found to have highest frequencies of these genotypes in 2016.

In the United Kingom, low frequencies of C-H134R (12% in one region), C-S135R and B-H277Y (<5% in a region, which consisted of 9 samples) were detected. In contrast to other countries with SDHI resistant variants, no C-G79R was detected in the United Kingdom. In Poland, low frequencies of resistance alleles (B-H277Y and D-D145G) were detected in 2016 for the first time.

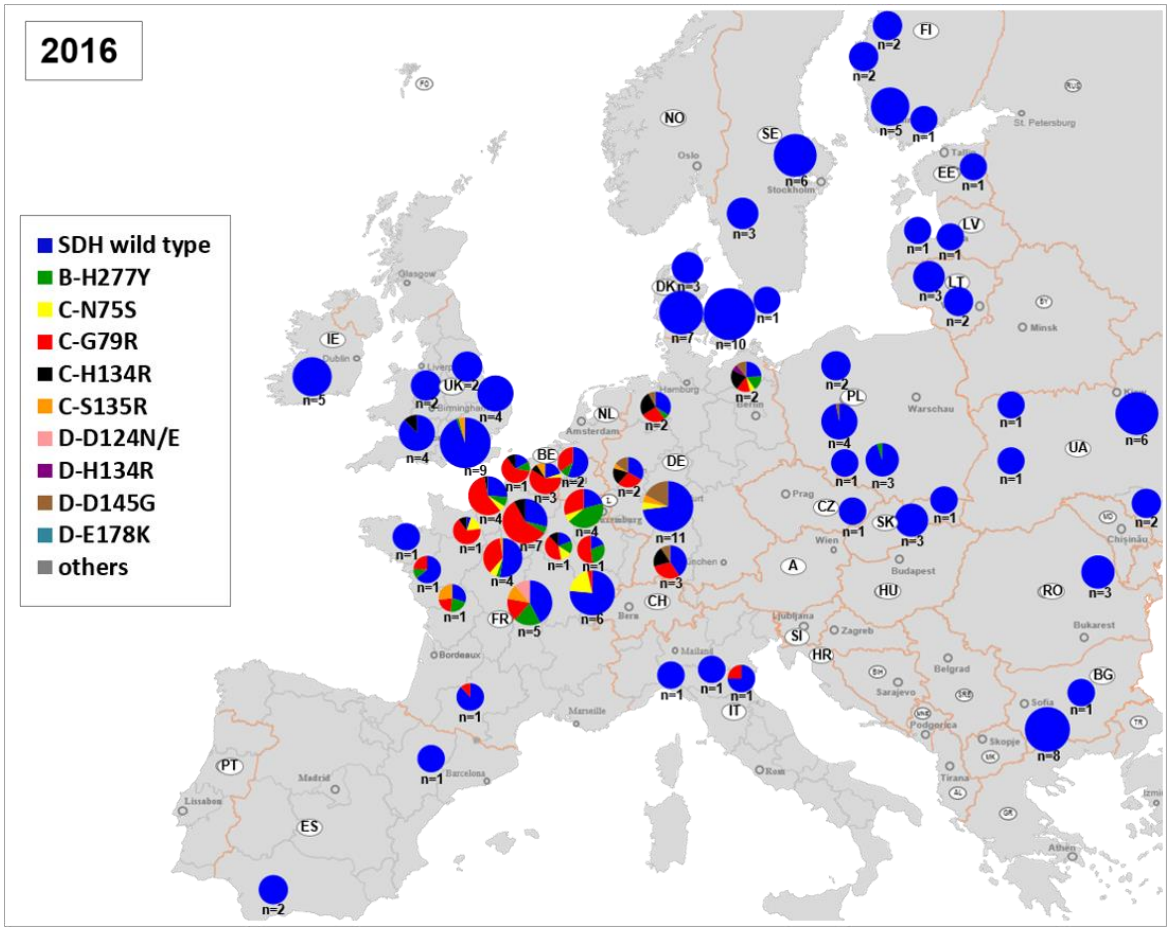


Figure 23: Frequency of genotypes causing SDHI resistance in *P. teres* collected in 'Field monitoring' from various European countries in 2016. Net blotch infected leaf samples (one sample includes a pool of 20 infected leaves randomly collected from one site) were taken from commercial sites and from untreated plots of trial sites. Frequency of alleles leading to SDHI resistance was measured using quantitative pyrosequencing. Colour code of SDH-variants and the number of samples are given in the figure. The size of the disc represents the number of analysed samples and the location of the disc indicates the origin of collected leaf samples. Samples are taken from Ireland (IE) (n=5), the United Kingdom (UK) (n=21), France (FR) (n=41), Spain (ES) (n=3), Belgium (BE) (n=2), Germany (DE) (n=20), Denmark (DK) (n=20), Sweden (SE) (n=10), Finland (FI) (n=10), Estonia (EE) (n=1), Latvia (LV) (n=2), Lithuania (LT) (n=5), Poland (n=10), Czech Republic (CZ) (n=1), Italy (n=3), Hungary (HU) (n=3), Slovakia (SK) (n=4), Ukraine (UA) (n=10), Romania (RO) (n=3) and Bulgaria (BG) (n=9).

In isolates obtained from 'Random monitoring', SDH-variant D-D145G was only found in Germany (see section 4.1.4.1). In accordance to that, the allele which causes D-D145G was also mainly detected in Germany in the 'Field monitoring'. In 2015, D-D145G was additionally detected in low frequencies in one sample from an eastern region in France.

The frequency of alleles, shown to cause SDHI resistance, is given in an overview in Figure 24. Here, all field samples which had been analysed in the years 2014 to 2016 are included. The proportion of resistance alleles in all samples from Europe was 26.2% in 2014, 27.4% in 2015 and 19.6% in 2016. C-G79R was shown to have the highest proportion within SDH-variants in field samples (77.5% in 2014, 71.7% in 2015 and 52.4% in 2015) (Figure 24 B). In 2016, other variants than C-G79R were found more frequently compared to the years before and the proportion of C-G79R within resistance alleles seems to have decreased over the years.

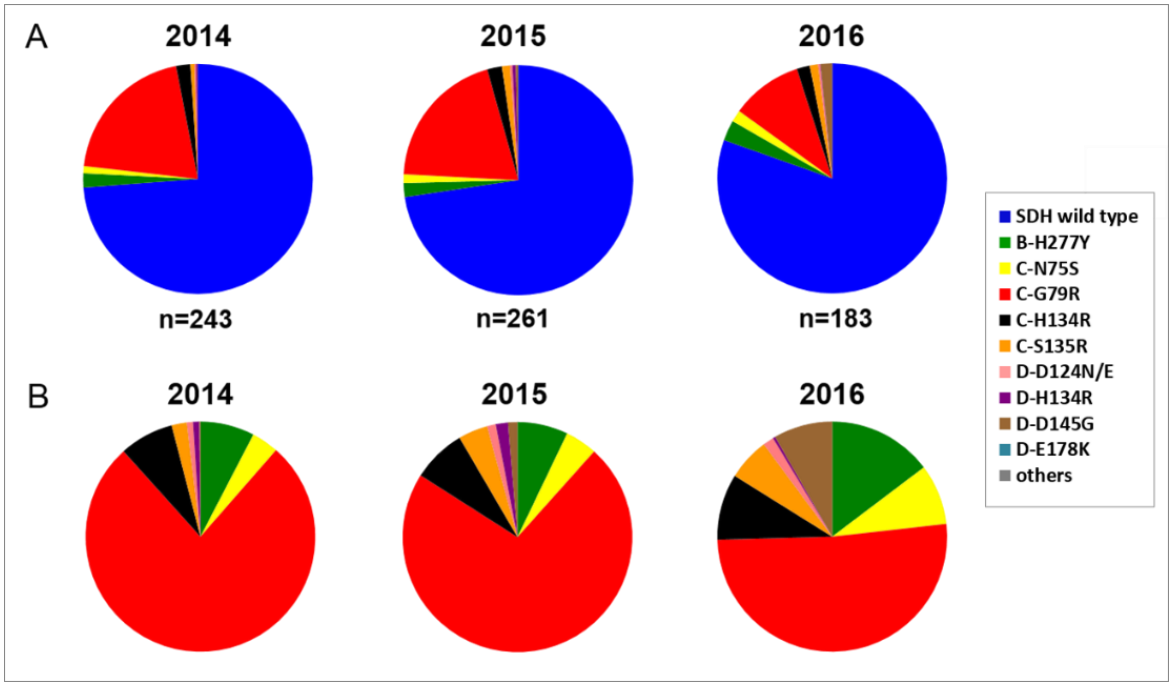


Figure 24: Frequency of alleles leading to SDHI resistance in *P. teres* in Europe from 2014 to 2016. Colour code of SDH-variants and the number of samples are given in the figure. **A**: Frequency of alleles leading to SDH variation in field samples from European countries from 2014 to 2016 (proportion of 'SDH wild type' was calculated by addition of resistance alleles and substraction from 100%). **B**: Frequency of alleles leading to different SDH-variants when 'resistant' proportion is regarded.

In the 'Field monitoring', samples were not taken balanced, which means that not the same number of samples from each region were analysed in every year. In 2014 and 2015, for example, more samples were collected in France and Germany compared to 2016. In 2016, countries with a still sensitive population of *P. teres*, such as Ukraine, sent a high proportion of samples compared to France and Germany. Therefore, the decrease of resistant alleles, which is observed in Figure 24, is probably a matter of unbalanced data.

The frequency of resistance genotypes is shown seperatly for the countries Germany and France (Figure 25) to see if there was a spread of resistant genotypes in both countries over the years. In Germany (Figure 25 A), the highest level of mutated alleles was observed in 2014. Here, C-G79R was the most frequent resistance mechanism. In 2015 and 2016, no further increase of resistance alleles was detected. However, sample numbers were low in 2015 and 2016, which was due to the relatively low infection pressure in many regions in these years. In 2015 and 2016, the proportion of alleles leading to C-G79R was lower than that found in 2014. The most frequent alteration in 2016 was D-D145G (14% in all samples), followed by C-G79R (11.6% in all samples) and thirdly C-H134R (9.6% in all samples). In France, an increase of alleles leading to SDHI resistance was observed (Figure 25 B). The frequency of resistance alleles raised from 42% in 2014 to 56% in 2015 and 59% in 2016. In contrast to Germany, the most frequent SDH-variant in France was shown to be C-G79R over all three years. However, a reduction of the C-G79R proportion within SDH-variants was observed in 2016 in France as well.

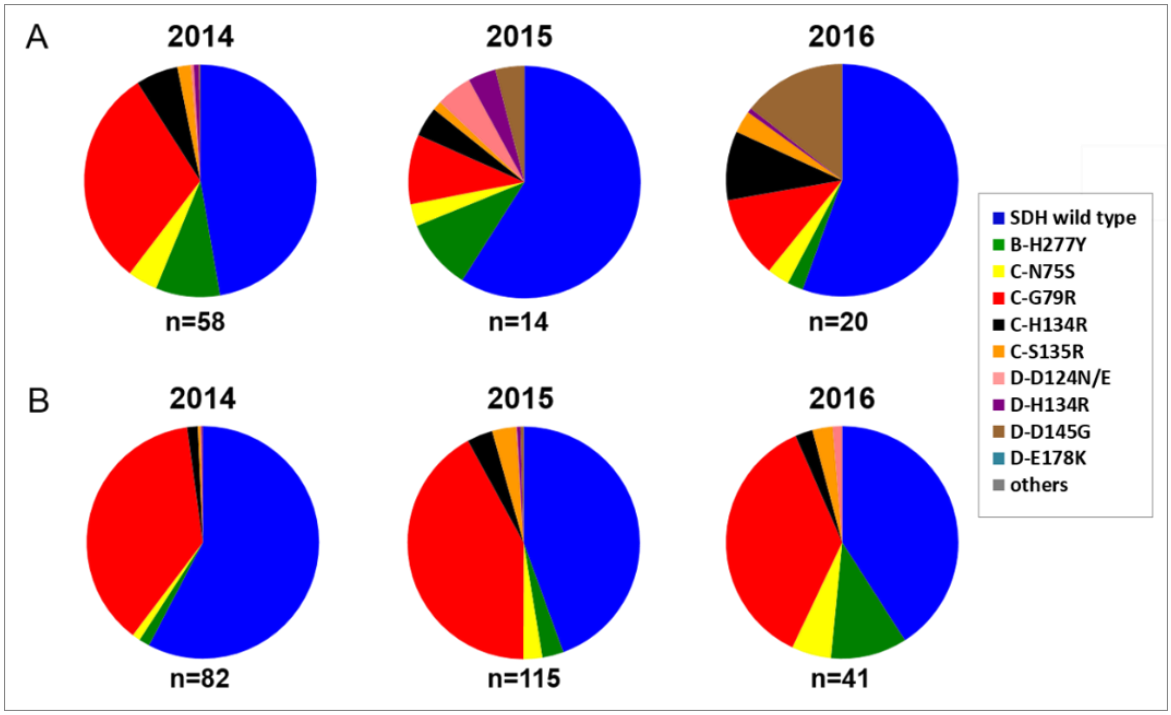


Figure 25: Frequency of alleles leading to SDHI resistance in *P. teres* in France and Germany from 2014 to 2016. Colour code of SDH-variants and the number of samples are given in the figure. Proportion of 'SDH wild type' was calculated by adding frequency of resistance alleles and substraction from 100%. **A:** Frequency of alleles leading to SDH-variation in field samples from Germany 2014 to 2016. **B:** Frequency of alleles leading to SDH variation in field samples from France 2014 to 2016.

In summary, C-G79R was the most dominant SDH-variant that was found in infected leaf samples from Europe. In 2015 and 2016, further mutations which cause SDHI resistance made up a significant proportion of the detected resistance alleles. The pattern of resistance alleles, even within a region, was shown to be a dynamic process.

4.1.4.3 Overview of frequency of SDHI resistant isolates

The frequency of SDH-variants connected to SDHI resistance in *P. teres* was determined by using two different sampling methods. On the one hand, the collection of air-borne spores from defined routes through European countries and the subsequent generation of isolates, and on the other hand, the collection of net blotch infected leaf samples and the determination of resistance alleles in DNA pools of 20 leaves per site. Both monitoring methods revealed that there was an increase, both in resistant isolates/alleles and the number of target-site mutations responsible for SDHI resistance, in some European countries since 2012. It was shown that amino acid substitution C-G79R predominantly was found in resistant isolates/samples. Both sampling methods indicated that the proportion of other SDH-variants than C-G79R increased in years 2014 to 2016. It was shown that most resistant isolates/samples were found in the northern and middle parts of France and Germany, but also in Belgium. The pattern of different SDH-variants was found to be highly comparable to each other when both monitoring methods were compared. However, some minor differences between 'Random monitoring' and 'Field monitoring' were found when countries such as the United Kingdom and Denmark were investigated.

4.1.4.4 Spot type of *P. teres* in Europe

The oligonucleotides that were used for pyrosequencing were designed on DNA of *P. teres* f. *teres* (PTT). In additional studies, it was tested whether amplification and detection of SNPs in *P. teres* f. *maculata* (PTM) type was possible as well. For this, two reference isolates of PTM and two reference isolates of PTT were obtained from CBS fungal isolate collection (Centraalbureau for Schimmelculturen, Utrecht, Netherlands). Furthermore, isolates were generated from leaf samples (obtained in 2014) which showed typical spot-type lesions instead of net-like lesions. The genes *SdhB*, *SdhC* and *SdhD* were sequenced.

Several nucleotide exchanges were found in the *Sdh* genes of PTM isolates compared to PTT isolates. The alignment of *SdhB* and *SdhC* sequences of PTT and PTM isolates is given in the Supplementary Material, Figure 49 and Figure 50. Most nucleotide exchanges did not affect amino acid sequence. However, two nucleotide exchanges were positioned at hybridisation site of primer KES 1847, which was used to detect B-H277Y in *P. teres*. Further, two nucleotide exchanges were detected at binding site of KES 1956 (C-N75S detection) and KES 2025 (C-G79R detection).

Pyrosequencing assays were tested for PTM reference isolates and in mixtures of 25%, 50% and 75% mixed with PTT isolates. No signals were obtained in case of PTM reference isolates when the B-H277Y assay was run. The other two assays for the detection of C-N75S and C-G79R resulted in robust signals that could be used for quantification. This means that molecular quantification of alleles which lead to SDHI resistance also includes spot-type genotypes of *P. teres*. One exception is B-H277Y, which was not detectable in PTM genotypes with primers used in this work. A list of samples and their origin, that showed spot-type lesions, and additionally had ‘robust’ signals in all assays with exception of B-H277Y assay are depicted in Supplementary Material, Table 34. These samples were mainly obtained from countries, such as Denmark and the UK, and eastern and southern countries, like Poland, Hungary, Italy and Spain. None of the SDHI resistant isolates from ‘Random monitoring’ showed PTM sequence, which is probably due to fact that PTT is the predominating type of *P. teres* in Germany, France and Belgium, where the highest proportion of SDHI resistant isolates was sampled.

4.1.5 Multiple resistance in *P. teres*

In addition to SDHIs, two other classes of single-site inhibiting fungicides have a predominant relevance in the protection of cereals against phytopathogenic fungi, the QoIs and the DMIs. In cereals, SDHI fungicides are often used in combination to both these classes for a better and broader disease control. *P. teres* isolates were additionally analysed with respect to QoI resistance and their DMI sensitivity in order to gain information about the occurrence of multiple resistance in *P. teres*.

4.1.5.1 QoI resistance in SDHI resistant isolates of *P. teres*

QoI resistance in *P. teres* is mainly mediated by mutations in Cyt *b* which lead to F129L exchange in complex III of the respiratory chain (Semar et al., 2007; Sierotzki et al., 2007). A few QoI resistant isolates from Ireland showed another mutation in Cyt *b* which cause G137R exchange (Sierotzki et al., 2007; FRAC, 2016). Both variants were shown to cause phenotypes which are moderately resistant to QoI fungicides (Semar et al., 2007). Existing pyrosequencing assays for F129L and G137R were used to analyse the QoI resistance status of SDHI resistant *P. teres* isolates. Additionally, the absence of G143A was verified using an existing qPCR assay. G143A and G137R were not detected in any of the tested isolates. F129L was detected in 18% of SDHI resistant isolates in 2013, 19% in 2014 and 34% in 2015. Thereby, differences of the F129L frequency was observed in isolates having different SDH-variants. The proportion of SDHI resistant isolates, which simultaneously had F129L exchange, is given in Figure 26 (seperately for each SDH-variant). The F129L frequency in Europe has been stable for many years within different regions (FRAC, 2016; internal data). In Germany, for example, a mean F129L frequency of around 20-30%, and in France around 30-40%, was measured for at least three years. This was not only measured in the QoI ‘Random monitoring’ conducted by EpiLogic but also in the pyrosequencing-based quantification of net blotch infected leaf samples performed during the present study (data not shown). However, the frequency of F129L in *P. teres* populations can vary between different regions and also within countries. Therefore, the proportion of F129L (frequencies obtained from QoI ‘Random monitoring’ carried out by EpiLogic) in isolates collected on the same sampling routes is shown in Figure 27.

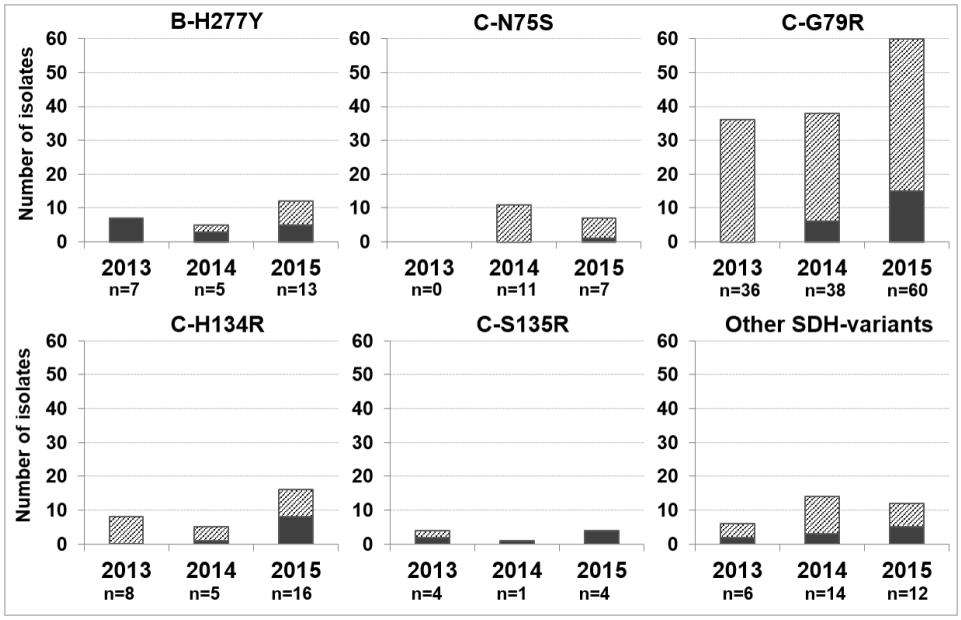


Figure 26: Proportion of SDHI and QoI double resistant *P. teres* isolates obtained from 'Random monitoring'. Each colomn represents collected isolates from year 2013, 2014 or 2015 having the same *Sdh* genotype. Colour code: Proportion of SDH-variant without F129L; Proportion of isolates simultaneously carrying an SDH-variant and F129L. Isolates containing different SDH-variants B-H277Y, C-N75S, C-G79R, C-H134R and C-S135R are shown separately, whereas SDH-D mutants (D-D124N/E, D-H134R, D-G138V, D-D145G, D-E178K) and newly found C-S73P in 2015 are shown together in one diagramme (other SDH-variants). The number of isolates having respective variant is given in the figure. F129L occurrence in SDHI resistant isolates was detected by pyrosequencing.

In 2013, only a few isolates had the F129L exchange in combination to an SDH-variant (mainly B-H277Y mutants). On routes, where C-G79R mutants had been collected, 25% of these isolates showed the F129L exchange. However, none of the 36 C-G79R mutants showed the F129L exchange in combination. In total, 11 SDHI resistant isolates carried F129L exchange in combination, whereas 50 SDHI resistant isolates were not QoI resistant. A slight increase of double mutants was observed in 2014. Thereby, C-G79R mutants showed the strongest increase of F129L in combination when the years 2013 and 2014 are compared. In 2015, the number of double resistant isolates further increased to 34% and was found to be highly comparable to frequencies obtained in the 'random' population on the same routes.

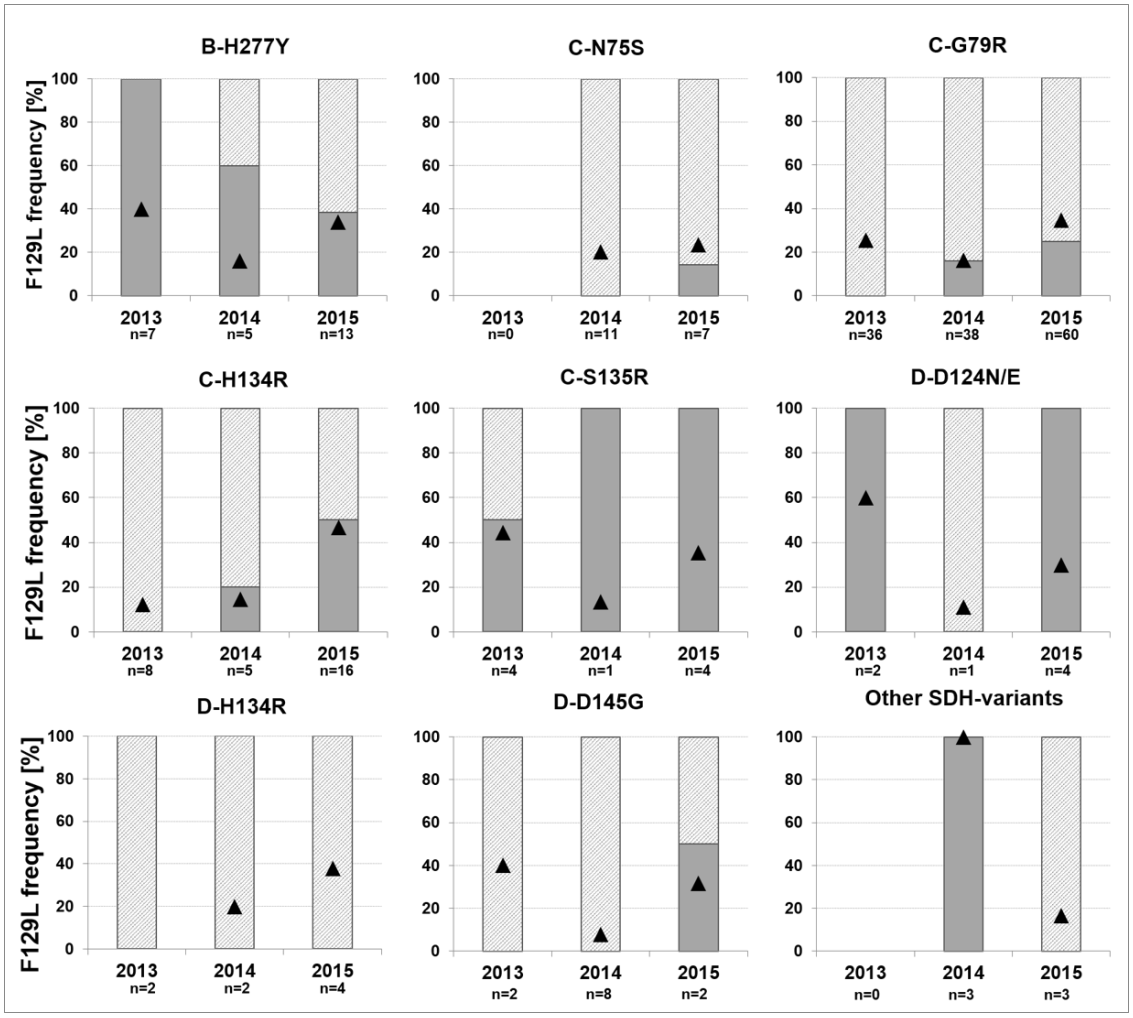


Figure 27: Frequency of F129L [%] in analysed SDHI resistant isolates of *P. teres* collected in 'Random monitoring' in the year 2013 to 2015. Each colomn represents collected isolates from year 2013, 2014 or 2015 having the same *Sdh* genotype. Colour code: Proportion of SDH-variant without F129L; Proportion of isolates simultaneously carrying an SDH-variant and F129L; ▲Frequency of F129L in all isolates collected from same routes in Europe where SDH resistant isolates were sampled (values are taken from QoI 'Random monitoring', internal unpublished data). Isolates containing different SDH-variants B-H277Y, C-N75S, C-G79R, C-H134R, C-S135R, D-D124N/E, D-H134R and D-D145G are shown separately, whereas newly found SDH-C and SDH-D mutants (D-E178K in 2014, C-S73P and D-G138V in 2015) are shown together in one diagramme. SDH-variant and the number of isolates having this variant is given in the figure. F129L occurrence in SDHI resistant isolates was detected by pyrosequencing. Frequency of F129L in 'random' population is missing in the case of D-H134R in 2013 because no data was available in QoI 'Random monitoring' from the same routes.

4.1.5.2 DMI sensitivity of SDHI resistant isolates of *P. teres*

Several isolates of *P. teres* from the year 2014 that had been collected in different European countries were investigated with respect to their sensitivity towards DMIs.

In order to establish whether there are differences in DMI sensitivity between SDH wild type and SDHI resistant isolates, the efficacy of the DMI prothioconazole against 29 SDHI sensitive isolates and 24 SDHI resistant isolates was measured in microtiter tests (Figure 28).

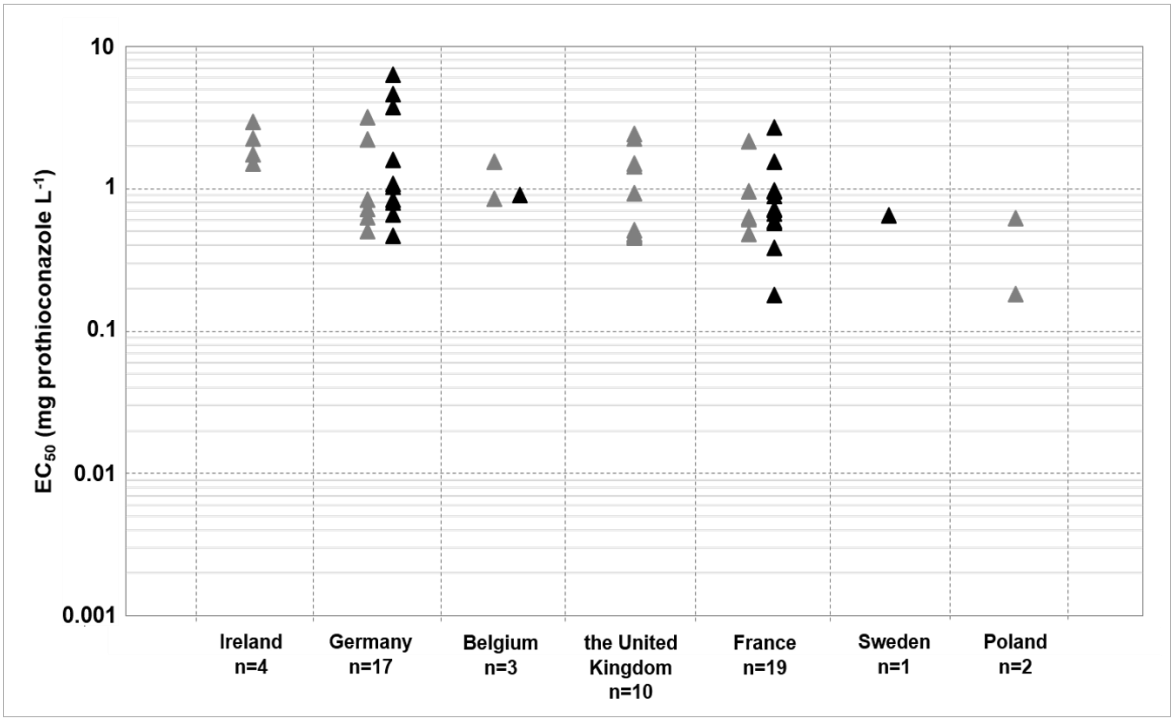


Figure 28: Prothioconazole sensitivity of SDHI sensitive and SDHI resistant isolates of *P. teres* obtained from 'Random monitoring' 2014. Photometric measurements (OD_{405}) detecting mycelial growth of *P. teres* in microtiter plates were used to determine EC_{50} values (probit calculation) of prothioconazole. Water control and seven concentrations of prothioconazole in four replicates were mixed with spore suspensions of SDH wild type isolates (n=29) and several SDH mutated isolates carrying different mutations in the *Sdh* genes (n=24).
Colour code: ▲ EC_{50} values of SDHI sensitive isolates, ▲ EC_{50} values of SDHI resistant isolates. The origin and the number of isolates is given in the figure.

EC_{50} values of all the isolates tested ranged from 0.179 to 6.28 mg prothioconazole L^{-1}. SDH wild types varied in their sensitivities from 0.182 to 3.15 mg prothioconazole L^{-1}. SDHI resistant isolates showed a slightly higher range of sensitivities from 0.179 to 6.28 mg prothioconazole L^{-1}. Within countries, isolates were highly comparable, independent of their SDHI resistance. Minor differences in EC_{50} values to prothioconazole were observed when isolates between different countries are compared. Although, *P. teres* isolates collected from different countries show a high range of different DMI sensitivities, no indication for a DMI 'shift' can be found. Isolates from earlier years show a similar range of EC_{50} values for prothioconazole (internal unpublished data). For example, isolates from 2001 collected from France, Germany and the Netherlands had EC_{50} values of 0.15 to 2.46 mg prothioconazole L^{-1} and isolates from 2010 collected in France, Germany and Ireland ranged from 0.073 to 15.79 mg prothioconazole L^{-1}. Highest EC_{50} values in 2010 were also observed for isolates sampled in Ireland, as detected in this study. Differences between countries should be studied in further experiments to see whether these differences are significant and have genetic reasons.

4.1.6 Competition studies on *Sdh* mutants of *P. teres* in *in planta* studies

Quantitative pyrosequencing assays were used to perform competition studies of SDHI-resistant isolates of *P. teres* in the glasshouse. Isolate mixtures which contained one resistant and one sensitive isolate (1:1) were prepared and applied to barley plants. Infected leaves of each mixture were harvested after 10-21 days and were used to prepare spore suspensions for the inoculation of new barley plants. Each mixture was transferred over several propagation cycles in the glasshouse without any use of fungicides. At the starting point, 2 mL of a spore suspension with 2.5E+04 spores mL^{-1} of each isolate or mixture were used to inoculate a pot with ~10 barley plants. Repetitions of each isolate and mixture were four to six pots, depending on the experiment. After 10-21 days, infected leaves were harvested and washed in a defined amount of water and were subsequently used to inoculate the next round of plants. An aliquot of each spore suspension was taken to extract DNA and was used to quantify the amount of the point mutations of interest. In addition to mixtures, each isolate was propagated individually and the infected leaf area was rated. DNA was also extracted from infected plants inoculated with single isolates, to check if there is cross-contamination between infected plants of each isolate. Four to six additional uninoculated plants were placed between replicates of isolates to see if there was any unintended spreading of isolates.

SDHI resistant isolates that carry exchanges B-H277Y, C-N75S, C-G79R and C-H134R were tested in a first approach. Each of these isolates were mixed with an isolate that carried the same *Sdh* mutation and additionally also F129L. This test was done to elaborate whether double resistant isolates show a 'fitness' disadvantage, compared to SDHI resistant isolates.

In Figure 29, a decrease of the F129L frequency can be seen in all mixtures when the starting point is compared to the second cycle. In this approach, a decrease of F129L frequency indicates a decrease of the double mutant in the mixture. F129L frequency decreases in the 'B-H277Y' mixture from 89 to 63%, in the 'C-N75S' mixture from 80 to 59%, in 'C-G79R' mixture from 60 to 46% and in the 'C-H134R' mixture from 32 to 0%. This indicates that SDHI resistant isolates of *P. teres* had an advantage in the growth on barley plants against SDHI/QoI double resistant isolates. After the third cycle, the experiment was stopped due to low infections pressures seen in many single isolates and mixtures, which did not give enough inoculum for another infection cycle.

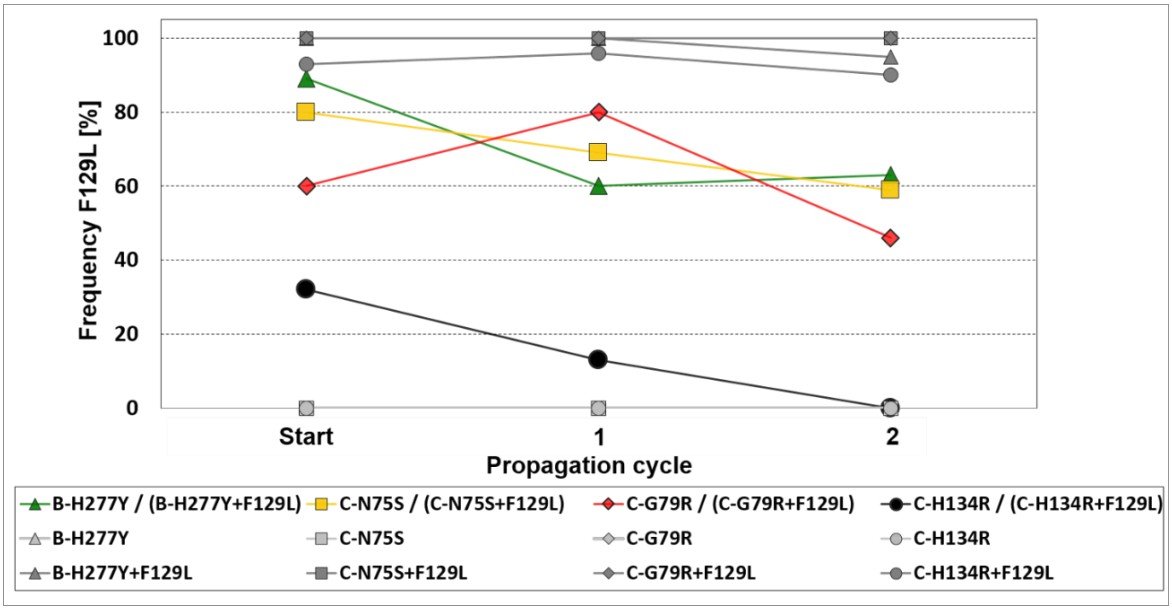


Figure 29: *In planta* competition studies of SDHI resistant isolates against SDHI and QoI double resistant isolates of *P. teres* under non-selective conditions. Four SDHI-resistant isolates, which carry B-H277Y, C-N75S, C-G79R or C-H134R and four SDHI/QoI resistant isolates (B-H277Y+F129L, C-N75S+F129L, C-G79R+F129L or C-H134R+F129L) were used to test the ability of double mutants to compete with single resistant isolates when propagated together over several rounds on barley cv. 'Astrid' (BBCH 11) without any fungicide pressure. Each mixture contains one SDHI-resistant and one SDHI/QoI-resistant isolate (B-H277Y mixture: green, C-N75S mixture: yellow, C-G79R mixture: red, C-H134R mixture black). In addition to mixtures, each isolate, which was used in a mixture, was transferred over the cycles alone. Spore suspensions were prepared from POA plates at the starting point or by washing infected leaves in Tween-water. Mixtures were subsequently transferred over two propagation cycles and DNA of spore suspension at each propagation cycle was analysed by pyrosequencing. F129L frequency [%] was measured in mixtures and single isolates (pool of six infected plants each mixture/isolate).

Table 24 shows the diseased leaf area [%] of all isolates and mixtures over the three infection cycles. In the first cycle, plants were inoculated with a defined number of spores (all isolates/mixtures were adjusted to 2.5E+04 spores mL^{-1}). Diseased leaf area was high in almost all inoculated plants. Lowest infection rates (22.5%) were observed in a double resistant isolate carrying B-H277Y and F129L exchange. Highest infection rates (80-87%) were observed for isolates carrying C-N75S variant, which was also seen in the mixture of these isolates.

In the second cycle, where infected leaves from the first round were used to inoculate new plants, the diseased leaf area decreased in many isolates and in corresponding mixtures of them. Infection rates decreased over the following propagation cycles to under 5% in case of B-H277Y and B-H277Y+F129L mutants, C-G79R and C-G79R+F129L mutants and in the double mutant C-H134R+F129L. Whereas infection rates of C-N75S and C-N75S+F129L mutants and single mutant carrying C-H134R remained high over all cycles. Spore number (mean of two agar plates, which were washed in a defined volume of water) was additionally analysed. Due to low number of isolates in this experiment, further experiments were conducted with more isolates of each genotype.

Table 24: Pathogenicity of SDHI resistant and SDHI/QoI double resistant isolates of *P. teres* on barley cv. 'Astrid'. Mean of four replicates, each replicate contained a pot with around 10 barley plants, shown for all propagation cycles and standard error is given. Each pot was inoculated with 2 mL of a spore suspension, which contained 2.5E+04 spores mL^{-1}. After 10-21 dpi, infected leaves of each isolate/mixture were harvested. Infected leaves within an isolate/mixture were pooled and washed in 10 mL of Tween-H_2O and applied to a next charge of plants.

Aa exchange(s)	Isolate ID	Number of spores [$x10^4$ spores mL^{-1}]	Diseased leaf area [%] (mean ± SE)			
			Cycle 1*	Cycle 2*	Cycle 3*	Mean (cycles)
B-H277Y	1710	27.5	52.5 ± 4.79	7.5 ± 1.44	1.3 ± 0.75	20.4
C-N75S	1688	40.5	80.0 ± 4.08	35.0 ± 2.89	40.0 ± 7.07	51.7
C-G79R	1678	25.5	60.0 ± 4.08	6.25 ± 1.25	1.2 ± 0.58	22.5
C-H134R	1707	30	67.5 ± 4.78	42.5 ± 2.50	80.0 ± 2.04	63.3
B-H277Y+F129L	1687	2.5	22.5 ± 2.5	5.0 ± 2.04	1.8 ± 0.63	9.8
C-N75S+F129L	1703	24.5	87.5 ± 2.5	32.5 ± 6.29	40.0 ± 4.08	53.3
C-G79R+F129L	1669	12	72.5 ± 2.5	15.0 ± 2.89	0	29.1
C-H134R+F129L	1672	29.5	47.5 ± 4.79	6.25 ± 1.25	1.3 ± 1.25	18.4
Mixture B-H277Y	1710+1687		40.0 ± 4.08	8.75 ± 1.25	0.8 ± 0.75	16.5
Mixture C-N75S	1688+1703		85.0 ± 2.89	32.5 ± 2.50	45.0 ± 6.45	54.2
Mixture C-G79R	1678+1669		70.0 ± 4.08	17.5 ± 4.79	21.3 ± 3.14	36.3
Mixture C-H134R	1707+1672		67.5 ± 2.50	12.5 ± 2.50	5.0 ± 1.02	28.3

* all spore suspensions from cycles 1-3 were analysed using quantitative pyrosequencing, in cycle 3 many samples showed undetectable levels of DNA, therefore, cycle 3 is not shown in Figure 29.

As described in section 4.1.4, C-G79R was the most frequent SDH-variant detected in SDHI resistant isolates in Europe. In addition to its high frequency, this SDH alteration gave one of the highest impacts of all variants on SDHIs. The following competition studies focused on the C-G79R mutants. When C-G79R mutants were analysed with respect to the occurrence of multiple resistance to QoI fungicides, F129L had been found in a lower number of C-G79R mutants than had been expected, particularly in the year 2013 (see section 4.1.5.1). Although the 'background' population in the same regions showed 25% F129L, none of the C-G79R mutants carried F129L in combination. On the one hand, this could be due to random effects or selection of mutants by products predominantely used in the field. On the other hand, this could be an indication of a possible 'fitness' penalty associated with multiple mutations in genes encoding for respiratory chain enzymes. In the following competition tests, C-G79R mutants (SDHIres), F129L mutants (QoIres) and C-G79R+F129L (SDHIres+QoIres) mutants were investigated. Isolates, resistant to one fungicide class, were mixed with completely sensitive isolates. Double resistant isolates (C-G79R+F129L) were tested in competition with sensitive isolates and in competition with isolates that were resistant to one of these fungicide classes. In first infection studies to check the pathogenicity of isolates, it was observed that reference isolates Pt 1013, Pt 1020

and Pt 1022, which have been used for many years as sensitive isolates in microtiter tests, showed a reduced pathogenicity on barley cv. 'Astrid' compared to some selected SDHI resistant isolates of *P. teres* and SDHI sensitive isolates from the year 2014 (data not shown).

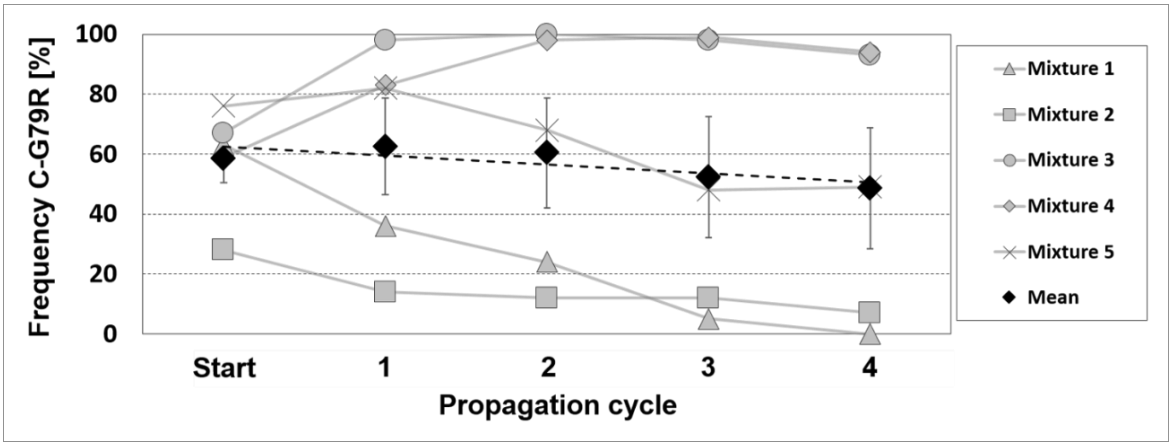


Figure 30: *In planta* competition studies of C-G79R mutants in mixtures with sensitive isolates of *P. teres* under non-selective conditions. Five SDHI-resistant (carrying C-G79R) and five SDHI sensitive isolates obtained from 'Random Monitoring' 2014 were used to test the ability of C-G79R mutants to compete with SDHI-sensitive isolates when propagated together over several rounds on barley cv. 'Astrid' (BBCH 11) without any fungicide pressure. Each mixture (mixture 1-5) contains one SDHI-resistant and one SDHI-sensitive isolate. All isolates used were shown to lack F129L and therefore are sensitive towards QoIs. Isolates were mixed according to their sampling origin. Isolates in mixture 1 and 4 were obtained from Germany, in mixture 2 from France, in mixture 3 from Belgium, and in mixture 5 the SDHI-resistant isolate from France and the SDHI-sensitive one from Germany. Spore suspensions were prepared from POA plates at the starting point or by washing infected leaves in malt-gelatine medium. Mixtures were subsequently transferred over four propagation cycles and DNA of spore suspension at each propagation cycle was analysed by pyrosequencing. In addition to different mixtures, which are shown in grey, the mean of all mixtures and their standard errors are given in black. The slope of the linear regression is -3.02 and was shown to not significantly vary from 0 (P=0.05). C-G79R frequency of single isolates was 0% (sensitive isolates) or 100% (C-G79R mutants) over all cycles (data not included in the figure).

Appropriate SDHI sensitive isolates for the competition studies were chosen according to the following criteria: Isolates (sensitive and resistant) were taken from the same sampling year (2014) and were mixed in pairs from same sampling country (in most pairs). Isolates and their origin that were used in mixtures are given in Supplementary Table 42. The competitiveness of C-G79R mutants compared to sensitive isolates over four propagation cycles under glasshouse conditions is shown in Figure 30. The frequency of C-G79R over all cycles and in all mixtures/isolates was measured using quantitative pyrosequencing. A slight reduction (not significant, P>0.05) of C-G79R frequency was observed in the mean of all mixtures. However, different mixtures revealed a highly diverse picture of increase and decrease. In two mixtures, C-G79R frequency increased to 100%, whereas it decreased in three other mixtures. These results indicate that field isolates show a high variability in their fitness, independent of their resistance status, and the outcome of competition tests can be dependent on the isolates/mixtures investigated.

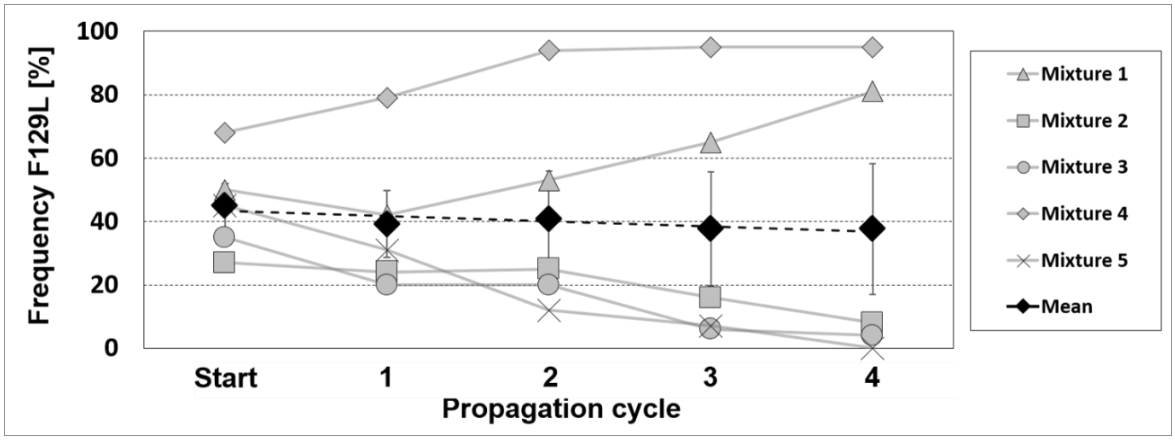


Figure 31: *In planta* competition studies of F129L mutants in mixtures with sensitive isolates of *P. teres* under non-selective conditions. Five QoI resistant (carrying F129L) and five QoI sensitive isolates obtained from 'Random Monitoring' 2014 were used to test the ability of F129L mutants to compete with QoI sensitive isolates when propagated together over several rounds on barley cv. 'Astrid' (BBCH 11) without any fungicide pressure. Each mixture (mixture 1-5) contains one QoI resistant and QoI sensitive isolate. All isolates used were shown to lack SDH exchanges and therefore are sensitive towards SDHIs. Isolates are mixed according to their sampling origin. Isolates in mixture 1 were obtained from Ireland (QoI resistant isolate) and the United Kingdom (QoI sensitive isolate), in mixture 2 from France, in mixture 3 from the United Kingdom, in mixture 4 from France (QoI resistant) or Germany (QoI sensitive) and in mixture 5 from the United Kingdom (QoI resistant isolate) and Germany (QoI sensitive). Spore suspensions were prepared from POA plates at the starting point or by washing infected leaves in malt-gelatine medium. Mixtures were subsequently transferred over four propagation cycles in the glasshouse and 2 mL of spore suspension at each propagation cycle was analysed by pyrosequencing in two repetitions. In addition to different mixtures, which are shown in grey, the mean of all mixtures and their standard errors are given in black. The slope of the linear regression is -1.62 and was shown to not significantly vary from 0 (P=0.05). F129L frequency of single isolates was 0% (sensitive isolates) or 100% (F129L mutants) over all cycles (data not included in the figure).

Additionally, the competitiveness of F129L field mutants of *P. teres* was analysed in mixtures of QoI resistant and QoI sensitive isolates. All the isolates used in this test did not show resistance towards SDHIs. The F129L frequency in mixtures was analysed over four propagation cycles and is given in Figure 31. A decrease of F129L frequency was observed in three mixtures (mixtures 2, 3 and 5). In contrast, an increase of F129L frequency was detected in mixtures 1 and 4, which indicates an isolate-dependent variability. In the mean of all mixtures, a slight but insignificant (P>0.05) decrease in F129L level can be seen.

Furthermore, the competitiveness of double resistant isolates (C-G79R+F129L) was studied in comparison to different isolates, which either showed no resistance (sensitive isolates) or were resistant towards SDHIs or QoIs. The results of these studies are depicted in Figure 32. Figure 32 A presents the C-G79R frequency in three mixtures with C-G79R+F129L mutants and F129L mutants. The frequency of F129L in three mixtures of C-G79R+F129L mutants with C-G79R mutants is shown in Figure 32 B, and the C-G79R frequency in four mixtures of C-G79R+F129L mutants with sensitive isolates is given in Figure 32 C. A slight decrease of the investigated resistance alleles was observed in all three approaches in the mean of all mixtures. However, as had been observed in fitness tests with single mutants, a high variability of different mixtures could be also observed in competition studies with double mutants. In the first approach, where double resistant isolates had been mixed with single QoI resistant isolates, C-G79R frequency decreased in two mixtures which indicated a decrease of double mutants, whereas C-G79R frequency

increased to 100% in the third mixture. In the second approach, where double resistant isolates had been mixed with single SDHI resistant isolates, two mixtures showed a decrease in F129L frequency which indicated a decrease of the double mutants and a slight increase of F129L level in a third mixture. In the last approach, where C-G79R+F129L mutants had been mixed with completely sensitive isolates, a decrease of C-G79R frequency was observed in two mixtures and a slight increase in two other mixtures. All isolates and mixtures over all cycles were analysed for both SNPs leading to C-G79R and F129L (only mutation of interest is shown in the figures).

In general, competition studies with field isolates of *P. teres* indicated a high variability of isolates, although the isolates involved had been collected in similar years and regions. In all cases of competition tests in the glasshouse, one or two mixtures showed the opposite trend to the majority of mixtures. In these mixtures, resistant field isolates were able to compete with sensitive isolates, and were shown to reach levels of up to 100% in mixtures. Taking all competition studies of *P. teres* into account, a slight decrease of the resistance alleles (in mean of all mixtures) was observed in each approach. This would indicate that the sensitive isolates (or single resistant isolates) used in these studies, showed a slightly higher competitiveness *in planta* than most resistant isolates (or double resistant isolates). However, a significant fitness penalty of C-G79R and C-G79R+F129L was not observed in the studies presented, which was due to the high variation.

All isolates (WT, F129L, C-G79R and C-G79R+F129L) that were used to prepare mixtures in fitness tests were analysed with respect to the number of spores that they produced on an artificial medium and their pathogenicity *in planta* during presented competition studies (Table 25).

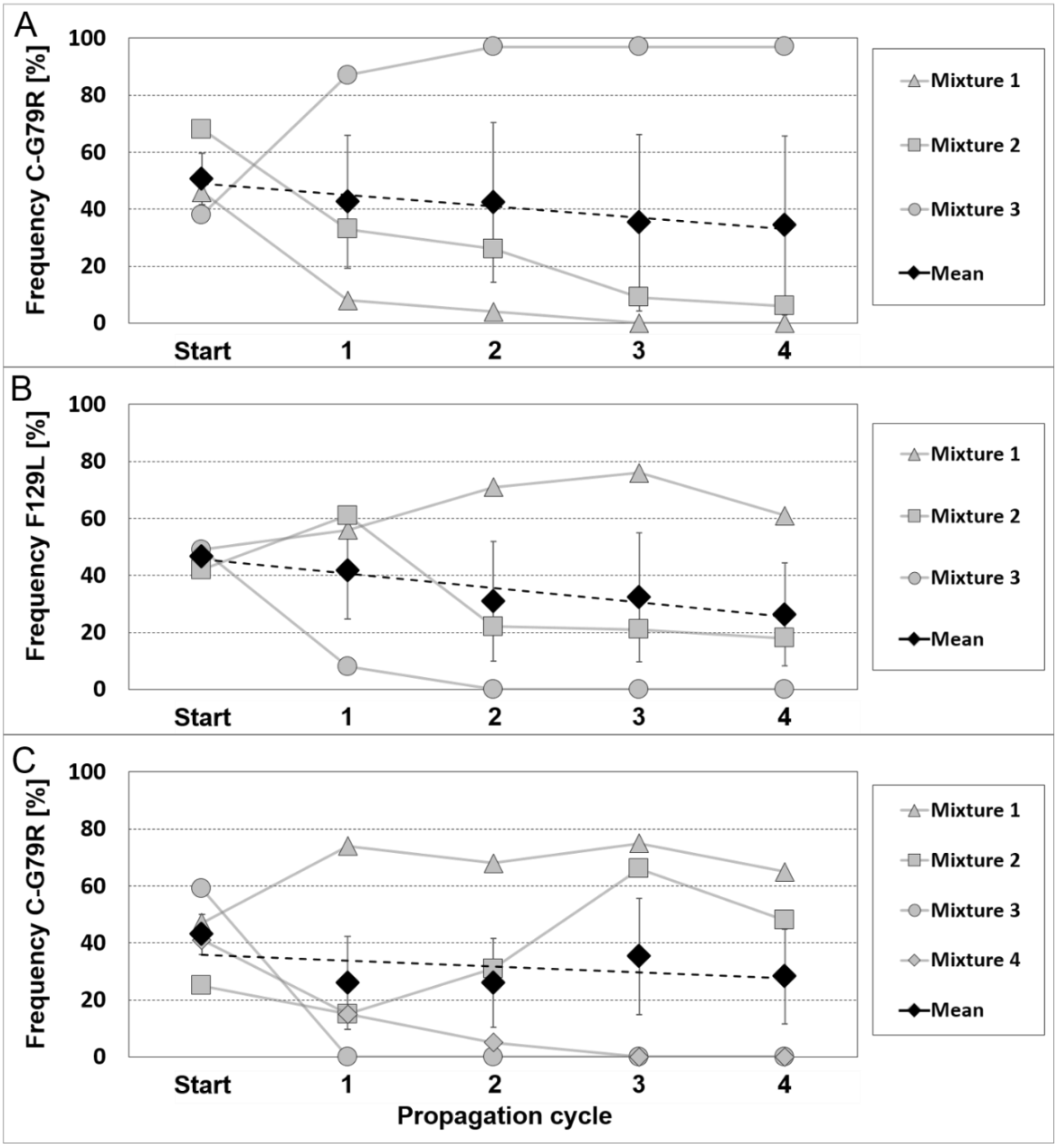


Figure 32: *In planta* competition studies of C-G79R+F129L double resistant isolates in mixtures with sensitive, QoI-resistant or SDHI-resistant isolates of *P. teres* under non-selective conditions. A: Double resistant (SDHIres+QoIres) isolates against QoI-resistant isolates (SDHIsens+QoIres); **B:** Double resistant (SDHIres+QoIres) isolates against SDHI-resistant isolates (SDHIres+QoIsens); **C:** Double resistant (SDHIres+QoIres) isolates against sensitive isolates (SDHIsens+QoIsens). The isolates included were six isolates (carrying C-G79R+F129L), four sensitive isolates (SDHIsens+QoIsens) and six isolates resistant to one fungicide class (3 isolates SDHIres, 3 isolates QoIres). Isolates were obtained from 'Random Monitoring' 2014 and were used to test the ability of double mutants to compete with sensitive isolates when propagated together over several rounds on barley cv. 'Astrid' (BBCH 11). Each mixture contains one double resistant and one other isolate. Isolates were mixed according to their sampling origin (in most cases). Spore suspensions were prepared from POA plates at the starting point or by washing infected leaves in malt-gelatine medium. Mixtures were subsequently transferred over four propagation cycles in the glasshouse and 2 mL of spore suspension at each propagation cycle was analysed by pyrosequencing in two repetitions. In addition to different mixtures, which are shown in grey, the mean of all mixtures and their standard errors are given in black. The slope of the linear regression is -4.0 (A), -5.0 (B), -2.03 (C) and was shown to not significantly vary from 0 (P=0.05).

Spore production on POA medium was highly dependent on the isolate that was analysed, independent of its resistance status. Spore numbers did not significantly vary (P=0.05) between sensitive and QoI-, SDHI or QoI and SDHI resistant isolates, although sensitive isolates (on average) produced slightly higher numbers of spores. Spore suspensions of each isolate were adjusted to 2.5E+04 spores mL^{-1} and were used to inoculate six pots with ~10 barley plants (2 mL each pot). Diseased leaf area was assessed 14 dpi (cycle 1- rating is given in Table 25). On average, the pathogenicitiy of sensitive isolates was not significantly different (P>0.05) to that of resistant isolates. Highest variation of infected leaf area was again observed in different isolates independent of their resistance situation, which further demonstrates the high variability of isolates collected from the field.

Table 25: Spore production on an artificial medium and pathogenicity of SDHI sensitive, C-G79R mutated and C-G79R+F129L double mutated isolates of *P. teres*. Each isolate was transferred to two POA plates. 10 days later, all spores on each plate were removed using a Drigalski spatula and 4 mL of water, and were filtered through gauze. The number of spores was counted in a Thoma cell chamber. Spore suspensions were adjusted to 2.5E+04 spores mL^{-1}. Six pots with ~10 barley plants per pot were inoculated with 2 mL of spore suspension each pot. Infected leaves were visually rated 14 dpi. Mean of six replicates shown for all isolates (n=33) and standard errors are given. Unpaired t-test (P=0.05) was used to calculate if mean of sensitive isolates (QoIsens+SDHIsens) significantly vary from that of mutated isolates showing F129L, C-G79R or C-G79R+F129L.

Aa exchange(s)	No. of isolates	Number of spores [x10^4 spores mL^{-1}]		Diseased leaf area [%] (mean ± SE)	
		Mean (two repetitions)	**Mean** (isolates ± SE)	**Mean** (four repetitions each isolate)	**Mean** (isolates ± SE)
no	9	53, 34, 40, 14.5, 23.5, 60, 16.5, 24.5, 73.5	37.7 ± 6.87[a]	43, 54, 39, 38, 58, 39, 35, 33, 58	44.1 ± 3.3[a]
F129L	8-10	48, 7.5, 24.5, 40.5, 14.5, 14.5, 18, 12.5, 10.5, 42	23.3 ± 4.68[a]	56, 39, 33, 57, 63, 58, 48, 45	49.9 ± 2.5[a]
C-G79R	10-11	16, 53.5, 45, 14.5, 29.5, 12, 37.5, 10.5, 10.5, 21, 13.5	23.9 ± 4.58[a]	68, 61, 43, 50, 48, 50, 43, 33, 47, 19	46.2 ± 4.1[a]
C-G79R+F129L	6	22.5, 25, 26.5, 18, 50.5, 13.5	26.0 ± 5.27[a]	53, 27, 53, 67, 43, 16	43.2 ± 7.7[a]

These data suggest that possible fitness costs of C-G79R and double mutants (C-G79R+F129L), that were investigated, were low under glasshouse conditions. Further tests with a higher number of isolates, to overcome the high natural variability of field isolates, or competition tests under more challenging conditions (e.g. resistant barley cultivar) could provide useful additional information about potential fitness penalties of resistant and multiple resistant isolates of *P. teres*.

4.2 Emergence of SDHI resistance in *Z. tritici* in Europe

4.2.1 Detection of resistant isolates

Z. tritici is a devastating pathogen of wheat, which can cause enormous yield losses in wheat cultivation in Europe (Eyal et al., 1987; Gurr and Fones, 2015). Intensive baseline studies before market launch of SDHIs and monitoring programmes after launch have been carried out since 2007. Sampling of *Z. tritici* isolates was done by collecting STB-infected leaves from various locations throughout Europe ('Random monitoring') or from field trials ('Trial site monitoring'). Subsequently, isolates were generated and tested for their sensitivity to fluxapyroxad in a microtiter test by EpiLogic. In 2012, one isolate from a northern region in France (Agnières) showed an EC_{50} value of 0.574 mg fluxapyroxad L^{-1} and was classified as 'suspicious'. This value was around 10-20-fold higher than that of sensitive reference isolates and outside a previously made baseline sensitivity. A mutation in the *SdhC* gene of *Z. tritici* which lead to C-T79N was detected in previous studies in our laboratory (FRAC, 2016). In 'Random monitoring', 484 isolates were analysed in 2012, followed by 456 isolates from 2013, 690 isolates from 2014, 630 isolates from 2015 and 504 isolates from 2016. In monitoring programmes of trial sites, 100 isolates from 2012, 487 isolates from 2013 and 25 isolates from 2015 were analysed in addition.

Analyses of 'suspicious' isolates after 2012 were done as a part of the present study. *Z. tritici* specific oligonucleotides, forward and reverse, were used to sequence the genes *SdhB*, *SdhC* and *SdhD* of SDHI resistant isolates in two independent reads. In the years 2012 to 2016, a total of 42 field isolates showed a target-site mutation in the *Sdh* genes, whereof three of them had two mutations in the *Sdh* genes in combination. These were B-N225I, B-T268I (one isolate carrying B-T268I in combination with C-I29V), B-T268A in combination with C-F23S, C-T79N/I (one isolate carrying C-T79N in combination with B-R240L), C-W80S, C-N86S/A, C-H152R and C-V166M. An overview of the different amino acid substitutions which were detected and that had been obtained from 'Random monitoring' and 'Trial site monitoring' in the whole of Europe, is given in Table 26. After the detection of C-T79N in 2012, an isolate from an additional 'Trial site monitoring' was found in 2013, which carried C-N86S. In 2014, no additional SDHI resistant isolates were detected in our monitoring programmes. In 2015, B-T268I, C-T79N, C-N86S, C-H152R and C-V166M were detected. One isolate from France, which had B-T268I, showed an additional exchange in SDH-C, namely C-I29V.

Table 26: Amino acid substitutions conferring SDHI resistance in field isolates of *Z. tritici.* Isolates were obtained from 'Random monitoring' and 'Trial site monitoring' in Europe from 2012 to 2016.

SDH-variant	Year of detection	Country	Codon WT >Codon *Sdh* mutant	Number of isolates
B-N225I	2016	NL	AAC>ATC	1
B-T268I	2015, 2016	IE, GB	ACC>ATC	2
B-T268I+C-I29V	2015	FR	ACC>ATC, ATC>GTC	1
B-T268A+C-F23S	2016	IE	ACC>GCC, TTC>TCC	1
C-T79N	2012, 2015, 2016	FR, IE, NL	ACC>AAC	17
C-T79N+B-R240L	2015	IE	ACC>AAC, CGA>CTA	1
C-T79I	2016	IE	ACC>ATC	1
C-W80S	2016	IE	TGG>TCG	1
C-N86S	2013, 2015, 2016	DE, IE, UK, NL	AAC>AGC	13
C-N86A	2016	NL	AAC>GCC	1
C-H152R	2015, 2016	IE, UK, NL	CAT>CGT	3
C-V166M	2015	UK	GTG>ATG	1

Most SDHI resistant isolates collected over the years, showed C-T79N exchange as the resistance mechanism. Most of them did not carry an additional mutation in the *Sdh* genes. However, one isolate from Ireland, was detected to have a second SDH-variant in SDH-B (B-R240L) in combination with C-T79N.

In 2016, new variants of SDH-B and SDH-C were detected. In addition to variants already known from single resistant isolates in 2012 to 2015, B-N225I, B-T268A in combination with C-F23S, C-T79I, C-W80S and C-N86A were found.

In the following sections, SDHI resistant isolates sampled in the years 2012 to 2015 were further characterised with respect to their impact on the SDHI efficacy and their competitiveness compared with sensitive isolates. *SdhB*, *SdhC* and *SdhD* genes of isolates collected in 2016 were sequenced but further testing in microtiter and glasshouse studies was not included in the present study.

4.2.2 Characterisation of mutations leading to SDHI resistance

4.2.2.1 Sensitivity of *Z. tritici* isolates *in vitro*

The sensitivity of *Z. tritici* isolates with mutations in the *Sdh* genes towards different SDHIs was determined in microtiter tests. The tested SDHIs are commercially available fungicides on the European market for control of Septoria tritici blotch, and represent the same set tested on *P. teres*.

Seven isolates showing *Sdh* wild type genes and 14 field isolates showing seven different *Sdh* genotypes were tested in two independent tests. In Figure 33, mean EC_{50} values towards benzovindiflupyr, isopyrazam, bixafen, fluxapyroxad, penthiopyrad and fluopyram are given. Standard errors were calculated from individual isolates with the same *Sdh* genotype. The EC_{50} values of all tested isolates ranged from 0.014 to 2.465 mg benzovindiflupyr L^{-1}, 0.030 to 5.447 mg fluxapyroxad L^{-1}, 0.019 to 6.921 mg isopyrazam

L^{-1}, 0.015 to 9.793 mg bixafen L^{-1}, 0.030 to 22.534 mg penthiopyrad L^{-1}, and 0.135 to 5.094 mg fluopyram L^{-1}.

The SDHI sensitive isolates that were analysed, originated from the years 1987-2015 (Origin and isolation year is given in Supplementary Table 43). Two of these SDH wild type isolates were moderately or highly resistant to QoIs caused by F129L (n=1) or G143A (n=1). Both QoI resistant isolates lay within the range of other SDHI sensitive isolates without QoI resistance. EC_{50} values of SDHI sensitive isolates varied from 0.014 to 0.038 mg benzovindiflupyr L^{-1}, 0.019 to 0.112 mg isopyrazam L^{-1}, 0.015 to 0.107 mg bixafen L^{-1}, 0.030 to 0.078 mg fluxapyroxad L^{-1}, 0.030 to 0.147 mg penthiopyrad L^{-1}, and 0.135 to 0.242 mg fluopyram L^{-1}.

The highest EC_{50} values measured for all SDHIs were found for isolates carrying the amino acid substitutions C-H152R and C-V166M. C-H152R has already been described in laboratory mutants of *Z. tritici* that had been generated by UV-light exposure (in most cases) and subsequent selection on SDHI-containing agar (Stammler et al., 2010; Fraaije et al., 2012; Scalliet et al., 2012). First field isolates that carried C-H152R were found in the season 2015 in Ireland (in the present work; Dooley et al., 2016) and the United Kingdom (FRAC, 2016). In the present study, mean EC_{50} values for C-H152R were 2.071 mg benzovindiflupyr L^{-1}, 5.250 mg fluxapyroxad L^{-1}, 6.715 mg isopyrazam L^{-1}, 8.252 mg bixafen L^{-1}, 17.330 mg penthiopyrad L^{-1}, and 4.386 mg fluopyram L^{-1}. C-H152R was shown to cause the highest loss of efficacy for all SDHIs tested, with exception of fluopyram. Fluopyram was impacted the most by SDH-variant C-V166M, which was found in another isolate from 2015.

Other amino acid substitutions, such as B-T268I, C-T79N and C-N86S were shown to confer a lower impact on all SDHIs compared with C-H152R. C-T79N showed mean EC_{50} values of 0.425 mg benzovindiflupyr L^{-1}, 0.980 mg fluxapyroxad L^{-1}, 0.590 mg isopyrazam L^{-1}, 0.754 mg bixafen L^{-1}, 0.929 mg penthiopyrad L^{-1}, and 1.312 mg fluopyram L^{-1}. One resistant C-T79N isolate showed another mutation in the *Sdh* genes, causing B-R240L exchange. In comparison to other T79N mutants, this isolate showed slightly higher EC_{50} values towards benzovindiflupyr and isopyrazam, but not for the others. However, EC_{50} values of the SDH double mutant (B-R240L+C-T79N) did not exceed factor 2 of those found for single C-T79N mutants. This means that the variation observed between C-T79N and C-T79N+B-R240L mutant is smaller than that found within sensitive isolates. These results indicate that the B-R240L variant causes no or only low additional effects on the SDHI efficacy. Similar results were obtained for B-T268I mutants and an isolate showing B-T268I in combination with C-I29V. This isolate showed slightly higher EC_{50} values compared to isolates which had only B-T268I. Due to the low number of isolates with two mutations in the *Sdh* genes, the effect of these additional mutations is difficult to determine. Both isolates had higher EC_{50} values compared with isolates with one SDH variation. However, reflecting

the range of SDHI sensitivities in 'baseline' isolates (SDH wild type), it is hard to predict whether higher EC_{50} values are caused by these additional mutations or on account of natural variation of isolates. Further studies, which would include more double SDH mutants, would be necessary to clarify smaller discrepancies of SDHI sensitivities within such isolates.

In general, cross resistance of SDH mutants was observed for all SDHIs tested. Small differences in the behaviour to different SDH-variants was observed for SDHIs in cases such as C-V166M and C-H152R. Here, fluopyram was more affected by C-V166M than by C-H152R, as it was observed for other SDHIs. Resistance factors of SDH-variants of field isolates are shown in Table 27. RF values were calculated as ratios of EC_{50} of resistant to EC_{50} of sensitive isolates. The strongest effect can be observed for C-H152R mutants and the isolate carrying C-V166M. C-N86S caused low to medium RF values which ranged from 8 to 39, depending on the substance. Lowest RF values were obtained for isolates with B-T268I and C-T79N exchange in all SDHIs tested. As had been observed in the studies on *P. teres* (see section 4.1.2.1), also wild type isolates of *Z. tritici* showed the lowest sensitivity towards fluopyram of the SDHIs tested. RF values of fluopyram were slightly lower compared with other SDHIs. However, low RF values of fluopyram were mainly based on the low activitiy of this substance towards wild type isolates. A decrease of SDHI efficacy was also observed for fluopyram when SDH mutated isolates are compared to wild type isolates.

Table 27: Resistance factors of *Z. tritici* isolates with different SDH-variants towards SDHIs. Resistance factors were calculated as ratios of mean EC_{50} of resistant isolates / EC_{50} of sensitive isolates shown in Figure 33. Benzovindiflupyr (benzo), isopyrazam (isopyra), bixafen (bixa), fluxapyroxad (fluxa), penthiopyrad (penthio) and fluopyram (fluo) are sorted due to their intrinsic activity on wild type isolates (left to the right). Colours indicate the strength of resistance with RF values ≤ 20 bright yellow, 21 to 50 dark yellow, 51 to 100 orange, and >100 red.

SDH-variants	RF values					
	Benzo	Isopyra	Bixa	Fluxa	Penthio	Fluo
B-T268I	19	13	19	17	15	7
C-T79N	17	11	14	16	14	7
C-N86S	39	18	18	17	25	8
C-V166M	58	28	36	23	47	29
C-H152R	82	128	148	88	262	25

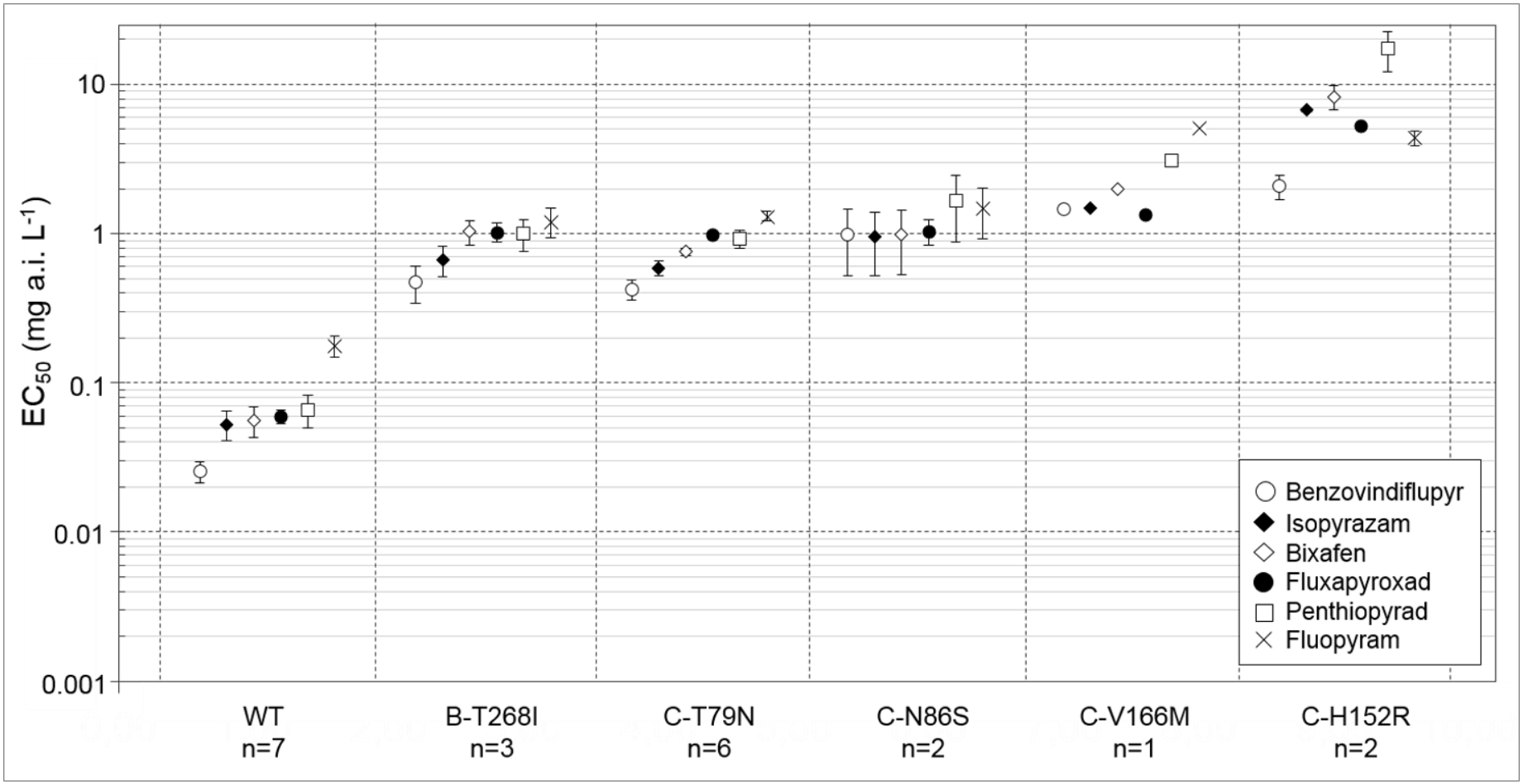


Figure 33: SDHI sensitivity of *Z. tritici* isolates carrying different mutations in the *Sdh* genes. Photometric measurements (OD_{405}) to detect mycelial growth of *Z. tritici* in microtiter plates were used to determine EC_{50} values (probit calculation) of SDHI fungicides. Water control and seven concentrations, up to 10 mg a.i. L^{-1} of benzovindiflupyr, isopyrazam, bixafen, fluxapyroxad, fluopyram and up to 30 mg a.i. L^{-1} of penthiopyrad, were applied in four replicates and were mixed with spore suspensions of SDH wild type isolates and several SDH mutated isolates. Fungicides are sorted beginning with the fungicide showing highest activitiy against SDH wild type isolates. SDH amino acid substitution, the number of isolates and standard errors are given. Some standard errors are smaller than the symbol itself and were calculated by mean of two individual experiments over isolates having the same *Sdh* genotype.

4.2.2.2 Sensitivity of *Z. tritici* isolates *in planta*

The SDHI efficacy was also investigated *in planta* under glasshouse conditions. In total, four glasshouse tests were carried out to test the response of SDHIs to SDH mutants. SDHIs were applied one-day preventative in two tests and four-day curative (4 dpi) in another two tests. In each test, wheat plants (BBCH 11) cv. 'Riband' were inoculated with two SDHI sensitive isolates and eight SDHI resistant isolates (B-T268I n=2, C-T79N n=2, C-N86S n=2, C-H152R n=2) in four replicates for each isolate and treatment. The infected leaf area on untreated and treated plants was visually rated 21 dpi. Not all SDHIs that had been tested in microtiter tests were available as solo products when studies were performed. In order to include all SDHIs in these glasshouse tests, all the SDHIs were obtained from Sigma Aldrich (with exception of benzovindiflupyr, see section 3.11.1) and were dissolved prior to application by using DMSO and Agnique AMD10 (see section 3.11.3.2). Agnique AMD10 is a solvent which is used in commercial formulations to increase the solubility of the fungicide. In cereal production, fungicides are applied in low spray volumes which range from 100 to 400 L ha^{-1}. Here, an appropriate formulation of active ingredients is necessary as SDHIs tend to precipitate in water solutions. A spray chamber that uses water volumes equal to 400 L ha^{-1} was used in the present studies.

In an initial experiment with different solvents it was shown that precipitation of all SDHIs was inhibited when a final concentration of 2% (v/v) DMSO and 0.15% (v/v) Agnique AMD10 in water was used (data not shown). The solvent system (2% DMSO, 0.15% Agnique AMD10) was solely applied in one preventative and one curative test to see whether these adjuvants show an effect on the growth of *Z. tritici*. In addition to DMSO/Agnique AMD10-dissolved SDHIs, Imbrex® was applied, which is a commercial SDHI-solo formulation containing fluxapyroxad. Fungicides were applied at full registered field rates of solo products or combination-products, these are 75 g a.i. ha^{-1} benzovindiflupyr, 100 g a.i. ha^{-1} of isopyrazam, 125 g a.i. ha^{-1} of bixafen, fluopyram, fluxapyroxad and Imbrex®, and 300 g a.i. ha^{-1} of penthiopyrad.

The inhibition of SDH wild type and SDH mutated isolates when SDHIs are applied one-day before inoculation is given in Figure 34. SDH wild type isolates were effectively controlled by all SDHIs tested. SDH-variants, which caused 'moderate' RF values in microtiter tests, such as B-T268I, C-T79N and C-N86S, were also shown to reduce the SDHI efficacy in glasshouse trials. Furthermore, C-H152R, which caused high RF values compared with other SDH-variants in microtiter, also gave the highest efficacy reduction of all SDHIs in glasshouse tests. The SDH-variants, B-T268I, C-T79N and C-N86S, all had similar effects on the SDHI efficacy. These mutants were controlled by mean inhibitions of 65 to 98%, depending on the SDHI under investigation. Significant effects were calculated by Lagrange-Multiplier test from two independent tests with the same set of isolates. Thereby, differences between wild type isolates and SDHI resistant isolates were calculated for each compound separately. The comparison of different SDHIs due to their efficacy was not

included in statistical tests. Imbrex® (commercial formulation with a.i. fluxapyroxad) showed the highest control of B-T268I, C-T79N and C-N86S mutants without significant differences to wild type isolates. However, when fluxapyroxad was dissolved in DMSO/Agnique AMD10, significant effects of these 'moderate' mutations were visible. The benefit of fluxapyroxad in its commercial formulation was observed in all glasshouse trials performed. This indicates that the optimised formulation of a substance can give an increase of efficacy. There are several reasons that could explain this, including a higher stability of molecules, a better uptake to plant tissue or fungal cells, or the spread on the plant surface in an optimised formulation.

In four-day curative tests, the same ranking of different SDH-variants was observed. The inhibition levels of both curative tests are given in Figure 35. However, in the curative situation, the effect of SDHI resistant isolates was more pronounced. SDHI sensitive isolates were effectively controlled by all SDHIs even when applied 4 dpi. SDHI resistant isolates of *Z. tritici* were significantly less controlled by all SDHIs used. The impact on the efficacy of SDHIs was highest in the case of isolates with C-H152R. Here, mean inhibition values of 0-15% were detected in different treatments. A high infection pressure in the untreated plants of both curative trials was observed for most isolates. Although the same number of spores were used to inoculate plants, the infected leaf area was observed to be different between isolates. Lowest infection rates in untreated plants were observed in all replicates of SDHI sensitive isolates (St 1965 and St 3718). Highest infection rates were observed for both C-T79N mutants and both C-N86S mutants. The high variation of inhibition values seen for isolates of the same genotype in the curative trials resulted mainly from the differences in the infection rate in the different experiments. In the first glasshouse test, mean growth rate [% infected leaf area] was 48% in the two sensitive isolates, 68% in B-T268I mutants, 97% in C-T79N mutants, 90% in C-N86S mutants and 71% in C-H152R mutants. In the second experiment, which was carried out in another chamber in the glasshouse with different glasshouse lightings, infection rates were even higher. The same set of isolates showed mean infection levels of 81% in the sensitive isolates, 97% in B-T268I mutants, 100% C-T79N mutants, 98% in C-N86S mutants and 96% C-H152R mutants. The diseased leaf area of different isolates in all four tests is given in Supplementary Material, Table 35.

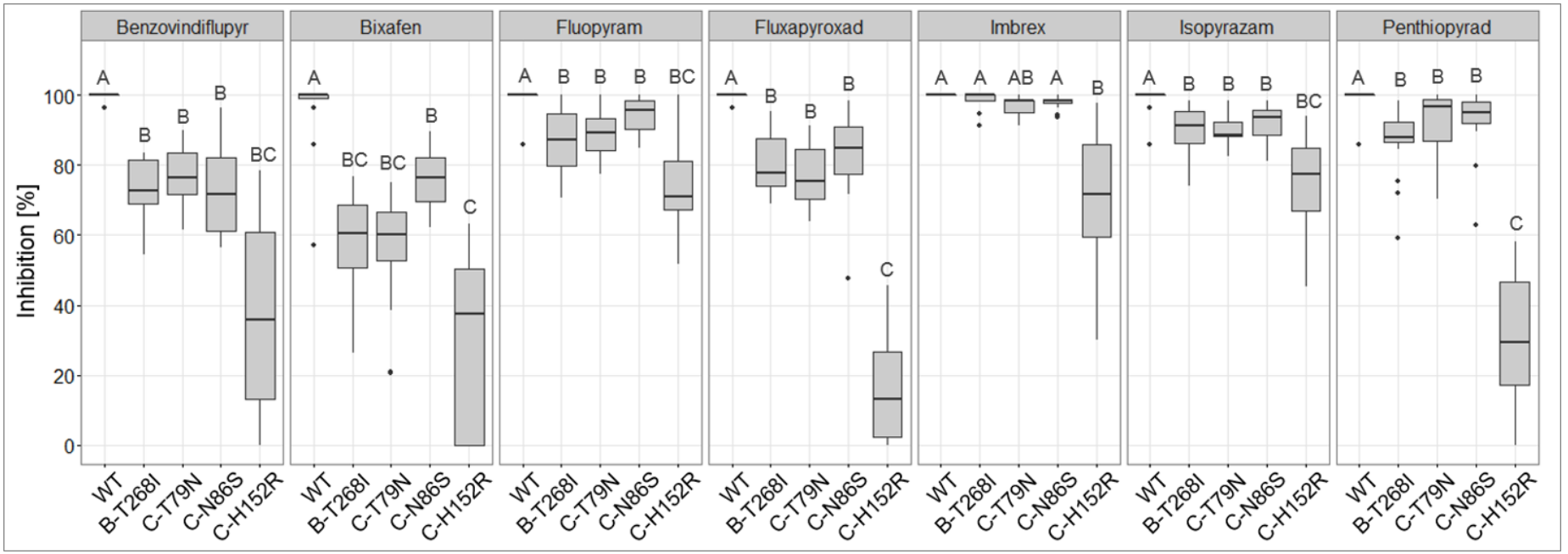


Figure 34: Inhibition [%] of wild type and *Sdh* mutated isolates of *Z. tritici* by several SDHIs under one-day preventative conditions in the glasshouse. Infected leaf area of two wild type isolates and eight *Sdh* mutated isolates with B-T268I (n=2), C-T79N (n=2), C-N86S (n=2), C-H152R (n=2) was determined. Untreated plants, plants treated with the blank formulation (2% DMSO, 0.15% Agnique AMD10)- and SDHI-treated plants, wheat plants cv. 'Riband' in BBCH 11, were rated 21 dpi. Each treatment and isolate was repeated in four replicates in each experiment. Two independent experiments were performed. Within a fungicide, same letters on top of Box-and-Whiskers mean that they do not differ significantly according to Lagrange multiplier to range test (P=0.05).

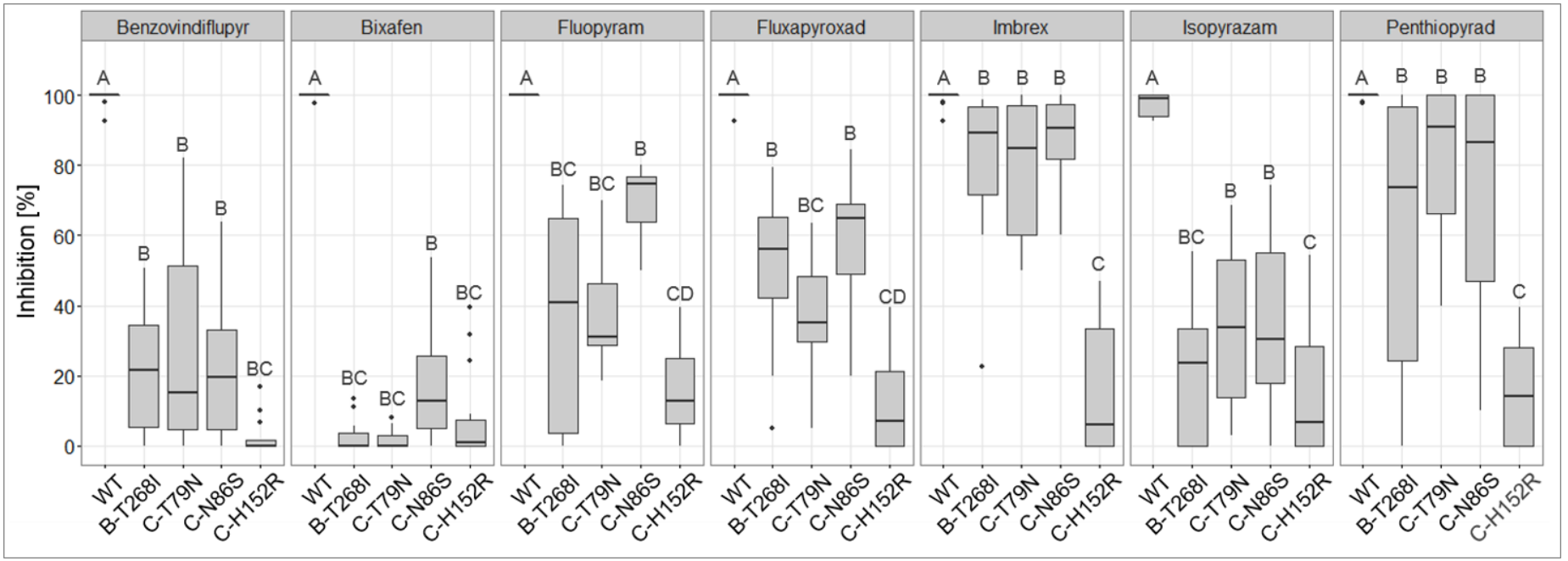


Figure 35: Inhibition [%] of wild type and *Sdh* mutated isolates of *Z. tritici* by several SDHIs under four-day curative conditions in the glasshouse. Infected leaf area of two wild type isolates and eight *Sdh* mutated isolates with B-T268I (n=2), C-T79N (n=2), C-N86S (n=2), and C-H152R (n=2) was determined. Untreated plants, plants treated with the blank formulation (2% DMSO, 0.15% Agnique AMD10)- and SDHI-treated plants, wheat plants cv. 'Riband' in BBCH 11, were rated 21 dpi. Each treatment and isolate was repeated in four replicates in each experiment. Two independent experiments were performed. Within a fungicide, same letters on top of Box-and-Whiskers mean that they do not differ significantly according to Lagrange multiplier to range test (P=0.05). These results will be partially published in Plant Pathology (Rehfus et al., accepted 2017).

4.2.3 Localisation of amino acid exchanges in the SDH enzyme

4.2.3.1 Alignments of *Z. tritici* SDH amino acid sequences to other phytopathogenic fungi

SDH-B and SDH-C amino acid sequences of *Z. tritici*, SDH wild type and SDHI resistant isolates, were aligned to sequences of other phytopathogenic fungi. The alignment in Figure 36 shows the amino acid positions in SDH-B, N225I, R240L, T268I/A, which were altered in one or more SDHI resistant isolates. All amino acid substitutions in SDH-B that were found in SDHI resistant isolates are located at highly conserved positions within phytopathogenic fungi.

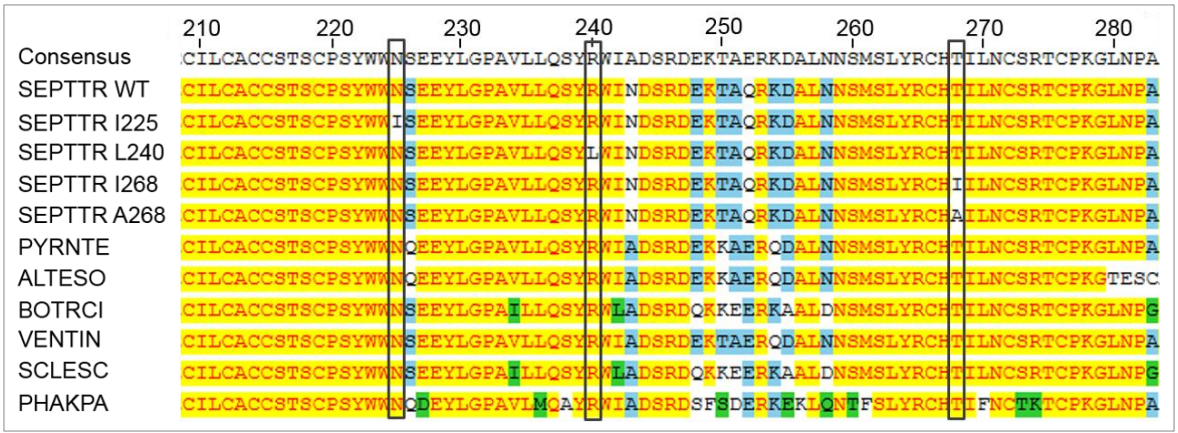


Figure 36: Alignment of partial SDH-B amino acid sequence of *Z. tritici* and other phytopathogenic fungi. Sequences of ascomycetous and basidiomycetous fungi are named by species EPPO code (SEPTTR: *Zymoseptoria tritici*, PYRNTE: *Pyrenophora teres*, ALTESO: *Alternaria solani*, BOTRCI: *Botrytis cinerea*, VENTIN: *Venturia inaequalis*, SCLESC: *Sclerotinia sclerotiorum*, PHAKPA: *Phakospora pachyrhizi*). In case of *Z. tritici*, a wild type sequence and the sequence of SDH-B N225I, R240L (which was found in combination to C-T79N), T268I and T268A mutants are included. The numbers of amino acids that are given in the figure are based on the sequence length of *Z. tritici*.
Colour code: >80% conserved, ≥50% conserved, ≥ 50% similar, not conserved. Sequence alignments were made by the multiple alignment tool on the bioinformatics webpage of BASF SE, which uses muscle-calculation.

In Figure 37, partial SDH-C amino acid sequences are shown for *Z. tritici* and its SDHI resistant field mutants in comparison to other plant pathogens. C-W80S and C-H152R are positioned at highly conserved positions within phytopathogenic fungi. Whereas in *P. teres*, the histidine at position 134 (which is orthologous to histidine at position 145 in *Z. tritici*) was replaced by arginine, in *Z. tritici* the histidine at position 152 (which is orthologous to *P. teres* H141) was shown to be replaced in SDHI resistant isolates. Amino acid substitutions C-T79N/I, C-N86S/A and C-V166M were placed at positions at which at least one other fungus was shown to have another amino acid. At position 79, in *B. cinerea* and *S. sclerotiorum* the amino acid proline was found. *S. sclerotiorum* was also found to be different at position 86, having F86, in contrast to other fungi that were included in this study.

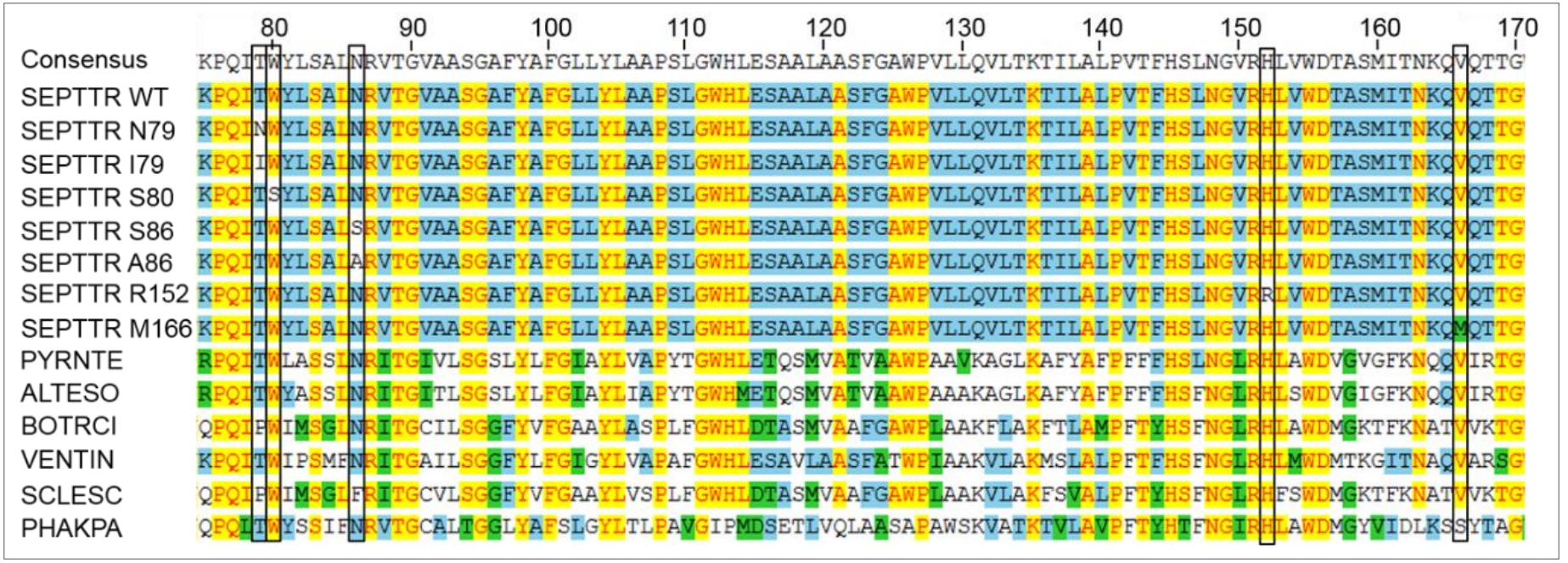


Figure 37: Alignment of partial SDH-C amino acid sequence of *Z. tritici* and other phytopathogenic fungi. Sequences of ascomycetous and basidiomycetous fungi are named by species EPPO code (SEPTTR: *Zymoseptoria tritici*, PYRNTE: *Pyrenophora teres*, ALTESO: *Alternaria solani*, BOTRCI: *Botrytis cinerea*, VENTIN: *Venturia inaequalis*, SCLESC: *Sclerotinia sclerotiorum*, PHAKPA: *Phakospora pachyrhizi*). In case of *Z. tritici*, a wild type sequence and the sequence of SDH-C T79N, T79I, W80S, N86S, N86A, H152R and V166M mutants are included. The numbers of amino acids that are given in the figure are based on the sequence length of *Z. tritici* SDH.
Colour code: > 80% conserved, ≥ 50% conserved, ≥ 50% similar, not conserved. Sequence alignments were made by the multiple alignment tool on bioinformatics webpage of BASF SE, which uses muscle-calculation.

Three isolates simultaneously showed two amino acid alterations in the SDH enzyme. B-R240L is located at a highly conserved position within fungal species and was found in addition to C-T79N in one isolate. One SDHI resistant isolate showed C-F23S in combination to B-T268A and another isolate had C-I29V simultaneously to B-T268I. C-F23S and C-I29V were found at positions which are highly variable within phytopathogenic species (not shown in the sequence alignment). Sequence alignment of SDHI resistant and sensitive isolates of *Z. tritici* revealed that C-I29V had also been found in a French isolate, which was shown to be sensitive towards SDHIs. Therefore, an effect of this SDH-variant on the SDHI sensitivity seems unlikely.

In general, the SDH-C subunit shows the lowest conservation within SDH subunits (Cecchini, 2003; Sierotzki and Scalliet, 2013; Mair et al., 2016). Even within *Z. tritici*, different *SdhC* variants exists, which show nucleotide polymorphisms at several positions. Most nucleotide exchanges were found to be silent and did not alter amino acid sequence. Two SNPs in *SdhC* gene that were frequently found (~50% of sequenced isolates), were shown to cause two alterations at the beginning of SDH-C amino acid sequence (C-N33T and C-N34T). Isolates showed either NN or TT but not mixed up. Complete DNA and protein sequence alignments of *Z. tritici* SDH-C can be found in the Supplementary Material, Figure 53 and Figure 54.

4.2.3.2 Homology modelling of the SDH enzyme of *Z. tritici*

Amino acid substitutions detected in SDHI resistant isolates were analysed with respect to their localisation within the SDH complex. The homology model of subunits SDH-B, SDH-C and SDH-D and altered amino acid residues are shown in Figure 38. SDH modelling of the wild type enzyme was performed by Dr. Antje Wolf (BASF SE), whereas mutagenesis and illustration of the SDH model was part of the present work.

Key substitutions (B-N225I, B-T268I, C-T79N, C-W80S, C-N86S, C-H152R and C-V166M) found in resistant field isolates in the present study, were all located in the neighbourhood of the Q-site. In docking studies, SDH-B residues P220, S221, W224, H267 and I269, together with residues in SDH-C (L71, W80, S83, A84 and R87) and in SDH-D (D129 and Y130) were shown to form the SDHI binding cavity in *Z. tritici* (Fraaije et al, 2012; Scalliet and Sierotzki, 2013). Therefore, most positions which could be substituted in case of SDHI resistance are located near positions that are important for SDHI binding to the SDH enzyme or are directly involved in the SDHI interaction to the target protein (in case of C-W80S).

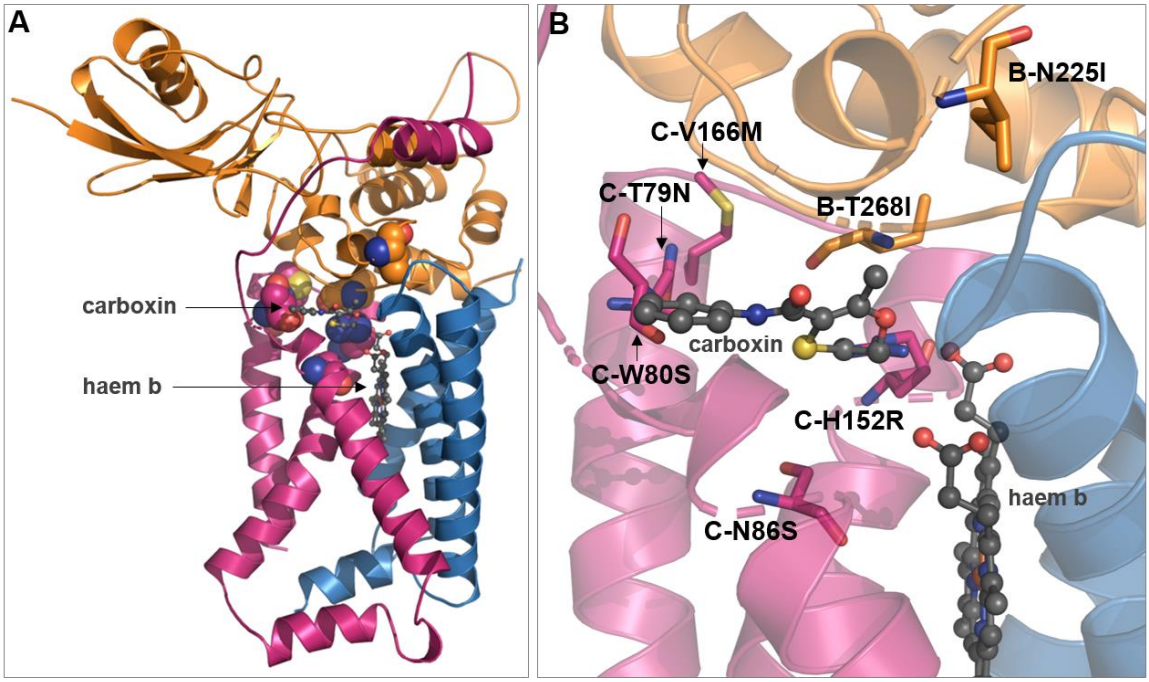


Figure 38: Localisation of amino acid substitutions which lead to SDHI resistance in homology model of *Z. tritici* succinate dehydrogenase enzyme. Homology model of SDH subunits SDH-B, SDH-C and SDH-D is based on X-ray from *G. gallus* (PDB 2WQY) with the docked pose of carboxin in ubiquinone-binding pocket and complexed haem b group between SDH-C and SDH-D. SDH-B (orange), SDH-C (lilac) and SDH-D (blue) subunits are shown in cartoon style (helices denote α-helical secondary structures and ribbons denote β-sheets). Carboxin and haem b are depicted in ball-and-stick models showing CPK colouring (carbon=grey, nitrogen=blue, oxygen=red, sulfur=yellow, iron=dark orange). Substituted amino acid residues of B-N225I, B-T268I, C-T79N, C-W80S, C-N86S, C-H152R and C-V166M are depicted in stick models showing CPK colouring except for carbon atoms, which are shown in the colour of corresponding subunit. Hydrogen atoms are not shown in any of the molecules. **A:** Overview of SDH-B, SDH-C and SDH-D subunits of SDH enzyme and localisation of substitutions (altered amino acid are shown as spheres). Alterations are found in direct neighbourhood of the Q-site. **B:** Closer view to alterations located in or near Q-site.

4.2.4 Frequency of SDHI resistance in Europe

The fluxapyroxad sensitivity of 3431 isolates of *Z. tritici* was analysed since 2012. In total, 42 isolates showed a reduced SDHI sensitivity in our monitoring programmes and these carried one or, in rare cases, two mutations in the genes *SdhB*, *SdhC* and *SdhD*.

Isolates obtained from SDHI ‘Random monitoring’ in the years from 2012 to 2016 have included 2819 isolates. First SDHI resistant isolates in ‘Random monitoring’ were collected in the year 2015. In 2015, 5 of 630 analysed isolates were shown to be SDHI resistant (0.8%). In 2016, 25 of 559 isolates carried SDH amino acid exchanges and were SDHI resistant (4.5%). This indicates that there was an increase of SDHI resistant isolates in 2015 to 2016, however, still at a very low level. SDHI resistant isolates in 2015 were collected in Ireland (n=2), the United Kingdom (n=2) and in France (n=1) and in 2016 in Ireland (n=12), the Netherlands (n=8) and the United Kingdom (n=5). Three additional isolates were detected in 2015 by molecular detection of known *Sdh* mutations in some isolates from DMI ‘Random monitoring’, which was conducted in our laboratory at the same time. These

isolates, one from the United Kingdom and two from Ireland, showed C-T79N exchange and were additionally included in the present study.

In addition to 'Random monitoring', 612 isolates were obtained from different trial sites in Europe ('Trial site monitoring'). These included 100 isolates from 2012 collected in France, 487 isolates from 2013 collected in Germany, and 25 isolates from 2015 collected in Ireland. In total, 9 of these isolates, most of them (n=7) collected in 2015 from Ireland, carried target-site mutations in the *Sdh* genes. The frequency of SDHI resistant isolates on the trial site in Ireland from 2015 was relatively high (7 of 25 isolates analysed, 28%) compared with frequencies observed in the 'Random monitoring'. This can be explained with the high SDHI usage at this site and that the trials which were performed on this field have focused on SDHI applications, even more than one SDHI application in a season.

The frequency of different amino acid substitutions found in isolates collected in a four-year period are shown in Figure 39. The most frequent SDH-variants are C-T79N (40.5%) and C-N86S (31%). Other substitutions, such as C-H152R (7.1%, n=3) and B-T268I (7.1%, n=3) were detected in a lower number of isolates. All other substitutions were detected in a single isolate (B-N225I, B-T268A, C-T79I, C-W80S, C-N86A and C-V166M).

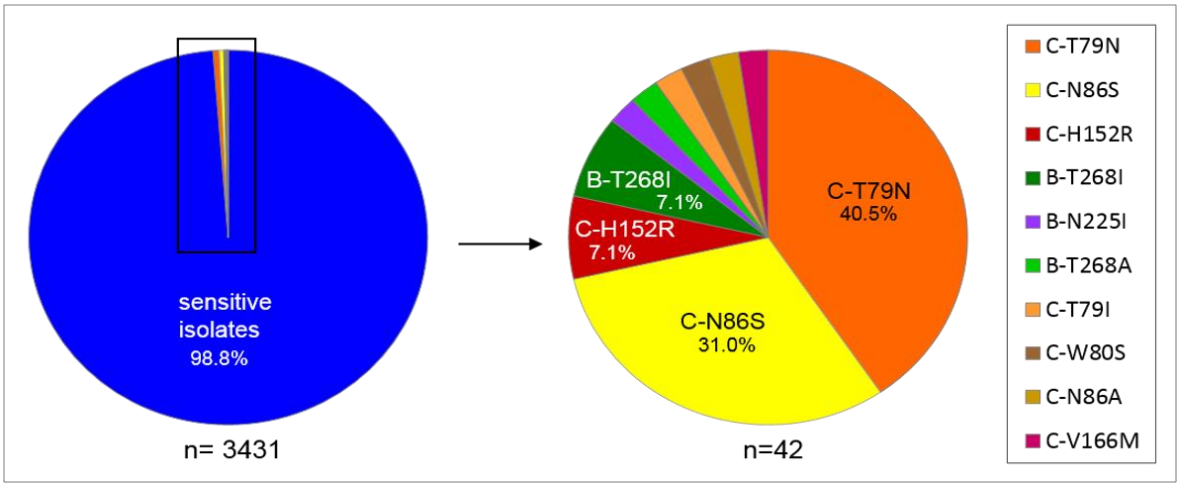


Figure 39: Frequency of amino acid substitutions in all *Z. tritici* isolates analysed and frequency of different substitutions within SDHI resistant isolates from European countries 2012-2016. All SDHI resistant isolates that were detected in the 'Random monitoring' and in 'Trial site monitoring' are included in the figure.

In addition to isolates, different locations were analysed by pyrosequencing and qPCR. The frequency of resistant alleles which cause C-H152R, B-T268I and C-T79N detected in early spring sampling in Ireland and the United Kingdom, is shown in Figure 40. In total, STB-infected wheat leaves were collected from 22 commercial locations. C-H152R was first found in 2015 on a trial site in Ireland. In spring 2016, C-H152R was not detectable by pyrosequencing in any of the samples from Ireland and the United Kingdom. In comparison, B-T268I was detected in one sample from southern Ireland and C-T79N was found in a low frequency (<10%) at different locations in Ireland and at a location in the United Kingdoms. Alleles causing C-T79N were find in a higher frequency only at one site in Ireland.

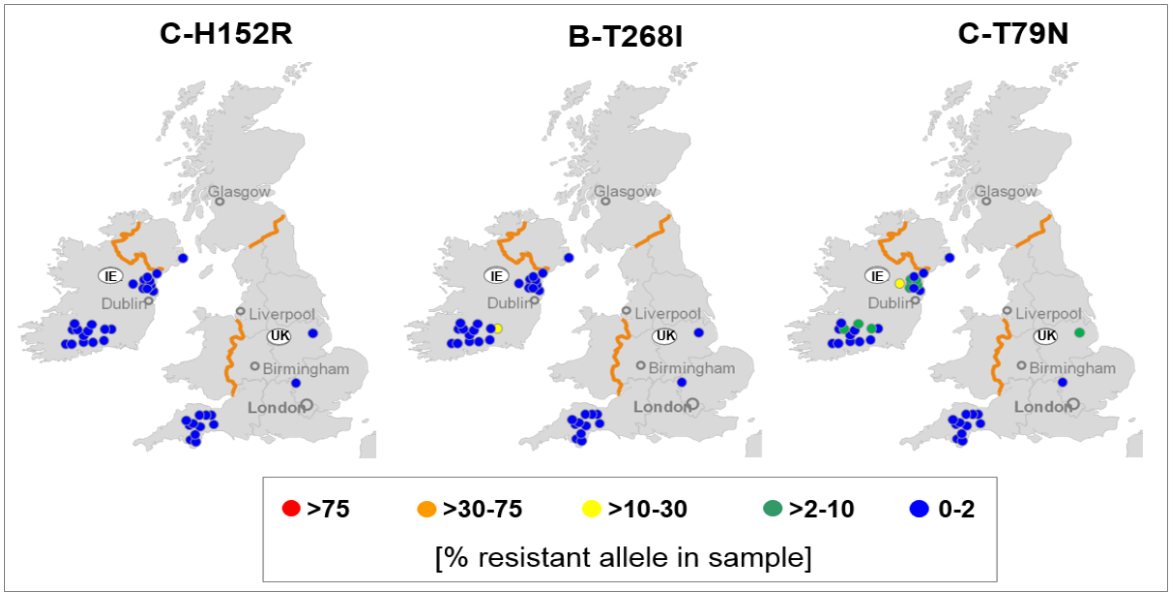


Figure 40: Frequency of resistant alleles in infected leaf samples of *Z. tritici* collected from commercial sites in Ireland (IE) and the United Kingdom (UK) in early spring in 2016. After the detection of different target-site mutations in SDHI resistant isolates of *Z. tritici*, the frequency of several mutations was quantitatively determined using pyrosequencing and qPCR. Infected leaves (~20 to 30 randomly collected leaves per sample) from 37 different fields in Ireland and the UK were analysed. All leaves within one sample were grinded. An aliquot was taken to extract DNA and was analysed by pyrosequencing (B-T268I, C-H152R) and qPCR (C-T79N).

These data indicate that SDHI resistant isolates are still detected at low frequencies within the European population of *Z. tritici*. Many countries, such as France and Germany, showed a completely sensitive population in 2016, although the two first resistant isolates (2012 and 2013) had been collected in these countries. In countries, such as Ireland, the United Kingdom and the Netherlands, few SDHI resistant isolates have been detected in 2016. In Ireland, the frequency of SDHI resistant isolates has increased from 6.1% in 2015 to 12.2% in 2016 (SDHI 'Random monitoring'). This increase is still low when compared with the rapid increase of SDHI resistant isolates in France and Germany in the case of *P. teres* (see section 4.1.4).

4.2.5 Multiple resistance in *Z. tritici*

SDHI resistant isolates, that had been sampled in European countries before 2016, were analysed for the occurrence of multiple resistance towards DMI and QoI fungicides. QoIs are severely affected by G143A exchange, which is present at high frequencies in European populations of *Z. tritici* (Gisi et al., 2002; Fraaije et al., 2005; FRAC, 2016). *Z. tritici* additionally shows adaptation against DMI fungicides by the accumulation of several amino acid substitutions in CYP51 enzyme (Cools and Fraaije, 2008; Stammler et al., 2008a, 2008b; Fraaije et al., 2012). Furthermore, SDHI resistant isolates were tested for a potential enhanced efflux of membrane transporters. Tolnaftate (a thiocarbamate), used to treat dermatomycoses (Ryder et al., 1986), is known as a preferred substrate for efflux transporter. It shows an altered MOA compared to cereal fungicides and can, therefore, be used to detect an enhanced efflux of unspecific toxic compounds (Leroux et al., 2002). QoI resistance was analysed in microtiter tests with pyraclostrobin (data not shown) and molecular detection of G143A by qPCR. DMI adaptation of isolates was analysed by cloning of *Cyp51* gene into pJet1.2 with the subsequent sequencing of clones (oligonucleotides pJet1.2fw, pJet1.2rev). A potentially enhanced efflux of isolates was determined in microtiter tests with tolnaftate and the detection of promotor insertions in *Mg*MFS1 transporter. The results for SDHI resistant isolates and two SDH wild type isolates are given in Table 28. The SDH wild type isolates included are reference isolate IPO323 and field isolate St 5950, which is highly adapted towards DMIs (*Cyp51* haplotype O3) and shows an enhanced efflux due to 512 bp promotor insertion in *Mg*MFS1 (as depicted in Omrane et al., 2015).

All SDHI resistant field isolates were shown to carry G143A and, therefore, are QoI resistant. *Cyp51* haplotypes indicate the adaptation level of isolates towards DMI fungicides. Most SDHI resistant isolates showed R7-R12 haplotypes, which is the nomenclature used in some publications (Leroux and Walker, 2011; Omrane et al., 2015). These haplotypes cause moderately to highly adapted phenotypes towards azoles frequently used in cereals, such as epoxiconazole and prothioconazole (Fraaije et al., 2012; http://eurowheat.au.dk/). In addition to known combinations of CYP51 exchanges, new combinations of alterations were detected in six SDHI resistant isolates. These haplotypes have been first identified in these isolates and some other isolates (SDHI sensitive) from DMI 'Random monitoring' in 2014 and 2015 (internal unpublished data). An enhanced efflux of SDHI resistant isolates could be excluded, with exception of one isolate which had slightly enhanced EC_{50} values for tolnaftate (St 6027, 0.87 mg tolnaftate L^{-1}). However, compared to St 5950 (carries 519 bp promotor insertion), which shows EC_{50} values of 1.69 mg tolnaftate L^{-1}, the enhancement of tolnaftate efflux in isolate St 6027 is lower.

Table 28: SDHI resistant isolates of *Z. tritici* collected in Europe from 2012 to 2015 and the fungicide sensitivity to QoIs, DMIs and tolnaftate. In addition to SDHI resistant isolates, IPO323, a sensitive reference isolate, and St 5950 are included. St 5950 shows no alteration in the SDH enzyme, but shows an enhanced efflux of tolnaftate. * St 6036 was kindly provided by Teagasc Institute, Ireland. ** These three isolates were detected by molecular detection method (qPCR) in isolates of the DMI 'Random monitoring', which were additionally screened for known SNPs during the present studies.

Isolate-ID	Year	Country	SDHIres	QoIres	DMI adaptation		Efflux	
			SDH-variant		CYP51-variants (haplotype)	Type	EC_{50} tolnaftate	Promotor insert[c]
IPO323	1981	NL	no	no	no alternations		0.495±0.052	no
St 5950	2014	PL	no	G143A	A379G, I381V, Del Y459/Y460, S524T	O3	1.690±0.009	yes
St 5548	2012	FR	C-T79N	G143A	I381V, Y461H	R6	0.434±0.062	no
St 5745	2013	DE	C-N86S	G143A	na	na	0.538±0.037	no
St 6027	2015	FR	B-T268I+C-I29V	G143A	D134G, V136A, I381V, Y461H	R11	0.867±0.294	no product
St 6028	2015	UK	C-V166M	G143A	V136A, I381V, Y461S, S524T	R12	0.572	no
St 6029	2015	IE	C-H152R	G143A	V136A, Y461S, S524T	R9	0.551±0.003	no
St 6030	2015	IE	B-R240L+C-T79N	G143A	na	na	0.543±0.009	no
St 6031	2015	IE	C-T79N	G143A	V136A, I381V, Y461H, S524T	R12	0.474±0.005	no
St 6032	2015	IE	B-T268I	G143A	V136A, Del Y459/Y460, S524T	new 2015[b]	0.548±0.002	no product
St 6033	2015	IE	C-T79N	G143A	V136A, I381V, Y461H, S524T	R12	0.537±0.012	no
St 6034	2015	IE	C-T79N	G143A	D134G, V136A, I381V, Y461H	R11	0.538±0.025	no
St 6035	2015	IE	C-N86S	G143A	I381V, Del Y459/Y460	R7	0.668±0.189	no
St 6036*	2015	IE	C-H152R+D-R47W	G143A	D134G, V136A, I381V, Y461H	R11	0.514±0.042	no
St 6037	2015	UK	B-T268I	G143A	V136C, A379G, I381V, Del Y459/Y460, S524T	new 2014[a]	0.527±0.058	no product
St 6038	2015	IE	C-T79N	G143A	V136C, I381V, Del Y459/Y460, S524T	new 2014[a]	0.552±0.003	no
St 6039	2015	IE	C-N86S	G143A	na	na	0.547±0.035	no
St 6127**	2015	UK	C-T79N	G143A	I381V, S524T	new 2015[b]	na	no
St 6149**	2015	IE	C-T79N	G143A	D134G, V136A, I381V, Y461S, S524T	new 2015[b]	0.457	no
St 6156**	2015	IE	C-T79N	G143A	D134G, V136A, I381V, Y461S, S524T	new 2015[b]	0.416	no

[a] these combinations of CYP51 alterations were first found in isolates in DMI 'Random monitoring' from 2014 (internal unpublished data)

[b] these combinations of CYP51 alterations were first found in isolates in DMI 'Random monitoring' from 2015 (internal unpublished data)

[c] promotor insertions of *Mg*MFS1 were studied by PCR using primers extracted from Omrane et al. (2015). Wild type promotor shows 700 bp product, isolates with *Mg*MFS1 overexpression show 1200 bp product.

Additonally, PCR of the promotor region did not show a product in this isolate. Omrane et al. (2015) reported this for three isolates in their studies and proposed new rearrangements in the promotor of *Mg*MFS1 in these isolates (no hybridisation of primer possible). St 6027 (B-T268I+C-I29V) also showed slightly higher EC_{50} values in microtiter tests with several SDHIs compared to mutants with B-T268I. Higher EC_{50} values for SDHIs in this isolate could also be explained by this slightly enhanced efflux. It was shown in preliminary studies that SDHIs are affected by *Mg*MFS1 overexpression (519 bp insertion) resulting in RF values of ≤10 (data not shown). In these studies, St 5950 (*Mg*MFS1 overexpressor) had EC_{50} values of 0.317 mg fluxapyroxad L^{-1}, 0.393 mg bixafen L^{-1} and 0.804 mg fluopyram L^{-1} without having a mutation in the *Sdh* genes. In comparison, IPO323 showed EC_{50} values of 0.054 mg fluxapyroxad L^{-1}, 0.038 mg bixafen L^{-1}, 0.157 mg fluopyram L^{-1}. The contribution of an enhanced efflux on the SDHI sensitivity and their different genetic mechanisms should be studied in the future. However, compared to high RF values that can be contributed by target-site mutations in the *Sdh* genes, low RF values of *Mg*MFS1 overexpression in case of SDHIs seems to have minor relevance. Alignments of CYP51 protein sequences and promotor insertion studies of SDHI resistant isolates are given in Supplementary Material, Figure 51 and Figure 52.

4.2.6 Competition studies of field mutants of *Z. tritici*

The competitiveness of SDHI resistant field mutants of *Z. tritici* was determined in glasshouse tests. Inoculation of wheat seedlings (cv. ‘Riband’) with mixtures of SDHI resistant isolates and appropriate SDHI sensitive isolates, and their subsequent propagation over three cycles was performed (see section 3.12.1). Quantitative pyrosequencing was used to detect the frequency of resistance alleles in each spore suspension of mixtures and single isolates over all propagation cycles. SDHI resistant field isolates were studied towards their DMI sensitivity (*Cyp51* haplotypes) and QoI resistance in section 4.2.5. Several SDHI sensitive field isolates were investigated with respect to their *Cyp51* haplotype and their QoI background to find appropriate mixing partners for SDHI resistant isolates. Other criteria considered were the sampling origin and the collection year. Thus, most isolates, with exception of two isolates, were collected in Ireland. One SDHI resistant isolate was from France (2015) and one sensitive isolate was from the United Kingdom (2016). The SDHI sensitive isolate from UK was used because it had a rare combination in CYP51, that had been first detected in 2015 in a B-T268I mutant.

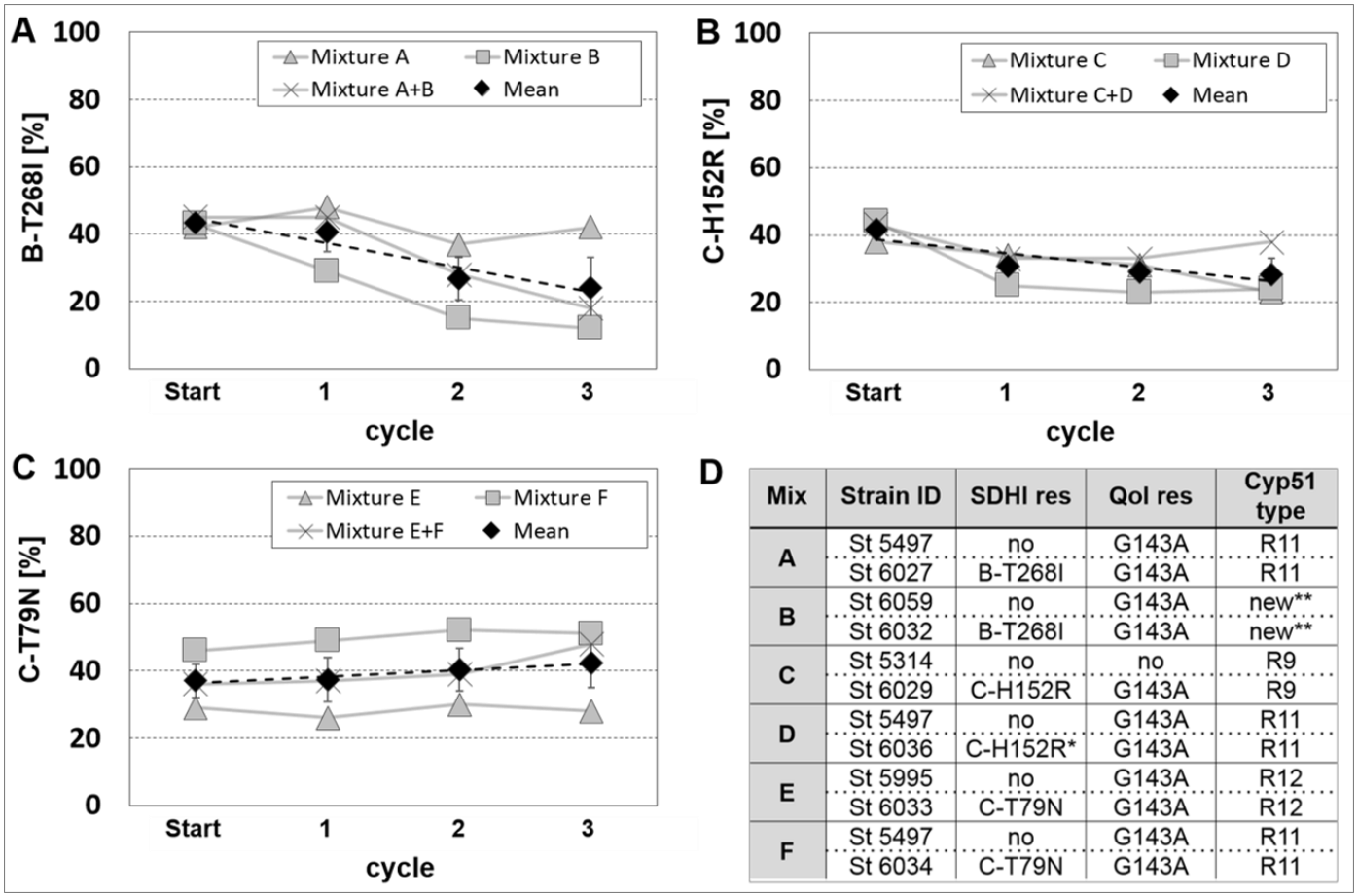
The SDHI resistant isolates investigated in competition studies, either carried a relatively rare SDH-variant in field isolates, B-T268I and C-H152R, or the more frequently detected C-T79N. Two isolates with the same SDH-variant were investigated and mixed in two independent mixtures with an appropriate SDHI sensitive isolate. In all mixtures, both isolates showed the same *Cyp51* haplotype and all isolates were QoI resistant (G143A), with exception of one SDHI sensitive isolate. This isolate was mixed with a C-H152R mutant

with G143A because they were shown to share the same *Cyp51* haplotype (R9). This haplotype had not been detected in any other SDHI sensitive isolate from Ireland. In addition to mixtures of two isolates (one SDHI sensitive and one SDHI resistant), mixtures of four isolates were also prepared (two SDHI resistant having the same SDH-variant and their SDHI sensitive mixing partners).

Pyrosequencing data of these competition experiments are given in Figure 41. In Figure 41 A, mixtures of B-T268I mutants and their sensitive partners are shown. In mixtures A and A+B a reduction of 20-25% of B-T268I frequency was observed. B-T268I frequency was stable in mixture B. In mean, a significant decrease of B-T268I was observed in this experiment with these isolates. A slight decrease of SDH-variant C-H152R was detected in mixtures C and D (15-20% reduction), whereas in mixture C+D C-H152R stayed more stable (Figure 41 B). C-T79N frequency was shown to be stable over all growth cycles in all mixtures with a tendency to increase (Figure 41 C).

Slopes of regression lines were statistically analysed to see whether the changes in resistance allele frequencies were significant. In summary, a significant decrease was observed for B-T268I ($P<0.001$) and C-H152R ($P<0.05$) frequency, however, C-T79N was shown to stay stable ($P>0.05$). Diseased leaf areas of different isolates over all cycles included in these competition studies are given in Table 29.

These results indicate that in the case of B-T268I and C-H152R, a reduction of the ‘fitness’ of these mutants could be observed, whereas C-T79N was shown to compete with SDHI sensitive isolates. Due to these fitness tests, it can be proposed that different target-site alterations can cause different fitness costs. These data are in line with the frequency of different alterations observed in the monitoring programmes. Most SDHI resistant isolates showed C-T79N exchange. B-T268I was detected in three isolates from 2015, however, was not detected in 2016 anymore. Instead, another alteration at the same position, B-T268A, was found in one isolate in 2016.



Mix	Strain ID	SDHI res	QoI res	Cyp51 type
A	St 5497	no	G143A	R11
	St 6027	B-T268I	G143A	R11
B	St 6059	no	G143A	new**
	St 6032	B-T268I	G143A	new**
C	St 5314	no	no	R9
	St 6029	C-H152R	G143A	R9
D	St 5497	no	G143A	R11
	St 6036	C-H152R*	G143A	R11
E	St 5995	no	G143A	R12
	St 6033	C-T79N	G143A	R12
F	St 5497	no	G143A	R11
	St 6034	C-T79N	G143A	R11

Figure 41: *In planta* competition studies of SDHI resistant field isolates of *Z. tritici* against SDHI sensitive isolates with similar genetic background. Three propagation cycles on wheat seedlings cv. 'Riband' were performed with mixtures of appropriate SDHI resistant (n=6) and sensitive (n=4) isolates without fungicide pressure. Quantitative pyrosequencing or qPCR (C-T79N) was used to detect frequency of *Sdh* mutations in spore suspensions that were used for inoculation. **A:** B-T268I frequency [%] in mixtures of B-T268I mutants (n=2) and sensitive isolates (n=2), two in mixture (A, B) or all four in mixture (A+B). **B:** C-H152R frequency [%] in mixtures of C-H152R mutants (n=2) and sensitive isolates (n=2), two in mixture (C, D) or all four in mixture (C+D). **C:** C-T79N frequency [%] in mixtures of C-T79N mutants (n=2) and sensitive isolates (n=2), two in mixture (E, F) or all four in mixture (E+F). **D:** Isolates in mixtures and their status of SDHI resistance (SDHI res), QoI resistance (QoI res) and *Cyp51* haplotype. *St 6036 was kindly provided by S. Kildea (Teagasc, Ireland, 2015) and had D-R47W exchange in combination to C-H152R. **This combination was first found in 2015. Single isolates were additionally propagated over all cycles. All mixtures and single isolates were tested for all mutations (including G143A) to detect possible contaminations between different treatments (data not shown in the figure).

Table 29: Pathogenicity of SDHI resistant and SDHI sensitive isolates of *Z. tritici* on wheat cv. 'Riband'. Mean of six replicates, each replicate containing a pot with around 10 wheat plants, shown for all propagation cycles and standard error is given. Each pot was inoculated with 2 mL of a spore suspension, which contained $3.0x10^6$ spores mL^{-1}. After 21-28 dpi, infected leaves of each isolate/mixture were harvested. Infected leaves within an isolate/mixture were pooled and washed in 10 mL of Tween-H_2O and applied to a next charge of plants.

SDH-variant	Isolate ID	Diseased leaf area [%] (mean ± SE)			
		Cycle 1	Cycle 2	Cycle 3	Mean (cycles)
no	St 5497	100 ± 0	90.8 ± 3.75	93.3 ± 4.94	94.7
	St 6059	100 ± 0	96.7 ± 1.67	98.3 ± 1.67	98.3
	St 5314	100 ± 0	78.3 ± 4.01	86.7 ± 3.33	88.3
	St 5995	96.7 ± 3.33	45.0 ± 3.14	100 ± 0.0	80.6
B-T268I	St 6027	78.3 ± 4.77	83.3 ± 2.11	91.7 ± 3.07	84.4
	St 6032	95.0 ± 5.0	78.3 ± 3.07	86.7 ± 3.33	86.7
C-H152R	St 6029	86.7 ± 6.15	73.3 ± 4.22	95.0 ± 2.24	85
	St 6036	90.0 ± 6.32	51.7 ± 6.01	78.3 ± 3.07	73.3
C-T79N	St 6033	83.3 ± 3.33	58.3 ± 3.07	100 ± 0.0	80.5
	St 6034	93.3 ± 6.67	90.8 ± 2.71	96.7 ± 3.33	93.6

4.2.7 SDHI resistant laboratory mutants of *Z. tritici*

4.2.7.1 SDHI sensitivity of laboratory mutants

Compared to *P. teres*, *Z. tritici* produces a high number of spores *in vitro*. Therefore, it is relatively easy to generate fungicide resistant isolates by selection on agar amended with fungicides. In previous studies, the generation of laboratory mutants of *Z. tritici* by UV-light exposure and fungicide selection has been carried out (Scalliet et al., 2012; Fraaije et al., 2012). The SDHI sensitivities of laboratory mutants generated in 2008 in our laboratory (Stammler et al., 2010), were investigated in the present study. Three SDH wild type isolates (S27, St 1965 and St 3718) were used to select SDHI resistant mutants on agar amended with fungicide by applying large numbers of spores after previous exposure to UV-light (in most tests). In these experiments, laboratory mutants were obtained by selection with different SDHIs (boscalid, fluopyram, fluxapyroxad and isopyrazam). The mutants investigated are B-H267Y (selected on 30 mg boscalid L^{-1}), B-H267L (selected on 30 mg isopyrazam L^{-1}), B-I269V (selected on 10 mg fluopyram L^{-1}), C-N86K and C-G90R (selected on 10 mg fluxapyroxad L^{-1} and 30 mg penthiopyrad L^{-1}) and C-H152R (selected on 30 mg boscalid L^{-1}). Several other laboratory mutants, which showed the same or other substitutions (or combinations of two SDH exchanges in rare cases), were detected (internal unpublished data).

During the present thesis, further laboratory isolates were produced to enlarge the set of SDHI resistant laboratory mutants. These additional isolates were produced in 2014 on agar amended with fluxapyroxad and were derived from the parental isolates IPO323, St 3718, St 3573 and St 5821. The latter two parental isolates are moderately or advanced resistant towards QoI fungicides, either showing G143A (St 3573) or F129L (St 5821). In comparison to most laboratory mutants from 2008, generation of mutants in the present work was not done by previous UV-mutagenesis (see section 3.12.2). This was done to reduce the likelihood of additional mutations to occur in the genome that do not have an impact on SDHIs. The list of selected isolates and SDH-variants that were found in this experiment is given in Supplementary Material, Table 36. At high fluxapyroxad concentrations (10 mg a.i. L^{-1}), C-H152R was present in at least one laboratory strain obtained by all parental isolates. Other amino acid substitutions, that were found in the field such as C-T79N, C-N86S, were not obtained even at the lower concentration of fluxapyroxad (1 mg a.i. L^{-1}). Most clones that were recovered from 1 mg fluxapyroxad L^{-1} were shown to be false positives (no growth visible on second fluxapyroxad selection).

C-H152R mutants generated in 2014 were included in sensitivity studies of laboratory mutants. The SDHI sensitivity of some laboratory mutants was determined in microtiter tests and EC_{50} values are shown in Figure 42. Laboratory mutants, which carried different SDH alterations, exhibited a range of different sensitivities. Some SDH-variants, such as B-H267Y, B-I269V and C-G90R, were shown to cause lower EC_{50} values compared to other variants in case of most SDHIs. However, fluopyram was less affected by B-H267Y than other SDHIs, which had also been observed in the orthologous exchange in *P. teres* (B-H277Y). On the other hand, fluopyram efficacy was more reduced in case of the B-I269V mutant compared to all other SDHIs showing EC_{50} of >3 mg a.i. L^{-1}. B-I269V and other SDH-variants were selected by fluopyram, however, B-I269V was exclusively selected by fluopyram and not by other SDHIs (internal unpublished data).

C-H152R was detected in many different isolates produced in laboratory studies. This variant, which was shown to cause highest EC_{50} values of all variants detected in the field, also gave high EC_{50} values in laboratory isolates. Two variants, B-H267L and C-N86K, were detected in laboratory studies, and which cause a high SDHI efficacy loss, but have not been found so far in the field. Both variants were shown to cause a high SDHI efficacy loss in microtiter tests, even higher than EC_{50} values of C-H152R mutants.

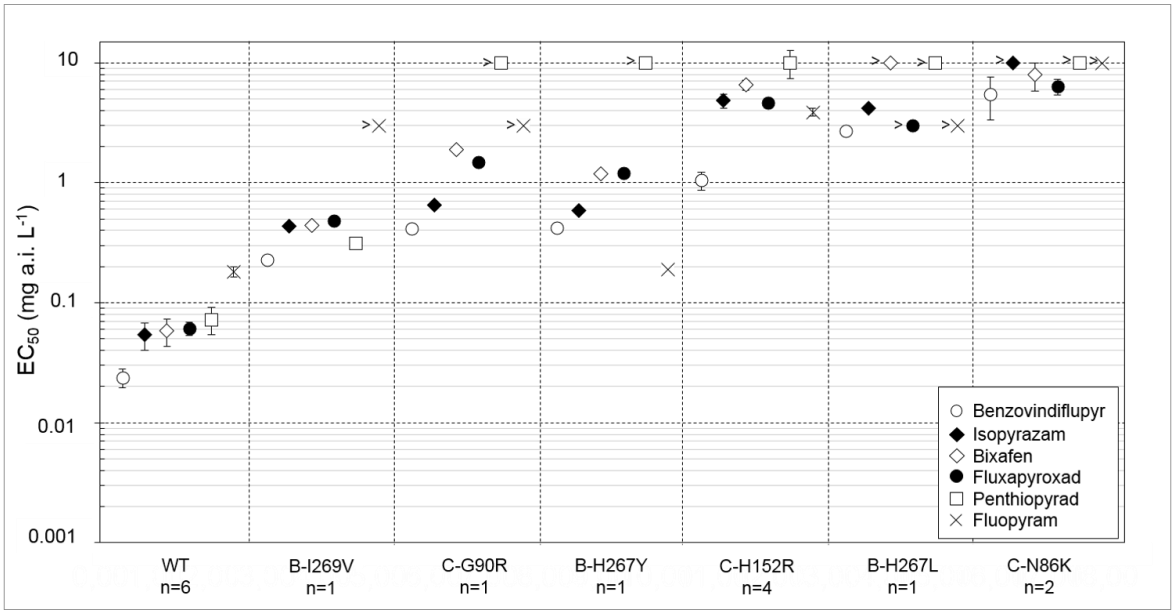


Figure 42: SDHI sensitivity of laboratory generated *Z. tritici* isolates carrying different mutations in the *Sdh* genes. Photometric measurements (OD_{405}) to detect mycelial growth of *Z. tritici* in microtiter plates were used to determine EC_{50} values (probit calculation) of SDHI fungicides. Water control and seven concentrations (up to 10 mg a.i. L^{-1}) of benzovindiflupyr, fluxapyroxad, isopyrazam, bixafen, penthiopyrad (up to 30 mg a.i. L^{-1} in case of C-H152R mutants) and fluopyram applied in four replicates were mixed with spore suspensions of *Sdh* wild type isolates and several *Sdh* mutated isolates. SDH amino acid substitution, the number of isolates and standard errors are given. Standard errors were calculated by mean values obtained from two independent tests over biological replicates (due to low number of investigated isolates (B-I269V, C-G90R, B-H267Y, B-H267L) some standard errors are missing). Some isolates in the case of fluopyram and fluxapyroxad were only tested up to 3 mg a.i. L^{-1} (B-I269V, C-G90R and B-H267L in the case of fluopyram and B-H267L in the case of fluxapyroxad).

In a direct comparison of parental isolates and C-H152R mutants, RF values were calculated for different pairs of isolates (Table 30). RF values calculated for different pairs of parental isolates and obtained C-H152R mutant ranged between 20 to 353, depending on the SDHI and isolate pair under investigation. It was observed that RF values of C-H152R mutants, although pairs of isolates have same/similar genetic background, can highly vary between different pairs of isolates. Lowest RF values were obtained for St 3718 and two corresponding C-H152R mutants (B3-6-18 generated in 2008, X3718-4 generated in 2014). St 3718 showed highest EC_{50} values in case of all SDHIs within sensitive, parental isolates. This demonstrates that RF values can show variation even within one SDH-variant depending on the sensitivity of the parental isolates.

Table 30: EC_{50} values and RF values of C-H152R mutants derived from different parental isolates. Parental isolates are S27 (SDHI sens, QoI sens), St 3718 (SDHI sens, QoI sens) and St 3573 (SDHI sens, QoI res). RF values were calculated by taking EC_{50} of corresponding parental isolates (EC_{50} of mutated isolate/ EC_{50} of parental isolate).

Isolates	Benzovindi.		Isopyrazam		Bixafen		Fluxapyroxad		Penthiopyrad		Fluopyram	
	EC_{50}	RF	EC_{50}	RF	EC_{50}	RF	EC_{50}	RF	EC_{50}	RF	EC_{50}	RF
S27	0.016		0.026		0.026		0.030		0.035		0.135	
B0-3-7	1.242	78	5.547	213	5.911	227	4.796	160	na	na	3.648	27
St 3718	0.038		0.112		0.088		0.078		0.147		0.165	
B3-6-18	1.087	29	6.224	55	8.155	93	4.728	61	15.608	106	3.899	24
X3718-4	0.836	22	3.648	33	6.616	75	3.421	44	15.950	109	3.314	20
St 3573	0.015		0.019		0.015		0.072		0.030		0.157	
X3573-2	1.310	87	3.885	204	5.295	353	5.570	77	7.985	266	4.661	30

4.2.7.2 Competition studies of C-H152R laboratory mutants in the glasshouse

C-H152R laboratory mutants were investigated with respect to their competitiveness to their parental strains in competition studies *in planta*. Thus, each SDHI sensitive parental isolate was mixed in two separate mixtures with two independent C-H152R mutants of it. These mixtures were transferred over several propagation cycles, as it was already described for field isolates (see section 4.2.6). In addition, each isolate or laboratory mutant was inoculated separately to rate diseased leaf area of isolates. In Figure 43, the frequency of C-H152R in mixtures is given for two independent experiments with the same set of isolates. These competition studies were performed in 2015 when glasshouse lights had not yet been adjusted to an optimised lighting for *Z. tritici* (see section 3.11.3.1) One experiment was carried out in early summer of 2015 (Figure 43 A, C, E). In this experiment, infection rates were high for most isolates, with the exception of the parental isolate IPO323, its C-H152R mutants and their mixtures. IPO323 and its corresponding C-H152R mutants showed diseased leaf area of <5%, which led to no or low pyrosequencing efficiencies and were excluded. In a second test, which was conducted in winter of 2015, infection rates were very low most probably due to a lower light exposure during the infection process. The experiment was, therefore, stopped after two cycles of propagation due to unsufficient spore yield to start a new inoculation cycle (Figure 43 B, D, F). Highest infection rates in all experiments were determined for parental isolate St 3718 and its corresponding laboratory mutants. All three isolates showed 80-100% of diseased leaf area in the first test over all cycles and 40-50% of diseased leaf area in the second study. St 3573 and St 5821, and mutants of them, showed medium infection rates (20-50%) in the first test and lower infection rates (0-15%) in the second trial.

A significant decrease of the C-H152R frequency was observed for mixtures containing St 3718 and its mutants. This was consistent in both experiments, however, in the experiment with lower infection pressure, the decrease was more pronounced (Figure 43 A+B).

A more diverse picture was observed when QoI resistant parental isolates were examined in mixtures with their C-H152R mutants. Due to the high occurrence of G143A in the *Z. tritici* population in Europe, it was seen as useful to examine multiple resistance and the resulting ‘fitness phenotype’ in this case.

Thus, mixtures of QoI resistant isolates with their appropriate mutants were replicated individually for each mixture. This means that e.g. mixture 1.1 and 1.2 are technical replicates (include the same C-H152R mutant and parental isolate) to elaborate if mixtures of same isolates behave similarly in a repetition. Replicates of mixtures behaved similar with respect to a decrease or increase of C-H152R frequency.

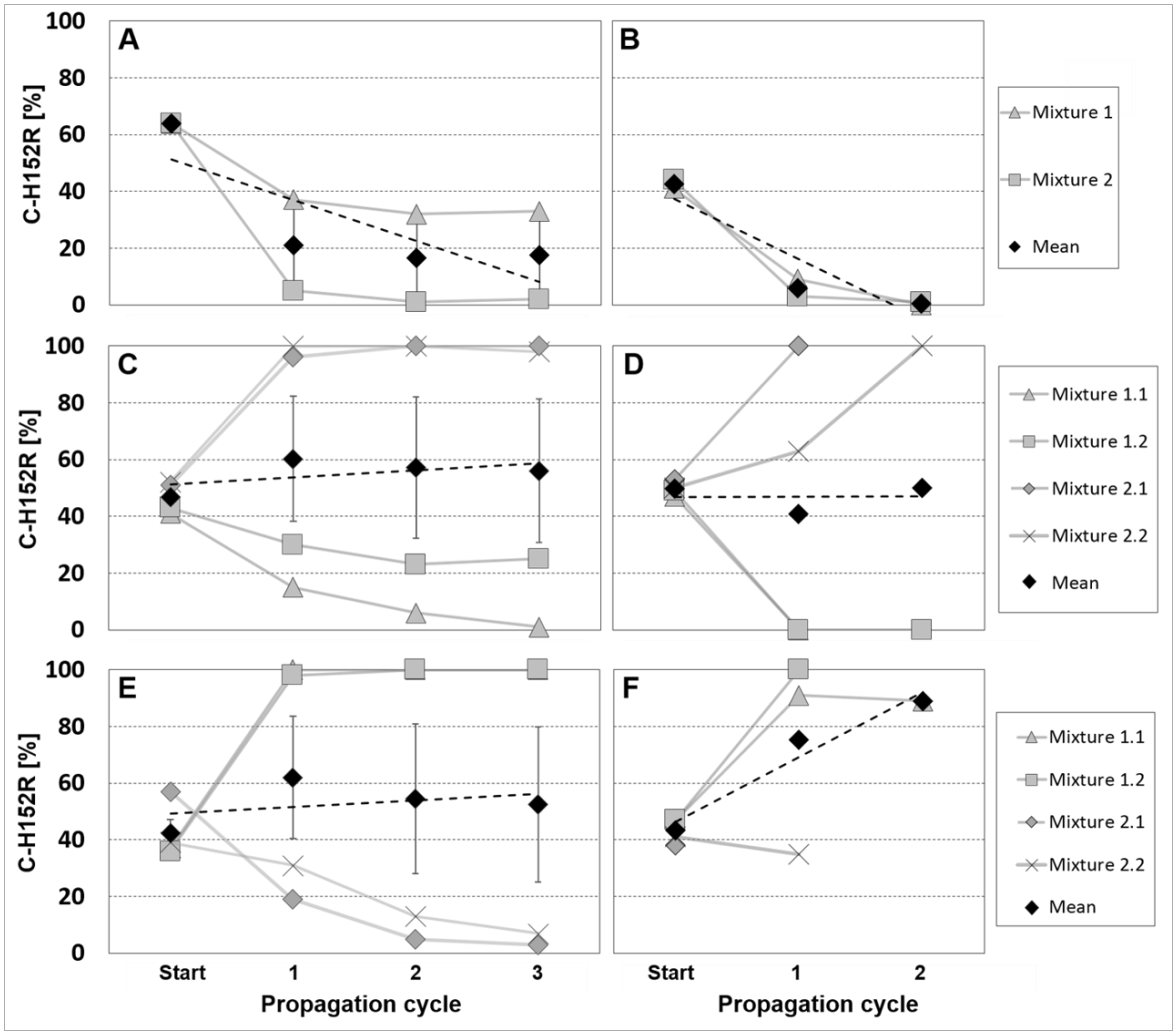


Figure 43 *In planta* competition studies of laboratory mutants of *Z. tritici* carrying C-H152R and their parental isolates under non-selective conditions. Two independent glasshouse tests were performed with the same set of isolates. C-H152R mutants, which were selected on fluxapyroxad-amended agar and were obtained from three parental isolates (St 3718, 3573 and 5821), were mixed with the corresponding parental isolate. Mixtures were subsequently transferred over three propagation cycles in a first trial (A, C, E) and two propagation cycles in a second trial (B, D, F) on wheat cv. 'Riband' (BBCH11). In the first trial (A, C, E), high infection rates were observed. In the second test (B, D, F), lower infection rates were observed for all isolates and mixtures. Some values are missing due to low infection rate and none-detectable signals in pyrosequencing procedure. **A** and **B:** Competition of two independent C-H152R mutants with their parental isolate 3718, which is QoI sensitive. **C** and **D:** Competition of two independent C-H152R mutants with their parental isolate 3573, which is QoI resistant (G143A). Each mixture was repeated. **E** and **F:** Competition of two independent C-H152R mutants with their parental isolate 5821, which is moderately QoI resistant (F129L). Each mixture was repeated. Spore suspensions were prepared from ISP2 plates at the starting point or by washing infected leaves in Tween-water. 2 mL of each spore suspension at each propagation cycle was used for analysis by pyrosequencing in two repetitions. In addition to different mixtures, which are shown in grey, the mean of mixtures and their standard errors are given in black. Single isolates were additionally inoculated and transferred over all cycles. The frequency of C-H152R was determined in mixtures and single isolates. Single isolates showed either 0% C-H152R for parental isolates 3718, 3573 and 5821 or 100% for all C-H152R mutants over all cycles (not shown in the figure).

Irrespective of an increase or decrease of C-H152R frequency in different mixtures, each mixture treated separately showed the same outcome in both experiments. One C-H152R mutant of St 3573 (G143A) was observed to compete with its parental isolate and was shown to reach frequencies of up to 100% in the mixture. The second mutant of St 3573 showed the opposite trend with a strong decrease of C-H152R frequency over propagation

cycles. The same results were obtained for C-H152R mutants of St 5821 (F129L), with an increase of the first mutant in the mixture but a decrease of the second mutant.

These competition studies with G143A/F129L parental isolates and their corresponding mutants showed controverse results, although parental isolates and their mutants should theoretically have a more similar genetic background than field isolates. In further studies, it would be necessary to sequence genomes of such mutants that show an advanced fitness compared to its parental isolate and thus to detect potential compensatory mechanisms that allow a higher pathogenicity compared to its parental isolate (although having C-H152R).

5 Discussion

SDHIs are today an important tool for disease management in cereals. To date, 14 plant pathogenic species show SDHI resistance under field conditions (FRAC, 2016). The most intensively studied fungal pathogens are described from various speciality and row crops, such as *B. cinerea* on strawberry (Stammler et al., 2007; Veloukas et al., 2011), *C. cassiicola* (Miyamoto et al., 2008, 2010), *Didymella bryoniae* (Avenot et al., 2012) and *Podosphaera xanthii* on curcurbits (Ishii et al., 2011; FRAC, 2016), *A. alternata* on pistachio (Avenot et al., 2008, 2009; Stammler, 2008), *A. solani* on potatoes (Fairchild et al., 2013), *S. sclerotiorum* on oilseed rape (Glättli et al., 2009), *V. inaequalis* on apple (Huf, 2016; FRAC, 2016) and *Stemphylium vesicarium* on asparagus (FRAC, 2016). Molecular studies have revealed that the resistance mechanisms are target-site mutations in the *Sdh* genes encoding for subunits SDH-B, SDH-C and SDH-D.

The emergence of SDHI resistant phenotypes of two cereal pathogens in Europe is described in the present work. First SDHI resistant isolates of *P. teres* and *Z. tritici* were detected in 2012. Therefore, these fungal species were the first important cereal pathogens with relevance in crop protection which acquired SDHI resistance. Several target-site mutations were detected and characterised in the present work. In the following sections, resistance mechanisms in *P. teres* and *Z. tritici* are compared to each other and with other phytopathogenic fungi. The frequency, the impact and the cross-resistance pattern of target-site mutations are highlighted. Finally, the competitiveness of resistant isolates is discussed with special attention to future efficacy of SDHI-containing products in cereal production systems.

5.1 Emerging situation of SDHI resistance in net blotch and STB in Europe

5.1.1 Frequency and distribution of SDHI resistant isolates of *P. teres* in Europe

The first SDHI resistant isolates of *P. teres* were detected in Germany in 2012. A target-site mutation in *SdhB* gene, leading to B-H277Y, was found (Rehfus et al., 2016; FRAC, 2016). Resistance factors (described as EC_{50} of mutated isolates/ EC_{50} sensitive isolates) for this mutation were low for all SDHIs tested in the present study. Accompanied by the introduction of more SDHI containing fungicides on the European market, the situation became more complex. In the following years, the number of resistant isolates and the responsible target-site mutations in resistant isolates has increased. In 2013, eight

additional SDHI resistance mutations leading to SDH-variants C-N75S, C-G79R, C-H134R, C-S135R, D-D124N/E, D-H134R and D-D145G were observed. However, sequencing of the genes *SdhB*, *SdhC* and *SdhD* of several SDHI resistant isolates revealed, that each resistant isolate showed only one of these mutations but not two or more in combination. Most resistant isolates collected in 2013 carried the C-G79R substitution. In addition to its high frequency, this SDH-variant gave one of the highest impacts on the efficacy of all SDHIs that were tested in microtiter tests. Most SDHI resistant isolates were collected in regions of Northern France and Northern Germany, which represent the main barley production areas in Europe. A further increase of SDHI resistant isolates was observed in Europe in 2014 and 2015. Regions, which show highest frequencies of resistant isolates, were north-eastern parts of France and middle and northern parts of Germany. Besides C-G79R substitution, other substitutions in the SDH enzyme gained in importance, e.g. C-N75S, C-H134R and D-D145G. Additionally, the SDH-variants C-S73P, D-G138V and D-E178K were detected in a few resistant isolates.

The situation of SDHI resistance in *P. teres* was studied using two different sampling methods (‘Random monitoring and ‘Field monitoring’). The pattern of SDH-variants that was observed in different regions was found to be highly comparable to each other. Both monitoring studies revealed that C-G79R was the most frequent SDH-variant, particularly in 2013 and 2014. In France, C-G79R was the most frequent alteration over all years (‘Random’- and ‘Field monitoring’). In Germany, the proportion of C-G79R mutants within resistant isolates was lower compared with France. For example, in the german ‘Field monitoring’ 2016, the most frequent SDH-variant was D-D145G then followed by C-G79R and C-H134R. This trend that other SDH-variants have gained in importance, was observed in France as well, however C-G79R was still the most frequent alteration within resistant isolates. France was shown to have the highest frequencies of SDHI resistant isolates in all countries that were analysed. Comparing the increase and frequency of SDH-variants in different countries of Europe, the ‘build-up’ of SDHI resistant isolates in France is hard to correlate with the use of SDHI-containing fungicides alone. Recommendations, that are followed in all countries with registered SDHI-containing products, restricted the usage of SDHIs to a maxium of two foliar applications per growing season (FRAC guidelines, FRAC, 2016). Particularly in France only one application of SDHIs per growing season is recommended (https://www.english.arvalisinstitutduvegetal.fr/). In addition, SDHIs are always applied in mixtures with fungicides which target an alternative MOA. Similarities in pattern could be found when the frequencies of SDHI resistant isolates in Europe were compared to a disease risk map for net blotch. The disease risk for net-blotch in winter barley production in Europe is given in Figure 44. In the north-east of France, where the highest level of resistant isolates was observed, the risk for net-blotch infection is high over large areas. This is due to various factors, such as climatic conditions that favour the propagation of the pathogen, the availability of host plants (main barley growing region in

France), and the use of susceptible barley cultivars in these regions (Born, 2013). The disease risk is also quite high in some parts of Germany and some regions in Poland (see Figure 44). In Poland, no SDHI resistant isolates were detected in 'Random monitoring'. Very low frequencies of resistance alleles were detected in infected barley leaves from trial sites in 2016. The absence or low levels of SDHI resistance in Poland, although showing high disease risk, can be explained by the more extensive agricultural systems there. Additionally, SDHI-containing products were introduced later to the polish market than in France and Germany. In the United Kingdom, the disease risk is mapped moderate to high for some regions. However, the areas which show 'high risk' are smaller than those regions in France and Germany and no regions are classified as 'very high' risk regions. This and the fact that SDHIs are probably used less in the UK than in Germany and France could explain why SDHI resistance in the UK is still at a low level. Fungicide inputs in barley in the UK are quite low compared to France and Germany and QoI/DMI fungicides have dominated (personal communication, Dr. R. Bryson, BASF SE).

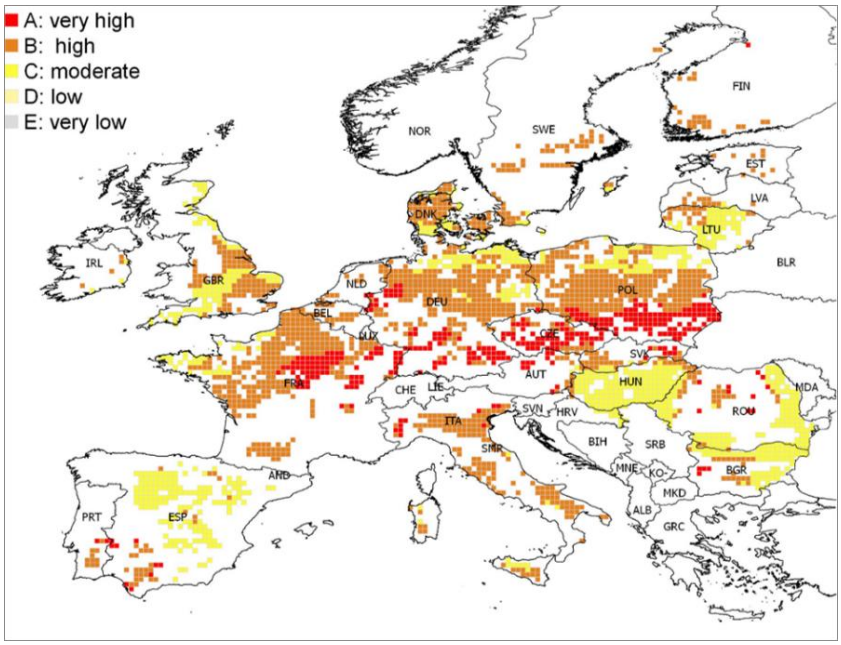


Figure 44: Disease risk for *P. teres* in winter barley (BBCH 25-69) in European cereal growing areas. Extracted from Born (2013) 'Optimal Zonal Trial Planning (OZTP) for evaluating fungicides in wheat and barley' for BASF by the company Spatial Business Integration. For analysing the disease risk in European countries, long-term information was derived from satellite images, weather records and field observations for regions in Europe where barley is grown. These data were compared to conditions *P. teres* requires for an optimal development. The data is computed in a grid of 25 km by 25 km and the results are compared to information available from scientific papers and reports of disease outbreaks. This map was also shown in Rehfus et al. (2016) in Figure 11.

This observation suggests that disease pressure and vitality of the pathogen are driving forces for resistance development and not solely due to fungicide selection pressure. In regions where the disease risk for net-blotch is high, *P. teres* shows an earlier onset of infections and a shorter latent period. Thus, more propagation cycles per season can be obtained, which implies that the risk of the random emergence of mutations could be higher, due to the higher number of spores that are produced. This is in line with the resistance risk

assessment by van den Bosch (2011) and Grimmer et al. (2015), which showed that the basic reproduction number and the latent period of a pathogen are significant factors for resistance risk development.

The distribution of the SDHI resistant isolates was found to be variable across Europe, with the highest level of resistant isolates and genetic diversity (with respect to SDH variation) in France and Germany. According to the FRAC Pathogen Risk List from 2013, *P. teres* is classified as a medium risk pathogen for the development of resistance to fungicides (FRAC, 2016). Nevertheless, a rapid occurrence and 'build-up' of resistant phenotypes towards SDHIs was observed between 2012 and 2014, six and five years (Germany and France, respectively) after the market launch of the first foliar-applied SDHIs. After the first strong increase from 2012 to 2013 in both countries, the proportion of SDHI resistant isolates further increased to ~70% in France or stayed at a level ~40-50% in Germany. In other countries, such as the United Kingdom, Denmark and Sweden, anomalies between different years were observed. For example, in the United Kingdom, SDHI resistant isolates were observed in a significant proportion in 2013, however in 2014 no SDHI resistant isolates were collected. In 2015, five of eight isolates collected in the UK were SDHI resistant. In net-blotch infected samples from the 'Field monitoring', very low frequencies of resistance alleles were observed in the UK in 2015 and even in 2016. This indicates that SDHI resistant isolates are detectable in some cases, but no real 'build-up' of resistant isolates in the population in the UK has occurred so far. Interestingly, in the case of QoI resistance, F129L reached the highest frequencies in the UK of all countries in Europe. Here, levels of up to 70% F129L were found in the population from the UK, whereas in Germany and France levels of more than 45% F129L were not exceeded in all years of observation (data not shown). An explanation could be a differential use of SDHIs and QoIs in these three countries. SDHIs are also broadly used in cereal cultivation in the UK. However, especially in the UK, barley cultivation is more extensive (compared with e.g. wheat) and fungicides with a lower price (e.g. QoIs) are often preferred (personal communication, Dr. R. Bryson, BASF SE) which is different to barley production in countries such as Germany and France. SDHI resistant isolates were also observed in countries such as Denmark (in 2013) and Sweden (in 2014) at low frequencies. In both countries, SDHIs registrations are very limited and most SDHIs are not yet approved for the use in cereals. A wind drift of spores from northern regions of Germany might additionally explain the occurence of resistant phenotypes in these countries rather than any selection pressure from fungicide applications.

Two further amino acid substitutions in the SDH complex, not detected in this study, have been reported in FRAC, namely C-K49E and C-R64K (FRAC, 2016).

5.1.2 Frequency and distribution of SDHI resistant isolates of *Z. tritici*

The first SDHI resistant isolate of *Z. tritici* was found in 2012 and carried C-T79N exchange (FRAC, 2016). Although high numbers of isolates were collected and tested for their sensitivity in the following years, only low numbers of SDHI resistant isolates have been found in Europe so far.

In our monitoring programmes (2012-2016), 42 of 3431 tested isolates were shown to be SDHI resistant. In 2013, another single isolate showing C-N86S in SDH enzyme was detected. In 2015, more SDHI resistant isolates were detected, however still at a very low level and most of them were collected from trial sites, which often show intensive 'SDHI application histories' and not in 'Random monitoring'. Genotypes which lead to SDHI resistance detected in 2015 showed B-T268I, C-T79N, C-N86S, C-H152R and C-V166M substitutions. Several other variants at the same or different positions were further detected in 2016 (B-N225I, C-T79I, C-N86A). Additionally, a low number of double *Sdh* mutated isolates were collected. These showed two altered positions in two different subunits, not two in the same subunit, B-R240L+C-T79N, B-T268I+C-I29V, B-T268A+C-F23S and C-H152R+D-R47W. Dr. B. Fraaije (personal communication, Rothamsted Research) has additionally reported of several isolates with more than one *Sdh* mutation but the contribution of such mutations that occur in combination to other resistance mutations with known impact has still to be determined. The knowledge of DNA/protein sequence and baseline information of wild type populations, in combination with sensitivity tests of sensitive and resistant isolates normally allows a reliable determination of resistance mutations. However, especially when a low number of isolates are available (at the beginning of emergence), baseline sensitivity shows high variation and the observed mutations cause only a low impact on the SDHI sensitivity, the determination is more challenging.

Particularly *SdhC* DNA sequence of *Z. tritici* shows a high variation, even within SDHI sensitive isolates. 35 nucleotide exchanges were detected in different SDHI sensitive isolates. The nucleotide sequence alignments of different wild type isolates can be found in the Supplementary Material, Figure 53. Most mutations were found to be silent, whereas three nucleotide exchanges led to the amino acid substitutions C-I29V, C-N33T and C-N34T. The amino acid sequence alignments are shown in the Supplementary Material, Figure 54. The first exchange was found in only one isolate from France, whereas C-N33T and C-N34T were found in ~50% of isolates, either showing NN or TT, but not mixed up. No differences in the SDHI sensitivity was discovered in isolates with NN or TT (in the present study; Dubos et al., 2013). Amino acid alterations at position 33 and 34 were also detected in SDHI resistant isolates. Amongst those SDHI resistant isolates which have the same resistance mutations, both types (NN and TT) could be observed in most cases. All three C-H152R field isolates showed TT at positions 33 and 34. However, B-T268I, C-T79N

and C-N86S isolates had both types. This gives evidence that these SDHI resistant isolates (showing same resistance mutation) have emerged in both types independently. This would indicate that resistance mutations evolved more than once (at least two times independently, probably even more often). Torriani et al. (2009) reported that G143A (QoI resistance) has emerged at least four times independently in European populations of *Z. tritici*; this was shown by phylogenetic analysis of mitochondrial sequences. Additionally, the F129L exchange in *P. teres* has been found to be coded by three different codons (TTC wild type to TTA, TTG or CTC, all coding for leucine) (Semar et al., 2007), also meaning an independent emergence. The occurrence of resistance in different geographical areas or in different genetic groups within a species was also shown in case of SDHI resistance in *B. cinerea* from apple (Yin et al., 2011).

Most amino acid substitutions found in SDHI resistant field isolates were shown to cause 'moderate' levels of resistance (see section 4.2.4). It is interesting to note that most of the 'moderate' mutations in field isolates, have not been detected in laboratory mutants before. Mutations, described by several studies with laboratory mutants (Skinner et al., 1998; Stammler et al., 2010; Fraaije et al., 2012; Scalliet et al., 2012), have not occurred in the field so far. Exceptions thereby are the 'strong' mutation leading to C-H152R and one 'moderate' mutation (C-N86S), which had been found in combination with B-H267Y in a laboratory isolate (Fraaije et al., 2012). One reason for the selection of mainly 'strong' mutations in laboratory screenings could be due to the high discriminatory doses chosen for the selection of SDHI resistant mutants. Thus, isolates carrying 'moderate' mutations were probably unable to grow in such experiments. On the other hand, isolates with 'strong' mutations which have been selected by SDHIs in several laboratory studies, such as B-H267L, C-N86K or C-G90R, have still not been found in the field population. A possible explanation could be the reduced fitness of these isolates. The amino acid substitution C-H152R, which has been described by three of the four laboratories which have generated SDHI resistant isolates (Stammler et al., 2010; Fraaije et al., 2012; Scalliet et al., 2012), was first detected in the field in 2015, three seasons after the first SDHI resistant isolate (C-T79N) had been detected. The monitoring results in 2016 indicate that, currently, the most frequent amino acid exchanges are C-T79N and C-N86S, and that others, such as B-T268I, C-W80S, C-H152R, B-N225I, B-T268A, C-T79I and C-N86A, have only been found in single isolates (the last four SDH-variants are described for the first time in the field). C-H152R was found in only two isolates in 2016, one from the Netherlands, the other from the United Kingdom. Since the C-H152R amino acid exchange causes the highest resistance level of all field mutants to all SDHIs, and thus should give an advantage at high or low exposures of SDHIs, it could be speculated that this alteration might be connected to fitness penalties as well. In addition, in a practical field situation, lower dose rates are often applied and selection of mutants is a result of lower, but in some cases, repeated

applications. This may favour selection of 'moderately' resistant isolates with a better level of fitness (fitness of *Sdh* mutants is discussed in section 5.4)

Other institutions and companies found other *Sdh* mutations. For example, C-W80S (first found in our monitoring in 2016) was sampled for the first time in UK in 2013 by another research group (FRAC, 2016). In 2014, no suspicious isolates were detected in our monitoring. However, in 2014, B-N225T exchange was detected in an isolate from Ireland in another monitoring programme (FRAC, 2016). The first report of C-H152R in an Irish field was published by Dooley et al. (2016).

5.1.3 Comparison of the emergence of SDHI resistance in *Z. tritici* and *P. teres*

In accordance to the emergence of QoI and DMI resistance, SDHI resistant isolates of *Z. tritici* and *P. teres* were first detected in Western Europe in countries such as Ireland, the United Kingdom, France and Germany. This phenomenon is probably based on the high disease pressures in these regions, which comes along with a high intensity of fungicide use in these areas (FRAC, 2016). After the first detection in 2012, a rapid increase of resistant isolates was observed in the following years in the European population of *P. teres*, particularly in countries such as France and Germany. In contrast, resistant isolates of *Z. tritici* in Europe were still observed at low frequencies, even in 2015 and 2016.

There are several reasons that could explain the much faster 'build-up' of resistant individuals in *P. teres* compared with *Z. tritici*. Factors such as the biology of both different fungal species, the intensity of fungicide usage and disease pressures in the years of detection could have had an impact on the emergence of resistance. *P. teres* shows a shorter latency phase in its host compared with *Z. tritici*. Up to 12 cycles of propagations can be observed in net blotch disease under favourable conditions per year, compared to 3-6 cycles of propagation in the case of STB (personal communication, G. Prigge, BASF SE; Gurr and Fones, 2015). Higher numbers of propagules and higher infection pressures could theoretically contribute to a faster emergence and 'build-up' of resistance in a population. However, field technicians and collectors, who sent infected leaf sampes, reported that the disease pressure of *P. teres* was low in most regions of Germany in 2015 (personal communication, Dieter Strobel, BASF SE). In contrast, significant disease levels of *Z. tritici* were observed in all years for most regions, which indicates that environmental conditions were favourable for *Z. tritici* in these years. Both pathogens are classified as 'medium-risk' pathogens for the development of fungicide resistance (FRAC, 2016) and, therefore, does not help to explain the faster 'build-up' of SDHI resistance in net blotch. QoI resistant isolates of *Z. tritici* emerged faster and developed in higher frequencies in European countries compared with *P. teres*, which seems to be in contrast to SDHI resistance emergence. However, QoI resistance is mediated by different CYTB alterations

in both species (G143A prevalent in *Z. tritici* and F129L in *P. teres*), which might also explain the differences of QoI resistance emergence in *P. teres* and *Z. tritici*. Additionally, the market launch and the range of SDHI containing products in barley and wheat production are very similar and, therefore, could also not really explain different numbers of SDHI resistant isolates in both fungi. In summary, a real explanation why *Sdh* mutations did evolve faster in net blotch than in STB is still missing and answering that question would require the analyses of detailed informations, such as the fungicide use patterns, weather conditions, crop patterns and several other factors that could affect the time of resistance emergence.

Further differences in the development of SDHI resistance in *P. teres* and *Z. tritici* were observed in the range of different target-site mutations. Mutations that were found in resistant isolates of *P. teres* and *Z. tritici*, and their orthologous positions in the respective other pathogen, are given in Table 31. Most amino acids in the SDH enzyme that could result in SDHI resistance when substituted, are conserved residues in both *P. teres* and *Z. tritici* and, therefore, show the same amino acid in sensitive isolates. Exceptions are positions 23 (21), 29 (27), 75 (64) and 84 (73) in SDH-C of *Z. tritici* (*P. teres*). Here, the wild typic amino acid is different in both species.

C-N86S in *Z. tritici* and orthologous C-N75S in *P. teres* is the only substitution that has been detected in both pathogens in the field so far. All other SDH-variants have been detected either in *P. teres* or *Z. tritici*. C-G79R amino acid substitution, which is the most frequent alteration in *P. teres*, was not detected in field isolates of *Z. tritici* but has been detected in some laboratory isolates (here C-G90R) (Stammler et al., 2010; Fraaije et al., 2012; Scalliet et al., 2012; in the present study). On the other hand, C-T79N, the most frequent exchange within SDHI resistant isolates of *Z. tritici*, has not been detected in *P. teres* or in any other plant pathogens so far.

For most mutations, there is no explanation based on nucleotide sequence, why different mutations develop in both pathogens. An exception thereby is C-S135R (*P. teres*), which is unlikely to occur in *Z. tritici* because serine is coded TCG (instead of AGC as in *P. teres*) and two nucleotide exchanges would be necessary to result in arginine. The same was observed for C-V166M (*Z. tritici*), which is unlikely to occur in *P. teres* due to different codons. On a protein level, active parts of the SDH enzyme are composed similar to each other, and conservation between both organisms is high (Cecchini, 2003). In Figure 45, the structural alignment of homology models of *P. teres* and *Z. tritici* is given (homology models were individually presented in the results for both pathogens in Figure 15 and Figure 38).

Table 31: Amino acid positions in *P. teres* and *Z. tritici* which can be substituted in the case of SDHI resistance. Positions included are found in SDHI resistant field isolates of either *P. teres* or *Z. tritici*. Wild type amino acid is given for all positions analysed. An altered amino acid is given, when this substitution was found in this species in the field or in laboratory mutants (*Z. tritici*). SDH-variants collected in the field are shown in bold. SDH-variants detected in the present study are underlined. Other SDH-variants included in the list were detected in laboratory mutants, either in the present study or in Stammler et al. (2010), Fraaije et al. (2012) and Scalliet et al. (2012). Laboratory mutants are marked with an asterix. SDH-variants not found as a single substitution but in combination with another, are given in brackets. D-R47W was detected in an isolate from Ireland which simultaneously carried C-H152R (Dooley et al., 2016); this isolate was provided to our laboratory for further studies.

Protein	AA position in *P. teres*	AA position in *Z. tritici*
SDH-B	N235	**N225I** /T
	(R250)	**(R240L)**
	H277Y	H267Y/L/R*
	T278	**T268I(A)**
SDH-C	(V21)	**(F23S)**
	(L27)	**(I29V)**
	K49E	K60
	R64K	K75
	T68	**T79N/I**
	W69	**W80S**
	S73P	A84V*
	N75S	**N86S/A** (K*)
	G79R	G90R*
	H134R	H145R*****
	S135R	S146
	H141	**H152R**
	V155	**V166M**
SDH-D	(R63)	**(R47W)**
	D124N/E	D108
	H134R	H118
	G138V	G122
	D145G	D129E*
	E178K	E162

The majority of altered amino acids found in *P. teres* are positioned in close proximity to the haem b group (exceptions are B-H277Y in binding pocket and D-D124N/E and D-E178K at a far distance). In contrast, all SDH variations of *Z. tritici* were located near to or directly in the binding pocket for ubiquinone and the SDHIs. Length differences and variable positions of amino acids in the SDH complex in plant pathogenic fungi could explain diverse biologcial profiles of SDHIs in different fungal species (Scalliet et al., 2012) and could further explain why different mutations evolve in different species.

Interestingly, amino acid substitution C-R64K in *P. teres* (FRAC, 2016) leads to the amino acid lysine, which is present in sensitive isolates of *Z. tritici* at that position. Such variable positions in the SDH enzyme in sensitive isolates of different plant pathogens species might explain why other mutations develop in different species and could give an explanation why RF values of the same substitution can vary between species.

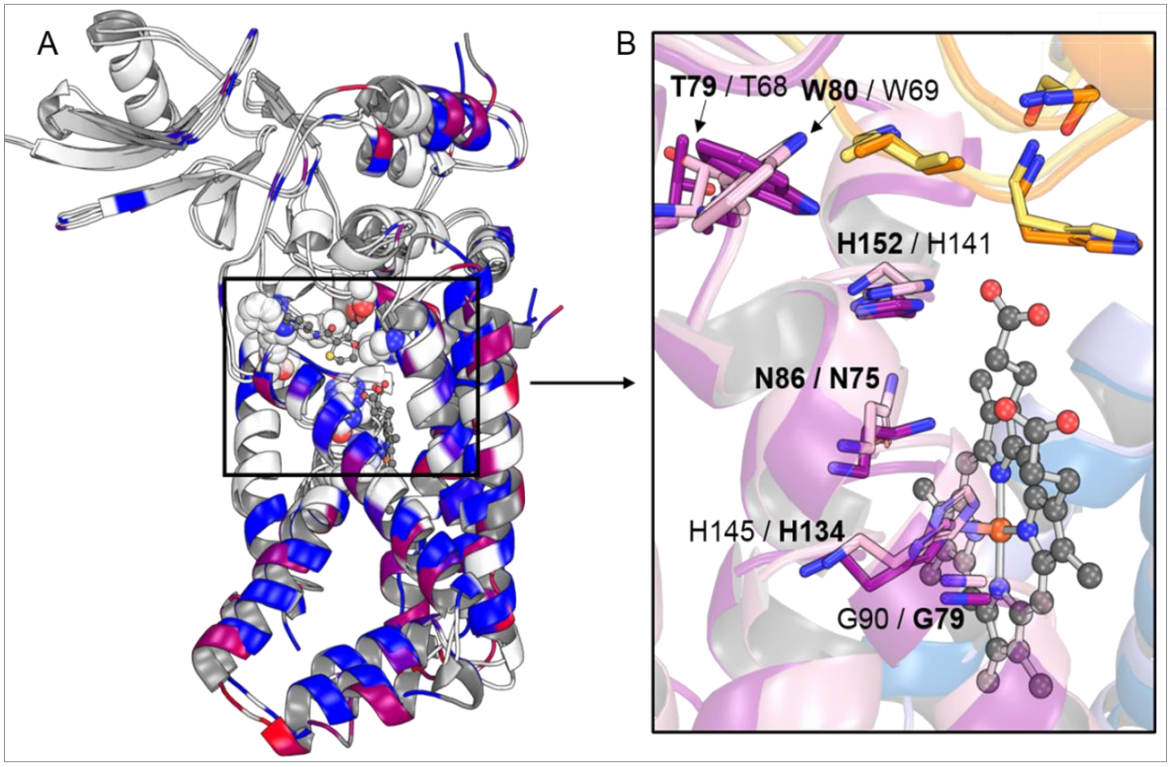


Figure 45: Structural alignment of *P. teres* and *Z. tritici* homology models. Homology models are based on X-ray from *G. gallus* (PDB 2WQY). **A:** Overlay of SDH-B, SDH-C and SDH-D subunits of both plant pathogens. Identical amino acid residues are shown in white, similar residues are shown in blue and dissimilar residues are shown in red. **B:** Zoom to some positions in SDH-C, which are known from SDHI resistant field isolates, and their overlay. Wild type amino acids are shown. Dark lilac shows the position of amino acids in *P. teres*, and light lilac the amino acids in *Z. tritici*. Positions that were found to be substituted in the field, are shown in bold.

EC_{50} values for SDHI sensitive isolates, that had been obtained in microtiter tests in the present study, ranged from 0.005 to 0.04 mg a.i. L^{-1} in the case of *P. teres*, and 0.05 to 0.18 mg a.i. L^{-1} in the case of *Z. tritici*, depending on the SDHI analysed. All SDHIs were shown to have higher EC_{50} values (~5-10 fold higher) for sensitive *Z. tritici* isolates than for sensitive *P. teres* isolates. However, if these differences are a result of altered positions in the SDH enzyme of different species, requires further detailed studies.

Resistance factors of mutations detected in *P. teres* and *Z. tritici* are shown in Table 32. Resistance levels in the case of the orthologous substitutions C-N75S and C-N86S are highly comparable in both pathogen species. However, C-G79R in *P. teres* showed slightly higher RF values for most compounds (penthiopyrad and fluopyram are exceptions) compared with C-G90R in *Z. tritici*. In contrast, B-H277Y in *P. teres* generally showed lower RF values than its orthologous variant in *Z. tritici*. Interestingly, laboratory mutants of *Z. tritici*, which had B-H267Y, did not show hypersensitivity to fluopyram, which was observed in B-H277Y in *P. teres*.

Table 32: Resistance factors of SDHI resistant isolates of *P. teres* and *Z. tritici* at corresponding substitutions. C-N75S, C-G79R, B-H277Y (*P. teres*) and C-N86S (*Z. tritici*) were detected in the field, C-G90R and B-H267Y are laboratory mutants of *Z. tritici*. RF values are based on microtiter tests shown in present study.

SDH-variants	RF values					
	Benzo	Isopyra	Bixa	Fluxa	Penthio	Fluo
C-N75S	24	19	14	20	34	5
C-N86S	39	18	18	17	25	8
C-G79R	31	37	84	86	234	7
C-G90R	26	25	73	50	790	>22
B-H277Y	9	6	9	14	13	0.25
B-H267Y	31	14	29	24	137	1

Some features of SDHI resistance were observed in common for both pathogens, although different target-site mutations were identified in *P. teres* and *Z. tritici*:

- A high number of different mutations were identified in the field
- Some mutations have a lower impact on SDHIs than others (cross-resistance of SDHIs on market, however, response not identical)
- Each resistant isolate showed one amino acid exchange in the SDH subunits; in a few cases two alterations were observed only in *Z. tritici*, but not two in the same subunit.

5.2 Orthologous SDH-variants in other plant pathogenic fungi

Well-studied examples of SDHI resistant plant pathogens are known from speciality and row crops. In most phytopathogenic species, which already showed SDHI resistant phenotypes in the field, several mutations at different positions in the SDH-B, SDH-C and SDH-D subunits have been detected (reviewed by Stammler et al., 2015; FRAC, 2016). Compared to plant pathogenic species which infect speciality and row crops, SDHI resistant isolates in cereal pathogens were detected later, which is most probably due to the earlier introduction of SDHI-containing products and the higher number of applications per season in speciality crops.

Many amino acid substitutions that were detected in *P. teres* and *Z. tritici* in the present study, have already been described in other plant pathogenic fungi. Orthologous (also called homologous) positions in different fungal species do not necessarily have the same amino acid number, which is due to length differences of amino acid sequences. Mair et al. (2016) recently published a proposal for using a unified nomenclature for SDH amino acid exchanges, which is based on *P. teres* SDH as archetype sequence. To date, the scientific community still uses the species-specific numbering of resistance amino acid alterations, which often leads to some confusion when resistance mechanisms in different species are compared. The numbering of orthologous amino acids in different species can be easily identified in the paper of Mair et al. (2016) and Stammler et al. (2015). In the following, SDH-

variants of *P. teres* and *Z. tritici* are compared to other published SDHI resistance mutations in other plant pathogens.

The histidine substitution in subunit B at position 277 in *P. teres* was found in many species that had acquired SDHI resistance. This position in *P. teres* is homologous to positions 249 in *Aspergillus oryzae*, 257 in *U. maydis*, 272 in *A. alternata* and *B. cinerea* and can be substituted to tyrosine, arginine, lysine and valine in resistant isolates of different species or even in the same species (FRAC, 2016). A well-studied case of SDHI resistance in plant pathogens is *B. cinerea*. Resistant isolates of *B. cinerea* were detected in apple (Yin et al., 2011), kiwi (Bardas et al., 2010) and strawberry (Stammler et al., 2007; Veloukas et al., 2011, 2013). B-H272Y/R/L/V and exchanges at other positions such as B-P225L/F/T, B-N230I, C-A85V and D-H132R have been detected in field isolates and laboratory isolates of *B. cinerea*. Thereby, different mutations were detected even in one sampling location (Stammler et al., 2007; Veloukas et al., 2011). D-H132R in *B. cinerea* is homologous to D-H134R in *P. teres* and B-N230I in *B. cinerea* is homologous to B-N225I in *Z. tritici*. Other variants known from *B. cinerea* were not present in field isolates of both investigated pathogens, however, were detected in some laboratory strains of *Z. tritici* in previous studies (e.g. B-H267Y/R/L and C-A84V) (Skinner et al., 1998; Stammler et al., 2010; Scalliet et al., 2012; Fraaije et al., 2012). Other plant pathogens, where intensive resistance research has been completed, are *A. solani* on potatoes (Gudmestad et al., 2013) and *A. alternata* on nut crops (e.g. pistachio) in the US (Avenot et al., 2008, 2009). Here, SDH-variants B-H277Y/R, C-H134R, D-D123E and D-H133R were detected in *A. alternata* and B-H278Y/R and D-H133R in *A. solani*. With the exception of B-H277R/B-H278R, orthologous mutations to those found in *Alternaria* species have also been identified in *P. teres* (B-H277Y, C-H134R, D-D124E and D-H134R). The question raises as to why *P. teres* evolved only B-H277Y at this position and not, for example, B-H277R, which was frequently found in resistant isolates of *A. solani*, *A. alternata*, *B. cinerea* and other SDHI resistant species. Another pathogen which showed similar mutations to some variants found in *P. teres* is *C. cassiicola*. Here, amino acid substitutions B-H278Y/R, C-S73P, D-S89P and D-G109V have been reported (Miyamoto et al., 2008, 2010). C-S73P in *P. teres* and *C. cassiicola* is placed at a position which has various possible amino acid residues in wild type sequences of other pathogenic fungi. In *B. cinerea* and *Z. tritici* alanine is found at this position, C-A84, and this was substituted to valine in laboratory mutants of both species. Current work in our laboratory has revealed an exchange at this position (C-I86F) which leads to a reduced SDHI sensitivity in *P. pachyrhizi*. This was communicated in the SDHI FRAC Working Group (FRAC, 2016). D-G138V, which was detected in two 2015 isolates of *P. teres*, is orthologous to D-G109V in *C. cassiicola*. The set of mutations detected in field isolates of *Z. tritici* was most comparable to *V. inaequalis* on apple. Here, C-H151R (C-H152R in *Z. tritici*) and B-T253I (ortholog to B-T268I in *Z. tritici*) was detected in our laboratory (Huf, 2016). However, C-T79N, which was the most frequently found SDHI resistance mutation

in *Z. tritici*, was not relevant in any other plant pathogen. C-T79I was detected at the orthologous position in *A. oryzae* (identical to one isolate from 2016 in *Z. tritici*). The most important substitution in *P. teres* (C-G79R) was not important in any other plant pathogen and has only been detected in laboratory mutants of *Z. tritici* so far. A second pathogen known from barley, *Ramularia collo-cygni*, evolved target-site mutations in the *Sdh* genes in the last two years. Here, research is still at the beginning, however, a few resistant isolates have been characterised and have shown C-N87S (homolog to C-N75S in *P. teres* and C-N86S in *Z. tritici*) in addition to C-H146R (homolog to C-H134R in *P. teres*) and C-H153R (homolog to C-H152R in *Z. tritici*) (FRAC, 2016). Few SDHI resistant isolates of *R. collo-cygni* were also analysed during the present study. Here, alterations B-H267R, B-T268I, B-I269V (numbering of alterations in SDH-B are based on *Z. tritici* sequence because beginning of *R. collo-cygni* SDH-B is still unknown), C-N87S, C-H146R and C-H153R were found in isolates from Germany (data not shown).

The observation of homologous mutations in other plant pathogenic fungi shows that, although the genetic modifications which lead to SDHI resistance are various, and large numbers of mutations have been found so far, they appear to be restricted to a smaller number of mutations that predominantly occur in the field (Stammler et al., 2015). Thereby, each fungal pathogen species shows its own set of mutations that were predominantely detected. Reasons for the diversity of mutations in different species can be seen in the use of SDHI-containing products in different crop/pathogen systems, an altered exposure, uptake and metabolisation of substances in plant pathogens and species-specific effects of mutations on the SDHI efficacy and pathogen fitness. Exact reasons for the development of different mutations remain so far unclear. However, it could be observed that similar SDH-variants were detected in species which are closely related to each other, e.g. *A. alternata* and *A. solani*. These species cause diseases in different crops and thus are present in different agricultural systems with other compounds/application intensities. Nevertheless they have evolved similar SDH-variants. Different species obviously have selected those *Sdh* mutations that provide the highest benefits regarding their specific SDHI environment and which cause the lowest fitness costs in their genetic background. This species-specific pattern of different resistance mutations clearly shows the importance of monitoring programmes of field populations. Rapid molecular detection methods of resistance alleles are only useful when the main resistance mechanisms (mutations) are known in a species (Stammler et al., 2015).

5.3 Impact of point mutations in the *Sdh* genes on the sensitivity to SDHIs

5.3.1 Structural changes of SDH enzyme caused by target-site mutations

The structure of *E. coli* SDH and several mitochondrial SDHs from eukaryotes display highly conserved residues which are involved in ubiquinone catalysis and haem b coordination (Yankovskaya et al., 2003; Sun et al., 2005; Horsefield et al., 2006). SDH-B subunit reveals relatively high conservation, whereas many variable positions have been observed in SDH-C and SDH-D (Cecchini, 2003; Maklashina et al., 2010). SDHI fungicides have been shown to strongly bind to the SDH complex at the ubiquinone reduction site (Keon et al., 1991; Matsson and Hederstedt, 2001; Yankovskaya et al., 2003; Horsefield et al., 2006; Huang et al., 2006; Glättli et al., 2009). Docking studies of modern SDHIs suggest that they bind deeper into the Q-site than ubiquinone itself, but show an overlapping binding mode to the natural substrate (Glättli et al., 2011; Sierotzki and Scalliet, 2013; Stammler et al., 2015). The Q-site is a hydrophobic pocket formed by highly conserved residues in many organisms (including bacteria and eukaryotes) of SDH-B, SDH-C and SDH-D subunits (Horsefield et al., 2004). Residues involved in ubiquinone binding are W224 in SDH-B, S83 in SDH-C and Y130 in SDH-D (*Z. tritici* numbering) (Tran et al., 2006; Silkin et al., 2007; Zhou et al., 2011). These residues are suggested to be also involved in SDHI interaction by the formation of hydrogen-bonds of SDHIs to B-W224 and D-Y130, and through a water molecule to C-S83 of the central amide bond, which is a common feature of all SDHIs. Modern SDHIs are thought to interact with the SDH enzyme at several additional positions (Horsefield et al., 2006; Huang et al., 2006; Ruprecht et al., 2009; Sierotzki and Scalliet, 2013; Stammler et al., 2015). A schematic view of the SDHI binding mode is highlighted in Figure 46.

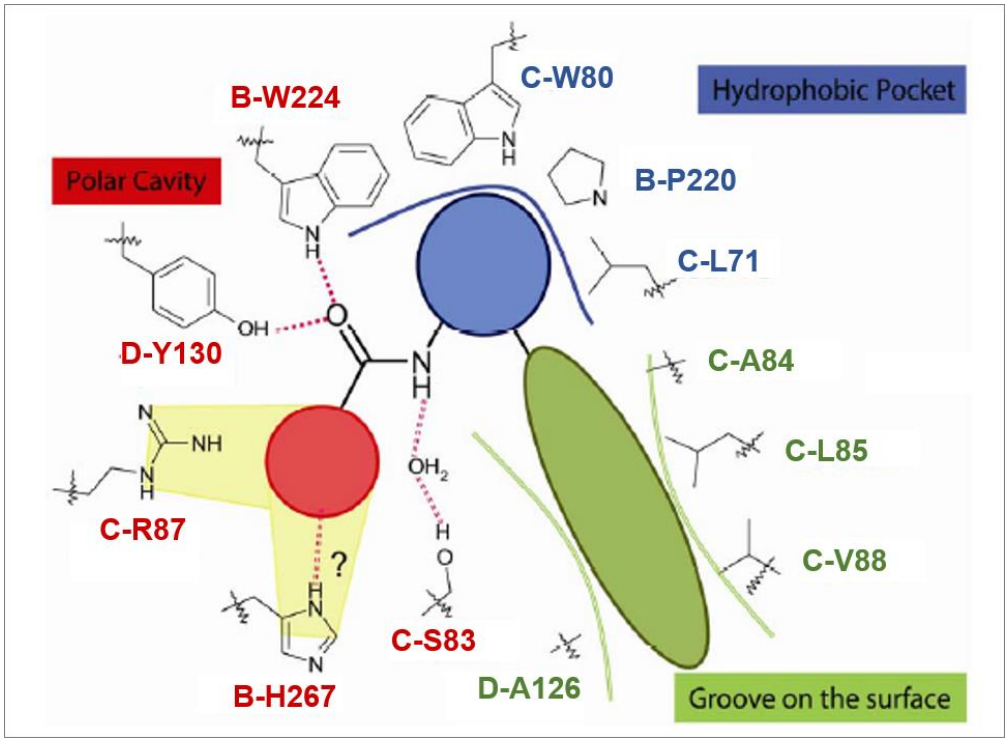


Figure 46: Schematic binding mode of SDHIs in *Z. tritici* ubiquinone-binding site. This picture is extracted from Sierotzki and Scalliet (2013) (modified). The polar cavity (red amino acid residues) is thought to interact with the acid core ring of SDHIs (red colour), the hydrophobic pocket (blue amino acid residues) are thought to interact with the linker of SDHIs (blue colour) and the groove on the protein surface (green amino acid residues) shows interaction with hydrophobic rest of SDHIs (green colour). Dashed lines show hydrogen-bonds (or electrostatic interaction), full lines depict hydrophobic contacts and yellow box gives putative interactions with π-clouds.

Some amino acid exchanges found in SDHI resistant plant pathogens are integral parts of the binding cavern of SDHIs. Thus, histidine at position 267 in SDH-B (*Z. tritici*, see Figure 46), which is often substituted in resistant isolates of different plant pathogens (e.g. B-H277Y in *P. teres*), is supposed to be involved in the interaction of SDHIs (Glättli et al., 2011; Sierotzki and Scalliet, 2013) and explains the reduced efficacies of SDHIs in the case of these mutants (no hydrogen bonds possible). Other examples of SDH-variants that are directly involved in SDHI binding, are C-W80S (in field isolates of *Z. tritici*) and B-P225L/T/F in *B. cinerea* (orthologous to B-P220 in *Z. tritici*). The resistance levels that are expressed by different exchanges is not correlated to the distance to SDHI interaction site (Stammler et al., 2015). This means that different exchanges even at one position can cause different resistance factors, which can also be dependent on the investigated organism (Scalliet et al., 2012). Several other SDH-variants detected in the present study are not directly involved in SDHI binding but are closely located to residues which form the binding cavern (e.g. B-T268I, C-T79N and C-N86S in *Z. tritici* and C-N75S and D-D145G in *P. teres*). It can be speculated, that as these substitutions are so closely located to the binding cavern that they could easily cause structural rearrangements within the binding cavern. Interestingly, substituted residues sometimes lead to a bulkier side chain (e.g. B-T268I and C-T79N), whereas in other examples substituted residues (e.g. C-N86S and D-D145G) are smaller, compared with the wild type amino acid.

Many substitutions found in SDHI resistant isolates of *P. teres* are located within SDH-C and SDH-D subunits near to the haem b group of the SDH enzyme (C-G79R, C-H134R, C-S135R, D-G138V and D-H134R). In the case of C-G79R substitution, the original position of the haem b group seems to be unlikely because the arginine residue is much bulkier compared to glycine, which would consequently lead to a 'clash' with the haem b group. Substituted residues in the cases C-S135R and D-G138V are also much bulkier compared to wild type amino acids. Histidine at position 134 in SDH-C and SDH-D are conserved residues, which coordinate the central iron atom of haem b group. Interestingly, these two (not both in combination) histidines can be substituted to arginine in SDHI resistant isolates of *P. teres*. Arginine is not known to act as coordination parter of the haem b group (Dokmanić et al., 2008), which means that one coordination partner is lost in the case of these mutants. It can be supposed that such changes in the enzyme can result in structural rearrangements, which indirectly affect the topology of the Q-site. To date, the exact role of the b-type haem in the SDH enzyme is still a matter of scientific debate (Horsefield et al., 2004; Oyedotun et al., 2004, 2007; Maklashina et al., 2010). It was demonstrated that haem b- lacking mutants of *E. coli* and *Saccharomyces cerevisiae*, which had been obtained by site-directed mutagenesis, still show ubiquinone reduction and correct assembly of the SDH complex (Oyedotun et al., 2007; Tran et al., 2007; Maklashina et al., 2010). On the other hand, there is strong evidence that haem b has a critical role in structural stabilisation of the enzyme and that it contributes to the maintenance of a high catalysis rate, which was suggested to result from an alternative electron pathway from [3Fe-4S] cluster to haem b and ubiquinone (Nakamura et al., 1996; Maklashina et al., 2001; Anderson et al., 2005; Tran et al., 2007; Stammler et al., 2015).

In addition, two positions at a far distance to the Q-site and haem b group, D-D124N/E and D-E178K, have been detected in *P. teres*. These alterations can most probably also lead to a structural rearrangement within the protein and thus affecting the Q-site topology, as described for amino acid substitutions near haem b. Although, these substitutions are at a far distance, they are observed at the same α-helices that span the membrane right up to the Q-site.

5.3.2 SDHI sensitivity of resistant isolates

In microtiter tests, the SDHI resistant isolates of *P. teres* and *Z. tritici* showed a 10 to 100-fold higher tolerance (in some cases even higher) to SDHIs, which was dependent on the mutation and the SDHI analysed. All SDHIs tested in this study were affected and confirm the statement by FRAC that in general cross-resistance exists between SDHIs (FRAC, 2016). This can be explained by the similar binding mode to the SDH enzyme by different SDHIs (Glättli et al., 2009; Fraaije et al., 2012; Sierotzki and Scalliet, 2013). *In vitro* sensitivity losses and *in planta* efficacy losses determined in the glasshouse do not necessarily reflect the situation in the field. Glasshouse tests are a step closer to field

conditions compared to microtiter studies, because the host plant is included. Therefore, glasshouse data can provide an estimation on the efficacy of fungicides under resistance conditions in the field.

In the present study, glasshouse tests were performed under preventative and curative applications of SDHIs at either the registered field rates or at one third of this rate. Lower dose rates or curative applications (four-days curative in the tests presented) challenge the fungicide performance. Two different approaches for the set-up of glasshouse trials were used. One approach was to use commercially available products (solo formulations) and a second was to use dissolved active ingredients. Since not all SDHIs are commercially available as solo formulations, it was decided to test all SDHIs dissolved in DMSO/Agnique AMD10 in *Z. tritici* for a representative comparison. Due to the very low water solubility of some SDHIs, it was necessary to optimise the solvent system to avoid precipitation of these compounds which could give inaccurate results. For comparison and as an evaluation of the effect of an individual optimised formulation, one compound (fluxapyroxad) was used both, as the commercial formulation in addition to the procedure with dissolved a.i. (*Z. tritici* glasshouse tests). Glasshouse data show that isolates carrying 'moderate' mutations (determined in microtiter tests) are generally better controlled than mutants, which carried 'strong' resistance mutations. The findings can be interpreted that the sensitivity of all compounds are impacted by all mutations, but that in most cases there is still a significant contribution to the control of isolates which contain the 'moderate' mutations B-T268I, C-T79N and C-N86S in *Z. tritici* and C-N75S, D-D124N/E and D-D145G in *P. teres*. Fluxapyroxad in its commercial formulation showed a better efficacy than in the standard solution used. This could indicate that there is potential for a higher control of all isolates by each SDHI in its commercial formulation compared to the standard solution as used in this study. There are several reasons that could explain this, including a higher stability of molecules, a better uptake to plant tissue or fungal cells, or the spread on the plant surface in an optimised formulation.

Under curative conditions or at lower dose rates of SDHIs, efficacies showed a strong reduction with some mutations, particularly in isolates carrying, for example, C-H152R in *Z. tritici* or C-G79R and C-H134R in *P. teres*.

It should also be considered that glasshouse trials were performed with single isolates representing 100% resistant genotypes, whereas in the field, lower frequencies of SDHI resistant isolates have been found so far. Therefore, the generally treated pathogen population in the field would be highly heterogenous. In addition, resistant isolates show a wide range of diverse mutation, even regarding one sample taken from one site. Therefore, the treated pathogen population in the field would be probably more heterogenous compared to the approach used in the current study. Glasshouse tests were also conducted using a high number of spores and optimal infection conditions for the fungus. Very high

infection pressures were observed in untreated control plants (mainly in curative trials of *Z. tritici*), which further challenged the fungicide performance. Therefore, the glasshouse trials indicate that SDHIs could still contribute to disease control in the field even if the frequency of mutations (at least for most mutations) is high in the population. It would be useful to determine the degree of this contribution in field experiments in the future.

Fungicide selection experiments (with solo products) and efficacy tests with artificially inoculated mutants in the field are interesting approaches, however, could possibly contribute to a faster 'build-up' of resistant isolates coupled with a more rapid decline of fungicides efficacy. This assumption is made because, particularly in *Z. tritici*, most SDHI resistant isolates were first collected from trial sites and not in the 'Random monitoring' (in the present study). Additionally, an increase of resistant isolates was first observed at trial sites in 2015 carried out by independent scientists in 2015 (where high doses of solo SDHIs were applied) (Dooley et al., 2016; personal communication, Dr. S. Kildea, Teagasc Institute). Additionally, samples taken from some Irish trial sites (untreated plots) showed higher frequencies of C-T79N compared to commercial sites in the same year, and even in 2016. A model for the evolution of fungicide resistance is given in Figure 47 (extracted from van den Bosch et al., 2011).

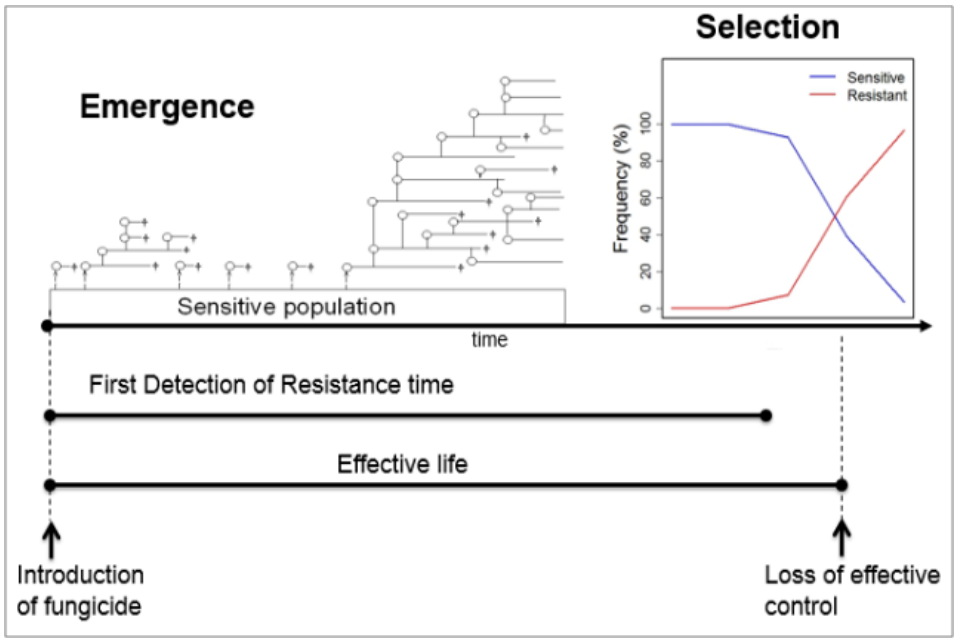


Figure 47: Emergence and selection phase of fungicide resistant populations. This model is extracted from van den Bosch et al. (2011) (modified). This model shows the emergence and selection phase of resistant individuals and depicts first time of resistance detection and the effective life of fungicides.

This model describes that single individuals within sensitive populations randomly acquire resistance by spontaneous mutation. However, natural selection can cause down selection of random occurred mutants. At the beginning of resistance evolution, levels of resistant individuals are so low that they are hardly detected. Under fungicide selective conditions, resistant individuals will spread until they exceed a critical point, whereafter the selection

phase with a strong increase of resistant isolates can occur. The rate of this increase in the resistant population will depend on a range of external factors, such as the use intensity of the given fungicide class, disease intensity, anti-resistance management (van den Bosch et al., 2011, 2014). In all phases of the resistance evolution, alternation and mixing of MOAs (still active MOAs) in addition to the use of multi-site fungicides, are useful tools to minimize the selection process and should be used in the field to avoid further spread and enrichment of resistant individuals. The relative fitness of resistant isolates compared with the wild type population will also impact on the resistance development in the population (fitness of SDHI resistant isolates is discussed separately in section 5.4). The relative slow increase of SDHI-resistant field mutants in *Z. tritici* and the noticeable difference between trial sites and 'Random monitoring' shows that in field situations resistance management can at least slow down the progress of SDHI resistance.

5.3.3 Cross-resistance of SDHI resistant phenotypes to various SDHIs

A general cross-resistance of SDHIs has been postulated, because they all share common chemical features and a similar binding mode to the target enzyme (Glättli et al., 2009; Scalliet et al., 2012; Fraaije et al., 2012; FRAC, 2016), although minor differences in the response to mutations can be observed for different compounds. However, there are exceptions with some mutations, where no clear cross-resistance seems to be present (Ishii et al., 2011; Veloukas et al., 2013). The magnitude of the impact conferred by a specific mutation can vary from species to species (Sierotzki and Scalliet, 2013; Stammler et al., 2015).

In cross-resistance studies, it needs to be carefully considered that RF values obtained from *in vitro* studies do not necessarily correlate in a linear way with the efficacies observed in the glasshouse particularly as the sensitivity of the wild type (reference isolates) can also vary. This was observed in the present study in case of fluopyram. Although fluopyram had lower RF values in microtiter tests compared with many other compounds with most SDH-variants, its efficacy was significantly decreased in glasshouse studies. For example, C-G79R mutants (*P. teres*) had a RF=7 in microtiter tests with fluopyram, but were shown to cause an efficacy reduction of 60% in fluopyram treated plants (full rates). In comparison, fluxapyroxad showed RF values of 86 (C-G79R) in microtiter tests, however, efficacy reduction was only 20% (full rates). This suggests that RF values obtained in microtiter tests are not generally a good indication of the *in planta* efficacy and/or field performance of compounds. Therefore, the impact of a specific mutation should be analysed in *in vitro* and *in planta* studies, and should be theoretically tested also under field conditions. Furthermore, each fungicide can have specific properties (e.g. stability and translocation properties in the plant), which can enhance performance, particularly under field conditions. Different SDHIs can also show an advantage or a disadvantage depending on the plant

pathogen, crop species, environmental conditions and agricultural practices, factors which also need to be considered when compounds are compared.

In laboratory mutants of *Z. tritici*, which had been obtained by selection on agar amended with an SDHI, numerous mutations were found (Stammler et al., 2010, Fraaije et al., 2012; Scalliet et al., 2012). Many mutations in the *Sdh* genes of these artificially created mutants conferred resistance to all SDHIs in a similar manner. Examples of such mutations, which were shown to cause high impacts on all SDHIs, are B-H267L and C-N86K. Additionally, mutations were detected which revealed different responses to some SDHIs compared to other SDHIs. Examples, thereof, are B-H267Y, which had no or just a low impact on fluopyram, and B-I269V and C-A84V, which had a high impact on fluopyram but low impact on several other SDHIs. A negative cross-resistance, which means that mutations confer sensitivity loss to one SDHI but hypersensitivity to another SDHI, was not detected in laboratory studies (Fraaije et al., 2012; Scalliet et al., 2012; in the present study).

In the present study, SDH-variants that were found in resistant field isolates were investigated. Thereby, cross-resistance to all SDHIs tested was observed for most mutations that were found. One exception was B-H277Y, which was shown to cause low resistance levels to most SDHIs, whereas it caused hypersensitivity to fluopyram. Hypersensitivity of the orthologous mutation, B-H272Y to fluopyram was also shown in field isolates of *B. cinerea* (Ishii et al., 2011; De Miccolis Angelini et al., 2015). Laboratory mutants of *Z. tritici*, which had homologous B-H267Y, did not show hypersensitivity *in vitro* (RF=1 in the present study and unpublished data from 2008, RF=5 in other studies from Scalliet et al., 2012). It could be concluded that hypersensitivity to fluopyram, in the case of B-H→Y, is dependent on the species where it occurs. However, in studies with B-H277Y mutants in *P. teres* and B-H272Y mutants in *B. cinerea*, field isolates were compared, and not parental isolate and corresponding mutants, as had been the case in *Z. tritici*. Additionally, B-H267Y laboratory mutants showed no hypersensitivity in *in vitro* tests, but low levels of hypersensitivity were observed once tested *in vivo* (Scalliet et al., 2012). This could lead to the conclusion that the vitality of an isolate can contribute to the impact observed in sensitivity tests. Efficacy levels of fungicides *in planta* (and to some extent *in vitro*) are dependent on the resistance level of the isolate, but are additionally impacted by the vitality of the isolate. Speed of fungal growth and aggressiveness of isolates (summarised as vitality) can give minor advantages or disadvantages in efficacy measurements, independent on their resistance status. Such differences were also observed in different C-H152R laboratory mutants in the present study (see Table 33). Here, a high variance of RF values was observed, although all isolates carried the same mutation. This was mainly due to different sensitivities of parental wild type isolates, however, even two mutants from the same parental isolate were shown to have slightly different RF values. Lowest RF values were observed for the two mutants of St 3718, which had the highest sensitivity towards SDHIs compared with all other parental isolates. Interestingly, these two

mutants also showed the lowest competitiveness in glasshouse studies when mixed with their parental isolates. This would imply that the parental isolate was much fitter compared with its mutants. RF values and the results of *in planta* competition studies are shown in Table 33.

Table 33: EC_{50} values of fluxapyroxad and RF values of C-H152R mutants used for competition studies in the glasshouse. Parental isolates are S27 (sens), St 3718 (sens), St 3573 (G143A) and St 5821 (F129L). RF values were calculated by taking EC_{50} of corresponding parental isolate. * Frequency of C-H152R in the last cycle determined in two independent competition tests (mixture of mutant and parental isolates).

Isolates	Competition studies *in planta* [% C-H152R in last cycle]*		Fluxapyroxad	
	test 1	test 2	EC_{50} [mg a.i. L^{-1}]	RF
S27	not used	not used	0.030	
B0-3-7	not used	not used	4.796	160
St 3718			**0.078**	
B3-6-18	**35**	**0**	**4.728**	**61**
X3718-4	**0**	**0**	**3.421**	**44**
St 3573			**0.072**	
X3573-2	**7.5**	**0**	**5.570**	**77**
X3573-5	**100**	**100**	**7.320**	**102**
5821			**0.057**	
X5821-1	**100**	**85**	**5.097**	**89**
X5821-3	**5**	**na**	**4.177**	**73**

It could be observed that mutants, which decreased within mixtures with its parental isolate, showed slightly lower RF values compared to those mutants with a higher competitiveness *in planta*. These results should not be (over)-interpreted because only a few pairs of parental isolate and according mutants have been investigated and differences were only small. Nevertheless, it could be assumed that the vitality of an isolate can also contribute to the outcome in microtiter tests. Individuals with a reduced fitness could possibly grow under untreated conditions, however, fitness cost will be more pronounced in treated conditions. If a resistance mechanism is causing a high degree of sensitivity loss, this effect is not clearly notable, however, looking at resistance mechanisms causing a lower reduction, thereby, a reduced fitness of an isolate could also result in lower EC_{50} values compared to more viable isolates.

In the determination of the effects of individual mutations, it is necessary to test several mutants with the same resistance mechanism. However, at an early stage of an emerging resistance often only small numbers of isolates are available. This can sometimes result in a misleading impression of the impact of mutants and the meaning for the field performance of the fungicides under investigation.

Therefore, gene-replacement mutants or artificially selected mutants could provide useful tools to determine ‘pure’ effects of mutations. Selection on fungicide-containing agar is not applicable for all pathogens, particularly in fungi that produce low numbers of conidia on artificial media (e.g. *P. teres*). Furthermore, in such forward-genetic approaches mutations are not directly introduced and selection can result in different mutations than those found in the field. Gene replacement mutants, on the other hand, would allow the direct introduction of a resistance allele in a defined background. However, vector construction,

transformation and selection techniques are time-consuming and would not allow a direct determination of the effect within the same season in which a mutation is detected (especially if there are as many target-site mutations as observed in the present study).

In summary, a high degree of cross-resistance of SDHIs was observed in the present study on SDHI resistant field isolates of *P. teres* and *Z. tritici*. In some cases, the magnitude of the effect was shown to vary between compounds. In the majority, mutations revealed an impact to all SDHIs, and some of them had a high impact to all compounds, e.g. C-G79R (*P. teres*) and C-H152R (*Z. tritici*). It can be assumed that the pathogen populations of both species can evolve resistance mechanisms that overcome or at least reduce the effects of all compounds that are present in their environment, and that these mutants will represent the most frequent mutations.

5.3.4 SDHI resistance- continuous or discrete evolution of fungicide resistance?

Fungicide resistance can be achieved by a single point mutation, e.g. G143A in QoI resistance. In *Z. tritici*, severe efficacy losses of QoIs in the field were obtained due to high frequencies of G143A within a short period in European populations (Fraaije et al., 2005; Torriani et al., 2009; FRAC, 2016). Such development of resistance is called a 'discrete' or 'single-step' evolution of fungicide resistance. QoI resistance is obtained by G143A, F129L or in rare cases G137R, depending on the plant pathogen (FRAC, 2016). G143A in CYTB is described for 22 fungal species to date (Mair et al., 2016; FRAC, 2016). Examples are *Z. tritici* (Fraaije et al., 2005), *B. cinerea* (FRAC, 2016), *A. alternata* (FRAC, 2016), *B. graminis* (Sierotzki et al., 2000), *P. viticola* (Heaney et al., 2000) and *V. inaequalis* (Steinfeld et al., 2002). Species which show F129L as QoI resistance mutation are, for example, *P. teres* (Semar et al., 2007; Sierotzki et al., 2007) and *P. pachyrhizi* (Klosowski et al., 2016). In some species, both mutations (F129L and G143A) were found to occur in the field, such as in *Z. tritici* and *P. viticola* (FRAC, 2016). RF values caused by F129L and G137R normally range between 5-15, in a few cases up to 50 (depending mainly on the pathogen), whereas G143A causes RF values greater than 100, and in some cases even greater than 1000 (FRAC, 2016). Therefore, G143A leads to high resistance levels and this is often referred to as 'complete' resistance, because when high frequencies of G143A are reached field efficacy of QoIs are dramatically reduced (FRAC, 2016). In contrast, F129L and G137R express moderate, also called 'partial' resistance because QoIs applied in appropriate rates still provide effective control, despite facing high mutation frequencies in the population (Semar et al., 2007). In other examples, first the accumulation of several mutations in the same gene or at different loci led to significant levels of resistance (Cools and Fraaije, 2008; Zhan et al., 2006). Such 'continous' evolvement of resistance was observed in the adaptation of *Z. tritici* towards DMI fungicides in Western Europe over many years (Stammler et al., 2008 a, b; Fraaije et al., 2012; Lucas et al., 2015). In contrast to QoI

resistance, this resistance emergence was slower and DMIs are still effective on *Z. tritici* populations (although showing DMI-adapted haplotypes in high frequencies) in many regions (http://eurowheat.au.dk/).

The mechanisms underlying SDHI resistance are more comparable to those found in QoI resistance than in DMI adaptation, but have been shown to be much more complex. SDHI resistance evolution could be regarded as an intermediate type between QoI and DMI resistance emergence such that genotypically it is more comparable to QoI resistance but phenotypically closer to DMI resistance. In accordance to QoI resistance, SDHI resistant isolates of *P. teres*, *Z. tritici* and many other pathogens in general had only a single *Sdh* mutation and not multiple mutations within target genes, as is the case in DMI adaptation. Only a small number of mutations are described in QoI resistant isolates. However, in the case of SDHI resistance, a high number of relevant mutations have been found, even within a single species. Some of these mutations in the SDH enzyme can mediate a high sensitivity reduction towards SDHIs. However, several other mutations that have been found in the field, express lower sensitivity losses. None of the sequenced *P. teres* isolates carried a second mutation in the *Sdh* genes, which indicates a minor role of such events in *P. teres* so far. However, a few double mutants in the *Sdh* genes have been reported from laboratory mutants (Fraaije et al., 2012; Scalliet et al., 2012) and at very low levels in isolates of *Z. tritici* collected in the field (in the present study). Therefore, the role of additional alterations in the SDH enzyme remains elusive and needs to be further studied in the future.

Of course, the question rises if SDHI resistance will 'build-up' as fast and as devastating as it has been observed in the case of G143A in *Z. tritici*. Considering the complexity of *Sdh* mutations that can occur in SDHI resistant fungi, and the to-date low frequency within the population, this would indicate that the emerging SDHI resistance is different to the 'black-white' scenario of QoI resistance due to G143A. In contrast to DMIs and SDHIs target enzyme, QoIs target CYTB, which is encoded by a mitochondrial gene. Therefore, several important differences in terms of resistance evolution are given. Mitochondrial DNA (mtDNA) is present in a high copy number within the fungal cell and mutates at a higher frequency than nuclear DNA (Bohr and Anson, 1999). This implies that point mutations occur more frequently in mtDNA and mutations in a single copy of mtDNA would not show direct effect on fungal vitality. Additionally, mitochondrial electron transfer can be obtained without complex III (and complex IV) by alternative oxidase (AOX) (Wood and Hollomon, 2003). This alternative pathway is regarded as a rescue mechanism and provides 40% of the normal efficiency of respiration (Fernández-Ortuño et al., 2008, 2010). An increase of reactive oxygen species was observed in mitochiondria as a result of QoI inhibition of CYTB (Bohr and Anson, 1999). Thus, alternative respiration might ensure ATP synthesis in the presence of sublethal concentrations of QoI fungicides, which theoretically could provide an opportunity for the fungus to select mutations in Cyt *b* (Fernández-Ortuño et al., 2010). It has been observed that different target-proteins (SDH, CYTB, CYP51) of fungicides can

have different levels of 'flexibility' with respect to their potential adaptation towards single-site inhibitors. CYTB, for example, is a highly conserved protein which is most probably more stringent in its structure than proteins with a lower conservation. G143A causes a relatively small change of amino acid residues from glycine to alanine. However, this exchange has a huge impact on the sensitivity towards QoI fungicides. In the case of SDHI resistance mutations, substituted amino acids partially result in much bulkier side chains, which are thought to cause severe structural rearrangements within the SDH enzyme. RF values are, however, often lower than for G143A in QoI fungicides (RF value comparison in the present study and reports on webpage of FRAC, 2016).

The fitness of G143A mutants has been elaborated in several fungal species by different working groups. Fitness penalties were observed in QoI-resistant populations of *P. viticola* and the rice pathogen *Pyricularia grisea* (Avila-Adame and Köller, 2003; Genet et al., 2006; Fernández-Ortuño et al., 2010). In contrast, in several other plant pathogens, such as *B. graminis*, no obvious fitness penalties were detected (Heaney et al., 2000). *S. cerevisae* has been used as a model to test the fitness associated with QoI resistance mutations. Residues that are involved in the Qo site of yeast were modified to mimic Qo binding site of several plant pathogenic species. These studies revealed that G143A led to a slight reduction in the activitiy of bc1 complex in most mimics of the Qo site with exceptions such as *B. graminis* f. sp. *tritici* (Fisher and Meunier, 2008). Controverse results were obtained in fitness studies on G143A in different species and even within a species. It seems likely that G143A causes a low impact on the fitness but not a severe impact. QoIs are still frequently used to control other plant pathogens in wheat and barley (e.g. rusts). In such an environment, the fitness cost of G143A could be regarded as too low to cause a decrease in frequency of QoI resistant individuals in the population. Therefore, in many pathogens such as *Z. tritici* high frequencies are still present in the field in Western Europe, although QoIs are not used to control STB anymore in these regions (FRAC, 2016; internal unpublished data).

Each mutation that causes fungicide resistance can, theoretically, negatively interfere with physiolocial and biochemical processes (Anderson, 2005). Although single effects of mutations could have a low impact on the fitness (e.g. G143A in some pathogens), it should be considered that there is an ongoing evolutionary process with the accumulation of resistances towards more than one fungicide class in some plant pathogens e.g. *Z. tritici*, *P. teres*, and others (Lucas et al., 2015). Each interaction of resistance alleles towards different fungicide classes theoretically can result in an altered fitness of these individuals, probably also depending on the pathogen species. The understanding of the evolution of (multi) resistance, and the impact on the fitness of such resistant organisms has key importance in the management of diseases in medicine, breeding of livestocks and in crop protection. The further spread of SDHI resistant isolates is highly dependent on their competitiveness under field conditions and this is highlighted in the following section.

5.4 Competitiveness of SDHI resistant isolates of *P. teres* and *Z. tritici*

The emergence and spread of fungicide resistant isolates is dependent on their advantage in a fungicide-selecting environment on the one hand and the presence of fitness penalties connected with this resistance on the other hand. Resistance towards antibacterial agents is well studied and fitness measurements are widely documented. Many mutations in antibiotic resistant strains of *E. coli* were shown to cause fitness costs in competitive studies without selection pressure (Enne et al., 2005; Trindade et al., 2009). Additionally, epistasis effects were observed when interaction of pairs of antibiotic resistance was analysed (Yeh et al., 2006; Chait et al., 2007). Epistasis happens when a phenotype of a mutation in one locus depends on which mutations are already present in other loci. In antibiotic resistant bacteria, epistatic effects were described as additive, synergistic, antagonistic or suppressive dependent on different combination of antibiotic resistance. It was observed that in certain drug combinations, which were shown to act suppressive, one of the antibiotics was more effective in the treatment against its resistant mutant than against wild types (Chait et al., 2007). Furthermore, it was suggested that suppressive interactions between antibiotics and their appropriate use of combinations could slow down or even counteract the evolution of drug resistance (Yeh et al., 2009). Little is known about epistatic effects in the interaction of multiple resistance towards fungicides in phytopathogenic fungi. However, to predict the evolution of multiple resistance, it is important to analyse possible fitness costs that are associated to single mutations and the accumulation of different mutations.

The fitness of a fungal plant pathogen is dependent on several parameters, which include spore production, spore dispersal, pathogenicity, mycelial growth, and survival between seasons (Mikaberidze and McDonald, 2015). Several studies on the fitness of SDHI resistant isolates of plant pathogenic fungi can be found in the literature (Kim and Xiao, 2011; Fraaije et al., 2012; Scalliet et al., 2012; Schmitz et al., 2014; Veloukas et al., 2014). Studies were conducted with artificial mutants and field isolates for mycelial growth, spore production and in dual inoculation tests with sensitive isolates. Furthermore, the enzyme activity was measured in response to several mutations that had been found in laboratory isolates. A reduced enzyme activity was detected in mitochondrial suspensions of recombinant strains of *Z. tritici* reference isolate IPO323 (Scalliet et al., 2012). All mutants showed a weaker ubiquinone reductase activity in these tests compared to IPO323 with different remaining enzyme activitites e.g. B-H267Y/L (9% or 13%), C-H152R (22%) and D-D129G (19%) (Scalliet et al., 2012). A high reduction of the enzyme activity could explain why these mutants have not been found so far in the field, or only at very low levels (e.g. C-H152R). Although they used homologous recombination constructs without the start codon to avoid functional expression from ectopic insertions, T-DNA and *Agrobacterium tumefaciens*-based transformation needs an additional introduced selection gene (e.g.

hygromycin resistance gene) which can lead to unintended, random insertion of the construct. This could consequently lead to disfunction of several other important pathways and could influence the vitality of the isolates (independent of the resistance mutation). In *B. cinerea*, fitness of SDHI resistant isolates has been investigated using recombinant mutants and field isolates in *in planta* studies (Lalève et al., 2014; Veloukas et al., 2014). Recombinant strains showed a reduced SDH activity and respiration rate, except for B-H272Y mutants (Lalève et al., 2014). This is contrary to the results obtained in *Z. tritici* (B-H267Y) (Scalliet et al., 2012). Multiple resistant field isolates of *B. cinerea*, which carried exchanges B-H272Y/L+G143A, B-N230I+G143A and B-P225F+G143A, showed reduced fitness values compared to the sensitive isolates, whereas B-H272R+G143A and isolates only showing G143A did not reveal a lower fitness (Veloukas et al., 2014). Fitness tests indicated that many investigated mutations caused a reduced fitness-phenotype and that the effect of orthologous mutations in species can be different, but even within one species the same mutation had different effects depending on the fitness test. However, studies on the fitness of SDHI resistant and multiple resistant isolates are rare, particularly for plant pathogens such as *Z. tritici* and *P. teres*.

Most competition tests do not cover all stages and stress conditions with which a fungus is faced in the field. 'Perfect' fitness tests would cover the ability of a resistant strain to compete with sensitive strains in an environment underlying natural conditions, which include fluctuating conditions (cold/heat, dry/wetness) in addition to the presence of different host genotypes and competing microorganisms (Mikaberidze and McDonald, 2015). However, field experiments are time and cost-intensive and the additional release of resistant spores of a fungal pathogen should be avoided. In the present study, the fitness of isolates was mainly studied *in planta* with field isolates, however, laboratory mutants (C-H152R) were also used. *In planta* competition studies are time and work consuming, compared to *in vitro* studies, and do not allow a high throughput of isolates. Infection and propagation studies in the glasshouse better reflect the situation which a resistant isolate is faced in the field, as such studies address the whole infection and asexual propagation process of the fungus. The pathogenicity of SDHI resistant isolates of *P. teres* and *Z. tritici*, and their competitiveness compared to sensitive isolates in dual inoculation, was investigated in the present study. Whenever field isolates were investigated, the sensitive isolates were taken mostly from the same country and year. Additionally, the occurrence of multiple resistance in these isolates was analysed, and the isolates were appropriately mixed. This was done to reduce the variability, which is automatically given in field isolates, to a minimum. In experiments conducted on *P. teres*, C-G79R, F129L and double mutants (C-G79R+F129L) were compared by using several different isolates from different countries. These studies revealed a high variability of isolates, despite the fact that the isolates were collected in similar years and regions. A high variability of *P. teres* field isolates was also observed in other studies when sporulation intensity was observed even in isolates

from the same field (Marzani, 2011). In the mean of all mixtures, a slight decrease of all resistance alleles was observed. However, a significant fitness penalty of C-G79R mutants and C-G79R+F129L mutants was not observed in the current study. This could be explained by the high variability of field isolates of *P. teres*, but also demonstrates that some resistant isolates show high competitiveness, at least under the conditions used. It could be speculated, that fitness penalites are low for those mutations that are detected in a high frequency in the field (e.g. C-G79R). On the other hand, C-G79R should, theoretically, lead to severe structural rearrangements in the SDH complex. Such structural rearragements should consequently lead to lower enzyme efficiencies. Therefore, further fitness tests should be performed under more challenging conditions for the fungus. These could include winter simulations, drought and heat stress or even fluctuating conditions, resistant host cultivars and the application of multi-site inhibitors (e.g. chlorothalonil). Considering the natural variability of field isolates, further fitness tests should include more isolates/mixtures especially when field isolates are compared. Since such tests would be time consuming and would require many resistant isolates (not given at the beginning of resistance evolution), it could be thinkable to screen a high number of sensitive isolates first to enhance the knowledge of the natural variability of the fungus in pathogenicity and growth tests. However, despite having this knowledge, unknown would remain the fitness of the parental isolate. Therefore, fitness tests on field isolates are challenging, particularly when a high variability is observed and the expected fitness cost is low for a particular mutation.

A lower variability compared to *P. teres*, was observed in competition tests with field isolates of *Z. tritici*. The results indicated that B-T268I and C-H152R exchanges have a negative effect on the fitness of isolates, whereas C-T79N mutants were shown to compete with SDHI sensitive isolates under the applied conditions. This is in line with the observation that C-T79N substitution is most frequently found within SDHI resistant isolates, whereas B-T268I and C-H152R are observed at very low levels. Of course, the number of investigated isolates was low and these results should be verified in additional studies. C-N86S exchange was also frequently found in SDHI resistant isolates but was not analysed in dual inoculation studies. However, in glasshouse sensitivity tests with SDHI resistant isolates, C-N86S and C-T79N mutants showed the highest infection rates of all SDHI resistant isolates. Thus, the impact on fitness caused by C-N86S seems to be low, but needs to be determined in future studies. Interestingly, C-T79N and C-N86S are both located at positions in the SDH enzyme that are not conserved throughout plant pathogenic species. Such variable positions seem to have a minor role for the enzymatic function, compared to those highly conserved, e.g. C-H152R, and could explain why these alterations have a lower impact on fitness. The fitness of C-H152R laboratory mutants of *Z. tritici*, which had been obtained on fungicide-amended agar, was tested with different parental isolates (QoI sens, G143A and F129L). C-H152R mutants of the wild type isolate St 3718, which shows high infection rates, were clearly less competitive than their parental isolate.

However, mutants of G143A and F129L parental isolates showed controverse results, which led to an increase of the first and a decrease of the second mutant in mixtures with their appropriate parental isolates. G143A and F129L parental isolates both showed lower infection rates compared to the QoI sensitive parental isolate (St 3718), which demonstrates that the outcome of such an experiment can be different when a different 'background' (isolate) is used. This shows the importance of analysing more than one parental isolate, also with respect to multiple resistant isolates. In this context, the possible occurrence of compensatory mutations that could modulate fitness losses of target-site mutations needs to be mentioned. Genome sequencing of such laboratory mutants that show benefical fitness compared to their parental isolates could provide useful insights to such mechanisms. Compensatory mechanisms could also explain the seldom occurrence of C-G79R in *P. teres* combined to F129L in 2013, and that this combination was found more frequently in 2014 and 2015. It seems possible that first compensatory mutations needed to occur to allow C-G79R and F129L to exist in combination. Genome sequencing of double resistant isolates (C-G79R+F129L) compared to single resistant isolates (C-G79R isolates and F129L isolates) could reveal such mechanisms.

In order to find an additional measurement tool for the fitness of isolates, the behaviour of different mutants of *Z. tritici* under stress was tested in initial tests in a microtiter approach (data not shown). The mycelial growth of isolates (measured in photometer) were tested in different concentrations of salt (sodium chloride and potassium chloride), sodium hypochlorite and were also exposed to low and high temperatures. Most conditions did not reveal a discrimination between sensitive and resistant isolates, heat stress was an exception. Here, minor (not significant) differences were observed, for example IPO323 still showed growth at higher temperatures compared to isolates with advanced resistance genotypes. Such tests could be additionally implemented to test the behaviour of mutants in a stress situation in a high throughput of isolates. However, in these preliminary tests the differences were small and conditions that more effectively reveal a fitness cost should be tested in addition (e.g. chlorothalonil).

The detection of fitness costs is a challenging effort and each approach (*in vitro* compared to *in vivo*, field isolates compared to laboratory mutants) reveals advantages and disadvantages. *In vitro* studies analyse single fitness parameters such as mycelial growth and sporulation, but do not include the host plant. *In vivo* studies are time and work consuming and do not allow high throughput, which would be necessary to show significant effects, particularly in studies with field isolates. The fitness associated with a mutation would be most precisely assessed in comparison to an isolate which shows the same genetic background except for the resistance allele. Laboratory mutants obtained by transformation or selection on fungicide-amended agar would allow the comparison of genetically similar isolates. However, random site effects during the transformation approach, and the stress caused by selection on fungicide-containing agar could lead to

unintended changes in the laboratory mutant compared to its parental isolate, thus, showing an effect on the fitness independently of the resistance mutation. In recent years, CRISPR/Cas-based manipulation of several organisms (including filamentous fungi) have demonstrated the advantage of this method (Jinek et al., 2012; O'Connell et al., 2014; Nødvig et al., 2015; Ochiai, 2015 and numerous others). The advantage of this method is the possibility to precisely modulate a genome (including point mutations, insertions, deletions) using RNA-based targeting. The method allows the introduction of mutations at a specific site, was shown to minimize off-target effects and can be performed without the use of selection markers. Having uniform sensitive and resistant isolates by a defined modulation of the genome with tools such as CRISPR/Cas9 could give further insights to the fitness of resistant (multiple) individuals in different organisms.

In summary, the resistance level and the fitness impact can be different for different target-site mutations within a phytopathogenic species and for orthologous mutations in different fungal species (in the current study; Lalève et al., 2014; Veloukas et al., 2014). Such differences might explain why the C-G79R amino acid substitution is the most important in *P. teres* and C-T79N was most frequently detected in SDHI resistant isolates of *Z. tritici*, and yet these two exchanges have not been reported from other plant pathogens from the field. Different species have obviously selected those *Sdh* mutations that gave the highest benefit regarding the specific SDHI environment, and which showed the lowest fitness impact in their genetic background (including parameters such as multiple resistance). Nowadays, an increasing number of plant pathogens accumulate resistance mechanisms against various fungicides, thereby, each species shows its specific pattern which could contribute to an altered fitness of SDHI resistant isolates within this background. It could be proposed that exchanges causing 'moderate' efficacy losses of SDHIs such as C-N75S/C-N86S or C-T79N allow the continuation of these individuals in the presence of SDHIs but do not impose severe fitness penalties as it could be the case with other alterations, such as C-H152R (only low frequencies in the field) and C-N86K (only in laboratory mutants of *Z. tritici*).

In higher organisms, e.g. nematodes and mammals, mutations in SDH-B, SDH-C and SDH-D subunits of SDH enzyme have been shown to cause tumors e.g. in hereditary paraganglioma (Niemann and Müller, 2000; Astuti et al., 2001; Douwes Dekker et al., 2003) or hypersensitive phenotypes to oxygen with a drastically shortened lifespan in *Caenorhabditis elegans* (Ishii et al., 1998). This clearly indicates that such alterations can cause severe fitness penalties. However, especially fungi can adapt rapidly to their environment. In *Z. tritici*, dispensable chromosomes and plasticity in meiosis has been detected (Goodwin et al., 2011), which could drive rapid adaptation to changing environments and potentially can cause a faster compensation of fitness costs compared to many other organisms.

5.5 Future perspective of SDHI efficacy

The potential comparisons with other plant pathogen species are limited, on account of the different resistance mechanisms (different mutations in the *Sdh* genes). Therefore, the prediction of the further evolution of SDHI resistance in *Z. tritici* and *P. teres* is challenging.

The presence of SDHI resistance in *P. teres* in some European regions requires that resistance management strategies are strictly followed. Guidelines are given by FRAC and these are reviewed yearly at the annual FRAC meetings. Effective resistance management strategies are still viable because QoI fungicides still contribute to net blotch control and the sensitivity to DMIs is stable over the last few years (FRAC, 2016). Different MOAs are useful tools, since the alternation of fungicides and particularly mixtures provide effective resistance management strategies (Hobbelen et al., 2014; van den Bosch et al., 2014). An emphasis on a preventative, rather than a curative application time point is also recommended, since an optimal application timing is also an effective resistance management approach (van den Berg et al., 2013). It seems likely that SDHIs can still contribute to net blotch control, even when high frequencies of mutants are present in the field. However, efficacy decrease of SDHI fungicides could be expected, particularly under suboptimal conditions (e.g. curative conditions) for the fungicide, if high frequencies of mutants, such as C-G79R and C-H134R, are present in the population.

Z. tritici is in an early phase of resistance development and the occurrence of numerous resistance mutations indicates a complex situation probably also in the future. The SDHI resistant isolates of *Z. tritici* described in this study were all QoI resistant, had *Cyp51* haplotypes with an advanced evolution in a DMI environment (i.e. different combination of mutations) and some of these were also benzimidazole resistant (data not shown). If such multiple resistant isolates have fitness penalties summarised from the different target-site mutations, should be further studied in detail with the purpose of developing effective resistance management strategies.

The efficacy of SDHIs against STB in the future is depending on several factors:

- Can double *Sdh* mutants evolve and cause higher resistance levels?
- Are C-T79N/C-N86S mutants competitive also in nature and develop high frequencies within the population?
- Are there compensatory mechanisms to enhance the fitness of mutants carrying mutations with severe impact on SDHIs (e.g. C-H152R)?
- Can (multiple) resistant isolates evolve other mechanisms that could enhance resistance level (e.g. overexpression of efflux pumps in higher frequencies in the population)?

Significant progress has been made in the last years in the understanding of fungicide resistance and how it can be managed (Brent and Hollomon, 2007; van den Bosch et al., 2011; Grimmer et al., 2015; Lucas et al., 2015). Effective anti-resistance strategies require effective and different MOAs. However, the number of MOAs is decreasing over the last years, which is due to the resistance build-up in some plant pathogens and stricter European legislation potentially reducing the availability of existing active ingredients and co-formulated products. In this background, future anti-resistance management should comprise integrated disease control, which uses conventional fungicides in addition to biofungicides and resistant cultivars (including classical breeding and GM technology) (reviewed Hollomon, 2015). Such integrated disease control measurements could help to challenge (multiple) resistant individuals to keep their subpopulation at a level as low as possible.

Future experiments to extend the research reported here might include:

- Competition studies of (multiple) resistant isolates exposed to stress conditions (e.g. simulation of winter, resistant host cultivars, multi-site inhibitors) in site-directed transformants (CRISPR/Cas) or in a high-throughput with field isolates
- Explore role and impact of double mutants of *Sdh* genes, e.g. site-directed mutagenesis using CRISPR/Cas
- Genome sequencing of resistant laboratory isolates which showed an enhanced competitiveness compared to parental isolates to identify possible compensatory mutations (which are thought to reduce or even eliminate fitness cost)
- Establish absolute quantification methods for relevant pathogens to investigate remaining pathogen numbers (resistant/sensitive in a mixture) after SDHI treatment (low and high concentrations) in terms to quantify absolute numbers of resistant individuals after treatments (how much can SDHIs still reduce also resistant individuals?)
- Investigate other potential mechanisms that are involved in SDHI resistance (e.g. efflux pumps and metabolism)

6 Literature

Abiko K, Kishi K, Yoshioka A (1977) Occurrence of oxycarboxin-tolerant isolates of *Puccinia horiana* P. Hennings in Japan. *Japanese Journal of Phytopathology* 43: 145-150.

Abbott WS (1925) A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology* 18: 265-267.

Ackrell BAC (2008) Progress in understanding structure-function relationships in respiratory chain complex II. *FEBS Letters* 466: 1-5.

Ahmadian A, Gharizadeh B, Gustafsson AC, Sterky F, Nyrén P, Uhlén M, Lundeberg J (2000) Single-nucleotide polymorphism analysis by pyrosequencing. *Analytical Biochemistry* 280: 103-110.

Ahmadian A, Ehn M, Hober S (2006) Pyrosequencing: history, biochemistry and future. *Clinica Chimica Acta* 363: 83-94.

Akar T, Avci M, Dusunceli F (2004) Barley, post-harvest operations. Available online: http://www.fao.org/fileadmin/user_upload/inpho/docs/Post_Harvest_Compendium_-_BARLEY.pdf [accessed 05-01-17].

Alcorn JL (1988) The taxonomy of '*Helminthosporium*' species. *Annual Review of Phytopathology* 26: 37-56.

Anderson JB (2005) Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. *Nature Reviews Microbiology* 3: 547-556.

Anderson RF, Hille R, Shinde SS, Cecchini G (2005) Electron transfer within complex II succinate:ubiquinone oxidoreductase of *Escherichia coli*. *Journal of Biological Chemistry* 280: 33331-33337.

Anke T, Oberwinkler F, Steglich W, Schramm G (1977) The strobilurins- new antifungal antibiotics from the basidiomycete *Strobilurus tenacellus* (Pers. ex Fr.) Sing. *Journal of Antibiotics* 30: 806-810.

Anke T, Hecht HJ, Schramm G, Steglich W (1979) Antibiotics from basidiomycetes. IX. Oudemansin, an antifungal antibiotic from *Oudemansiella mucida* (Schrader ex Fr.) hoehnel (Agaricales). *Journal of Antibiotics* 32: 1112-1117.

Anonymous (2002) A history of crop protection and pest control in our society: Analyzing the risks, balancing the benefits: the facts on pesticides and human safety. Available online: http://croplife.ca/ [accessed 15-08-2015].

Arvalis (2017) Fungicide treatments: still aiming for diversity. Available online: https://www.english.arvalisinstitutduvegetal.fr/fungicide-treatments-still-aiming-for-diversity-@/view-1352-arvstatiques.html [accessed 05-04-2017].

Astuti D, Latif F, Dallol A, Dahia PL, Douglas F, George E, Sköldberg F, Husebye ES, Eng C, Maher ER (2001) Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma. *The American Journal of Human Genetics* 69: 49-54.

Avenot HF, Michailides TJ (2007) Resistance to boscalid fungicide in *Alternaria alternata* isolates from pistachio in California. *Plant Disease* 91: 1345-1350.

Avenot HF, Sellam A, Karaoglanidis G, Michailides TJ (2008) Characterization of mutations in the iron-sulphur subunit of succinate dehydrogenase correlating with boscalid resistance in *Alternaria alternata* from California pistachio. *Phytopathology* 98: 736-742.

Avenot HF, Sellam A, Michailides TJ (2009) Characterization of mutations in the membrane-anchored AaSDHC and AaSDHD of succinate dehydrogenase from *Alternaria alternata* isolates conferring field resistance to the fungicide boscalid. *Plant Pathology* 58: 1134-1143.

Avenot HF, Thomas A, Gitaitis RD, Langston Jr DB, Stevenson KL (2012) Molecular characterization of boscalid-and penthiopyrad-resistant isolates of *Didymella bryoniae* and assessment of their sensitivity to fluopyram. *Pest Management Science* 68: 645-651.

Avila-Adame C, Köller W (2003) Characterization of spontaneous mutants of *Magnaporthe grisea* expressing stable resistance to the Qo inhibiting fungicide azoxystrobin. *Current Genetics* 42: 332-338.

Bach E, Christensen S, Dalgaard L, Larsen PO, Olsen CE (1979) Structures, properties and relationship to the aspergillomarasmines of toxins produced by *Pyrenophora teres*. *Physiological Plant Pathology* 14: 41-46.

Badr A, Sch R, El Rabey H, Effgen S, Ibrahim HH, Pozzi C, Rohde W, Salamini F (2000) On the origin and domestication history of barley (*Hordeum vulgare*). *Molecular Biology and Evolution* 17: 499-510.

Bakonyi J, Justesen AF (2007) Genetic relationship of *Pyrenophora graminea*, *P. teres* f. *maculata* and *P. teres* f. *teres* assessed by RAPD analysis. *Journal of Phytopathology* 155: 76-83.

Baldwin BC (1983) Fungicidal inhibitors of ergosterol biosynthesis. *Biochemical Society Transactions* 11: 659-663.

Baldwin BC, Rathmell WG (1988) Evolution of concepts for chemical control of plant-disease. *Annual Review of Phytopathology* 26: 265-283.

Baldwin BC (1990) Inhibitors of ergosterol biosynthesis as crop protection agents. *Biochemical Society Transactions* 18: 61-62.

Baloch RI, Mercer EI, Wiggins TE, Baldwin BC (1984) Inhibition of ergosterol biosynthesis in *Saccharomyces cerevisiae* and *Ustilago maydis* by tridemorph, fenpropimorph and fenpropidin. *Phytochemistry* 23: 2219-2226.

Bardas GA, Veloukas T, Koutita O, Karaoglanidis GS (2010) Multiple resistance of *Botrytis cinerea* from kiwifruit to SDHIs, QoIs and fungicides of other chemical groups. *Pest Management Science* 66: 967-973.

Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M, Parr-Dobrzanski B (2002) The strobilurin fungicides. *Pesticide Science* 58: 649-662.

Bassetti M, Merelli M, Temperoni C, Astilean A (2013) New antibiotics for bad bugs: where are we? *Annals of Clinical Microbiology and Antimicrobials* 12: 22.

Bateman GL, Gutteridge RJ, Gherbawy Y, Thomsett MA, Nicholson P (2007) Infection of stem bases and grains of winter wheat by *Fusarium culmorum* and *F. graminearum* and effect of tillage method and maize-stalk residues. *Plant Pathology* 56: 604-615.

Ben-Yephet Y, Dinoor A, Henis Y (1975) The physiological basis of carboxin sensitivitiy and tolerance in *Ustilago hordei*. *Phytopathology* 65: 936-942.

Bødker L, Schulz H, Kristensen K (1990) Influence of cultural practices on incidence of take all (*Gaeumannomyces graminis* var. *tritici*) in winter wheat and winter rye. *Tidsskrift for Planteavl* 94: 201-209.

Bohr VA, Anson RM (1999) Mitochondrial DNA repair pathways. *Journal of Bioenergetics and Biomembranes* 31: 391-398.

Born K (2013) Optimal Zonal Trial Planning (OZTP) for evaluating fungicides in wheat and barley. For BASF by the company Spatial Business Integration, Darmstadt, Germany.

Bouma E (2005) Development of comparable agro-climatic zones for the international exchange of data on the efficacy and crop safety of plant protection products. *EPPO Bulletin* 35: 233-238.

Brent KJ, Hollomon DW (2007) *Fungicide Resistance in Crop Pathogens: How can it be managed? (2nd revised edition)*. Fungicide Resistance Action Committee (FRAC), Brussels, Belgium. Available online: http://www.frac.info/docs/default-source/publications/monographs/monograph-1.pdf [accessed 2017-04-04].

Brown JS, Kellock AW, Paddick RG (1978) Distribution and dissemination of *Mycosphaerella graminicola* (Fuckel) Schroeter in relation to epidemiology of speckled leaf blotch of wheat. *Australian Journal of Agricultural Research* 29: 1139-1145.

Brown MP, Steffenson BJ, Webster RK (1993) Host range of *Pyrenophora teres* f. *teres* isolates from California. *Plant Disease* 77: 942-947.

Brown J, Hovmøller MS (2002) Aerial dispersal of pathogens on the global and continental scales and its impact on plant disease. *Science Magazine* 297: 537-541.

Brown JKM (2002) Yield penalties of disease resistance in crops. *Current Opinion in Plant Biology* 5: 339-344.

Brunner PC, Schürch S, McDonald BA (2007) The origin and colonization history of the barley scald pathogen *Rhynchosporium secalis*. *Journal of Evolutionary Biology* 20: 1311-1321.

Brunner PC, Torriani SF, Croll D, Stukenbrock EH, McDonald BA (2013) Coevolution and life cycle specialization of plant cell wall degrading enzymes in a hemibiotrophic pathogen. *Molecular Biology and Evolution* 30: 1337-1347.

Bryant D (2004) *Glutathione Conjugation of Herbicides and Fungicides in Plants and Fungi: Functional Characterization of Glutathione Transferases from Phytopathogens*. Doctoral thesis, Durham University, Durham, UK. Available online: http://etheses.dur.ac.uk/3117/ [accessed 17-01-2017].

Campbell R (1989) *Biological Control of Microbial Plant Pathogens*. Cambridge University Press, Cambridge, UK, pp. 70-76.

Campbell GF, Lucas JA, Crous PW (2002) Evidence of recombination between net- and spot-type populations of *Pyrenophora teres* as determined by RAPD analysis. *Mycological Research* 106: 602-608.

Campbell GF, Crous PW (2003) Genetic stability of net x spot hybrid progeny of the barley pathogen *Pyrenophora teres*. *Australasian Plant Pathology* 32: 283-287.

Cecchini G (2003) Function and structure of complex II of the respiratory chain. *Annual Review of Biochemistry* 72: 77-109.

Cha RS, Zarbl H, Keohavong P, Thilly WG (1992) Mismatch amplification mutation assay (MAMA): application to the c-H-ras gene. *Genome Research* 2: 14-20.

Chait R, Craney A, Kishony R (2007) Antibiotic interactions that select against resistance. *Nature* 446: 668-671.

Chapeland F, Fritz R, Lanen C, Gredt M, Leroux P (1999) Inheritance and mechanisms of resistance to anilinopyrimidine fungicides in *Botrytis cinerea* (*Botryotinia fuckeliana*). *Pesticide Biochemistry and Physiology* 64: 85-100.

Chen RS, McDonald BA (1996) Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola*. *Genetics* 142: 1119-1127.

Colbach N, Saur L (1998) Influence of crop management on eyespot development and infection cycles of winter wheat. *Field Crops Research* 69: 47-67.

Conway KE (1996) An overview of the influence of sustainable agricultural systems on plant diseases. *Crop Protection* 15: 223-228.

Cools HJ, Fraaije BA (2008) Are azole fungicides losing ground against Septoria wheat disease? Resistance mechanisms in *Mycosphaerella graminicola*. *Pest Management Science* 64: 681-684.

Cools HJ, Fraaije BA (2013) Update on mechanisms of azole resistance in *Mycosphaerella graminicola* and implications for future control. *Pest Management Science* 69: 150-155.

Cools HJ, Hawkins NJ, Fraaije BA (2013) Constraints on the evolution of azole resistance in plant pathogenic fungi. *Plant Pathology* 62: 36-42.

Cowger C, McDonald BA, Mundt CC (2002) Frequency of sexual reproduction by *Mycosphaerella graminicola* on partially resistant wheat cultivars. *Phytopathology* 92: 1175-1181.

Cuomo CA, Güldener U, Xu JR, Trail F, Turgeon BG, Di Pietro A, Walton JD, Ma L-J, Baker SE, Corby Kistler H, et al. (2007) The *Fusarium graminearum* genome reveals a link between localized polymorphism and pathogen specialization. *Science* 317: 1400-1402.

Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. *Nature* 411: 826-833.

Darwin C (1859) *On the Origin of Species by Means of Natural Selection (1st edition)*. Murray, London, UK, pp. 426.

Dean R, Van Kan JAL, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Foster GD, et al. (2012) The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology* 13: 414-430.

De Jonge R, van Esse HP, Kombrink A, Shinya T, Desaki Y, Bours R, Thomma BP (2010) Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science* 329: 953-955.

Delp CJ, Dekker J (1985) Fungicide resistance: definitions and use of terms. *EPPO Bulletin* 15: 333-335.

De Miccolis Angelini RM, Pollastro S, Faretra F (2015) Genetics of fungicide resistance. In: Ishii H, Hollomon DW (eds.) *Fungicide Resistance in Plant Pathogens. Principles and a Guide to Practical Management*, Springer, Tokyo, Japan, pp. 13-34.

De Wolf E (2008) Septoria tritici blotch. *Kensas State Plant Pathology EP-133*, Kansas State University, US. Available online: http://www.plantpath.k-state.edu/extension/publications/septoria-tritici-blotch.pdf [accessed 2017-04-08].

Dokmanić I, Šikić M, Tomić S (2008) Metals in proteins: correlation between the metal-ion type, coordination number and the amino-acid residues involved in the coordination. *Acta Crystallographica Section D: Biological Crystallography* 64: 257-263.

Dooley H, Shaw MW, Mehenni-Ciz J, Spink J, Kildea S (2016) Detection of *Zymoseptoria tritici* SDHI-insensitive field isolates carrying the SdhC-H152R and SdhD-R47W substitutions. *Pest Management Science* 72: 2203-2207.

Douiyssi A, Rasmusson DC, Roelfs AP (1998) Responses of barley cultivars and lines to isolates of *Pyrenophora teres*. *Plant Disease* 82: 316-321.

Dubos T, Pasquali M, Pogoda F, Casanova A, Hoffmann L, Beyer M (2013) Differences between the succinate dehydrogenase sequences of isopyrazam sensitive *Zymoseptoria tritici* and insensitive *Fusarium graminearum* strains. *Pesticide Biochemistry and Physiology* 105: 28-35.

Duncan KE, Howard RJ (2000) Cytological analysis of wheat infection by the leaf blotch pathogen *Mycosphaerella graminicola*. *Mycological Research* 104: 1074-1082.

Douwes Dekker PB, Hogendoorn PC, Kuipers-Dijkshoorn N, Prins FA, van Duinen SG, Taschner PE, van der Mey AG, Cornelisse CJ (2003) SDHD mutations in head and neck paragangliomas result in destabilization of complex II in the mitochondrial respiratory chain with loss of enzymatic activity and abnormal mitochondrial morphology. *Journal of Pathology* 201: 480-486.

Enne VI, Delsol AA, Davis GR, Hayward SL, Roe JM, Bennett PM (2005) Assessment of the fitness impacts on *Escherichia coli* of acquisition of antibiotic resistance genes encoded by different types of genetic element. *Journal of Antimicrobial Chemotherapy* 56: 544-551.

Epilogic (2016) Available online: http://www.epilogic.de/ [accessed 01-10-2016].

Eriksen L, Munk L (2003) The occurrence of *Mycosphaerella graminicola* and its anamorph *Septoria tritici* in winter wheat during the growing season. *European Journal of Plant Pathology* 109: 253-259.

Eurowheat (2017) Available online: http://eurowheat.au.dk/ [accessed 23-01-2017].

Eyal Z, Scharen AL, Prescott JM, van Ginkel M (1987) *The* Septoria *diseases of wheat: concepts and methods of disease management*. Mexico City, Mexico, DF: CIMMYT.

Eyal Z (1999) *Septoria* and *Stagonospora* diseases of cereals: a comparative perspective. In: Lucas JA, Bowyer P, Anderson HM (eds.) Septoria *on Cereals: A Study of Pathosystems*, CABI Publishing, Wallingford, UK, pp. 1-25.

Fairchild KL, Miles TD, Wharton PS (2013) Assessing fungicide resistance in populations of *Alternaria* in Idaho potato fields. *Crop Protection* 49: 31-39.

Fent K, Hunn J (1996) Cytotoxicity of organic environmental chemicals to fish liver cells (PLHC-1). *Marine Environmental Research* 42: 377-382.

Fernández-Ortuño D, Torés JA, de Vicente A, Pérez-García A (2008) Field resistance to QoI fungicides in *Podosphaera fusca* is not supported by typical mutations in the mitochondrial cytochrome *b* gene. *Pest Management Science* 64: 694-702.

Fernández-Ortuño D, Torés JA, de Vicente A, Pérez-García A (2010) Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. *International Microbiology* 11: 1-9.

Fisher N, Brown AC, Sexton G, Cook A, Windass J, Meunier B (2004) Modeling the Q_o site of crop pathogens in *Saccharomyces cerevisiae* cytochrome *b*. *European Journal of Biochemistry* 271: 2264-2271.

Fisher N, Meunier B (2008) Molecular basis of resistance to cytochrome *bc*1 inhibitor. *FEMS Yeast Research* 8: 183-192.

Food and Agriculture Organization of the United Nations (FAO) (2017) Available online: http://www.fao.org/faostat/en/ [accessed 04-01-2017].

Fraaije BA, Butters JA, Coelho JM, Jones DR, Hollomon DW (2002) Following the dynamics of strobilurin resistance in *Blumeria graminis* f. sp. *tritici* using quantitative allele-specific real-time PCR measurements with the fluorescent dye SYBR Green I. *Plant Pathology* 51: 45-54.

Fraaije BA, Lucas JA, Clark WS, Burnett F (2003) QoI resistance development in populations of cereal pathogens in the UK. In: *The BCPC Conference Pests and Diseases*, British Crop Protection Council (BCPC), Alton, UK, pp. 689-694.

Fraaije BA, Cools HJ, Fountaine J, Lovell DJ, Motteram J, West JS, Lucas A (2005) Role of ascospores in further spread of QoI-resistant cytochrome *b* alleles (G143A) in field populations of *Mycosphaerella graminicola*. *Phytopathology* 95: 933-941.

Fraaije BA, Bayon C, Atkins S, Cools HJ, Lucas JA, Fraaije MW (2012) Risk assessment studies on succinate dehydrogenase inhibitors, the new weapons in the battle to control Septoria leaf blotch in wheat. *Molecular Plant Pathology* 13: 263-275.

Friesen TL, Faris JD, Solomon PS, Oliver RP (2008a) Hostspecific toxins, effectors of necrotrophic pathogenicity. *Cellular Microbiology* 10: 1421-1428.

Friesen TL, Zhang Z, Solomon PS, Oliver RP, Faris JD (2008b) Characterization of the interaction of a novel *Stagonospora nodorum* host-selective toxin with a wheat susceptibility gene. *Plant Physiology* 146: 682-693.

Fungicide Resistance Action Committee (FRAC) (2016) Available online: http://www.frac.info/ [accessed 06-02-2017].

Gadher P, Mercer EI, Baldwin BC, Wiggins TE (1983) A comparison of the potency of some fungicides as inhibitors of sterol 14-demethylation. *Pesticide Biochemistry and Physiology* 19: 1-10.

Genet JL, Jaworska G, Deparis F (2006) Effect of dose rate and mixtures of fungicides on selection for QoI resistance in populations of *Plasmopara viticola*. *Pest Management Science* 62:188-194.

Georgopoulos SG, Chrysayi M, White GA (1975) Carboxin resistance in the haploid, the heterozygous diploid, and the plant-parasitic dicaryotic phase of *Ustilago maydis*. *Pesticide Biochemistry and Physiology* 5: 543-551.

Germer S, Holland MJ, Higuchi R (2000) High-throughput SNP allele-frequency determination in pooled DNA samples by kinetic PCR. *Genome Research* 10: 258-266.

Gisi U, Sierotzki H, Cook A, McCaffery A (2002) Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Management Science* 58: 859-867.

Glaab WE, Skopek TR (1999) A novel assay for allelic discrimination that combines the fluorogenic 5' nuclease polymerase chain reaction (TaqMan®) and mismatch amplification mutation assay. *Mutation Research/ Fundamental and Molecular Mechanisms of Mutagenesis* 430: 1-12.

Gladders P, Paveley N, Barrie IA, Hardwick NV, Hims M, Langton S, Taylor M (2001) Agronomic and meteorologic factors affecting the severity of leaf blotch caused by *Mycosphaerella graminicola* in commercial wheat crops in England. *Annals of Applied Biology* 138: 301-311.

Gladders P, Langton SD, Barrie IA, Hardwick NV, Taylor MC, Paveley ND (2007) The importance of weather and agronomic factors for the overwinter survival of yellow rust (*Puccinia striiformis*) and subsequent disease risk in commercial wheat crops in England. *Annals of Applied Biology* 150: 371-382.

Glättli A, Stammler G, Schlehuber S (2009) Mutations in the target proteins of succinate-dehydrogenase inhibitors (SDHI) and 14α-demethylase inhibitors (DMI) conferring changes in the sensitivity- structural insights from molecular modelling. In: *9th International Conference on Plant Diseases*, Association Française de Protection des Plantes (AFPP), Tours, France, pp. 670-681.

Glättli A, Grote T, Stammler G (2011) SDH-Inhibitors: History, biological performance and molecular mode of action. In: Dehne HW, Deising HB, Gisi U, Kuck K-H, Russell P, Lyr H (eds.) *Modern Fungicides and Antifungal Compounds Vol. VI*, Deutsche Phytomedizinische Gesellschaft (DPG), Braunschweig, Germany, pp. 159-169.

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology* 43: 205-227.

Goodwin SB, Ben M'Barek S, Dhillon B, Wittenberg AH, Crane CF, Hane JK, Antoniw J, Foster AJ, van der Lee TAJ, Kema GHJ, et al. (2011) Finished genome of the fungal wheat pathogen *Mycosphaerella graminicola* reveals dispensome structure, chromosome plasticity, and stealth pathogenesis. *PLoS Genet* 7: e1002070.

Grasso V, Sierotzki H, Garibaldi A, Gisi U (2006a) Characterization of the cytochrome *b* gene fragment of *Puccinia* species responsible for the binding site of QoI fungicides. *Pesticide Biochemistry and Physiology* 84: 72-82.

Grasso V, Palermo S, Sierotzki H, Garibaldi A, Gisi U (2006b) Cytochrome *b* gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Management Science* 62: 465-472.

Gray GG (1966) *Genetic Systems in the Net Blotch Disease Complex of Barley*. Doctoral thesis, North Dakota State University, Fargo, US.

Grewal TS, Rossnagel BG, Pozniak CJ, Scoles GJ (2008) Mapping quantitative trait loci associated with barley net blotch resistance. *Theoretical and Applied Genetics* 116: 529-539.

Grimmer MK, van den Bosch F, Powers SJ, Paveley ND (2015) Fungicide resistance risk assessment based on traits associated with the rate of pathogen evolution. *Pest Management Science* 71: 207-215.

Gudmestad NC, Arabiat S, Miller JS, Pasche JS (2013) Prevalence and impact of SDHI fungicide resistance in *Alternaria solani*. *Plant Disease* 97: 952-960.

Gurr SJ, Fones H (2015) The impact of Septoria tritici blotch disease on wheat: an EU perspective. *Fungal Genetics and Biology* 79: 3-7.

Hägerhäll C (1997) Succinate: quinone oxidoreductases: Variations on a conserved theme. *Biochimica et Biophysica Acta (BBA)- Bioenergetics* 1320: 107-141.

Hammond-Kosack KE, Jones JDG (2000) Response to plant pathogens. In: Buchannan B, Gruissem W, Jones R (eds.) *Biochemistry and Molecular Biology of Plants*, American Society of Plant Physiologists, Rockville, US, pp. 1102-1157.

Hayes PM (1992) Economic trait loci (quantitative trait loci-QTL) analysis progress report. North American Barley Genome Mapping Project (NABGMP), *Barley Genetics Newsletter (US)* 21: 30-31.

Heaney SP, Hall AA, Davies SA, Olaya G (2000) Resistance to fungicides in the QoI-STAR cross-resistance group: current perspectives. In: *The Brighton Crop Protection Conference (BCPC): Pests and Diseases (Vol. 2)*, British Crop Protection Council, Brighton, UK, pp. 755-762.

Herbicide Resistance Action Committee (HRAC) (2017) Available online: http://hracglobal.com/herbicide-resistance/overview [accessed 03-03-2017].

Hewitt HG (1998) *Fungicides in Crop Protection*. CABI Publishing, Wallingford, UK, pp. 221.

Highwood DP (1990) Fungicide Resistance Action Committee. *Pesticide Outlook* 1: 30-32.

Hilu HB, Bever WM (1957) Inoculation, oversummering and suscept-pathogen relationships of *Septoria tritici* on *Triticum* species. *Phytopathology* 47: 474-480.

Hobbelen PH, Paveley ND, van den Bosch F (2014) The emergence of resistance to fungicides. *PloS One* 9: e91910.

Hoffmann GM, Schmutterer H (1999) Septoria Blatt- und Ährenkrankheiten. In: *Parasitäre Krankheiten und Schädlinge an landwirtschaftlichen Kulturpflanzen*, Verlag Eugen Ulmer, Stuttgart, pp. 59-64.

Hollomon DW, Brent KJ (2009) Combating plant diseases-the Darwin connection. *Pest Management Science* 65: 1156-1163.

Hollomon DW (2015) Fungicide resistance: 40 years on and still a major problem. In: Ishii H, Hollomon DW (eds.) *Fungicide Resistance in Plant Pathogens. Principles and a Guide to Practical Management*, Springer, Tokyo, Japan, pp. 3-11.

Holmes SJI, Colhoun J (1974) Infection of wheat by *Septoria nodorum* and *Septoria tritici* in relation to plant age, air temperature and relative humidity. *Transactions of the British Mycological Society* 63: 329-338.

Horsefield R, Iwata S, Byrne B (2004) Complex II from a structural perspective. *Current Protein and Peptide Science* 5: 107-118.

Horsefield R, Yankovskaya V, Sexton G, Whittingham W, Shiomi K, Ōmura S, Byrne B, Cecchini G, Iwata S (2006) Structural and computational analysis of the quinone-binding site of complex II (succinate:ubiquinone oxidoreductase): a mechanism of electron transfer and proton conduction during ubiquinone reduction. *Journal of Biology and Chemistry* 281: 7309-7316.

Huang L-s, Sun G, Cobessi D, Wang AC, Shen JT, Tung EY, Anderson VE, Berry EA (2006) 3-nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation by complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme. *Journal of Biological Chemistry* 281: 5965-5972.

Huf A (2016) *Untersuchungen zur Sensitivität von* Venturia inaequalis *gegenüber Succinat-Dehydrogenase Inhibitoren*. Master thesis, Universität Hohenheim, Stuttgart, Germany.

Hunter T, Coker RR, Royle DJ (1999) The teleomorph stage, *Mycosphaerella graminicola*, in epidemics of Septoria tritici blotch on winter wheat in the UK. *Plant Pathology* 48: 51-57.

Ishii N, Fujii M, Hartman PS, Tsuda M (1998) A mutation in succinate dehydrogenase cytochrome *b* causes oxidative stress and ageing in nematodes. *Nature* 394: 694.

Ishii H, Miyamoto T, Ushio S, Kakishima M (2011) Lack of cross-resistance to a novel succinate dehydrogenase inhibitor, fluopyram, in highly boscalid-resistant isolates of *Corynespora cassiicola* and *Podosphaera xanthii*. *Pest Management Science* 67: 474-482.

Ito S, Kuribayashi K (1931) The ascigerous forms of some graminicolous species of *Helminthosporium* in Japan. *Journal of Faculty of Agriculture of the Hokkaido Imperial University, Sapporo* 29: 85-125.

Ito Y, Muraguchi H, Seshime Y, Oita S, Yanagi SO (2004) Flutolanil and carboxin resistance in *Coprinus cinereus* conferred by a mutation in the cytochrome b_{560} subunit of succinate dehydrogenase complex (complex II). *Molecular Genetics and Genomics* 272: 328-335.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337: 816-821.

Jones JDG, Dangl JL (2006) The plant immune system. *Nature* 444: 323-329.

Jonsson R, Sall T, Bryngelsson T (2000) Genetic diversity for random amplified polymorphic DNA (RAPD) markers in two Swedish populations of *Pyrenophora teres*. *Canadian Journal of Plant Pathology* 22: 258-264.

Joosten MH, Hendrickx LJ, De Wit PJ (1990) Carbohydrate composition of apoplastic fluids isolated from tomato leaves inoculated with virulent or avirulent races of *Cladosporium fulvum* (syn. *Fulvia fulva*). *Netherlands Journal of Plant Pathology* 96: 103-112.

Jordan VWL (1981) Aetiology of barley net blotch caused by *Pyrenophora teres* and some effects on yield. *Plant Pathology* 30: 77-87.

Jordan VWL, Allen EC (1984) Barley net blotch: influence of straw disposal and cultivation methods on inoculum potential, and on incidence and severity of autumn disease. *Plant Pathology* 33: 547-559.

Jørgensen HJL, Lübeck PS, Thordal-Christensen H, de Neergaard E, Smedegård-Petersen V (1998) Mechanisms of induced resistance in barley against *Drechslera teres*. *Phytopathology* 88: 698-707.

Jørgensen LN, Olsen LV (2007) Control of tan spot (*Drechslera tritici-repentis*) using host resistance. Tillage methods and fungicides. *Crop Protection* 26: 1606-1616.

Jørgensen LN, Jahn M, Clark B, Antichi D, Góral T, Schepers H, Lucas P, Rolland B, Gouache D, Hornok L (2008). Best control practices of diseases in winter wheat. Available online:
http://www.eurowheat.org/upload/eurowheat/document/ENDURE_wheat_case_report.pdf [accessed 05-12-2016].

Jørgensen LN, Hovmøller MS, Hansen JG, Lassen P, Clark B, Bayles R, Rodemann B, Flath K, Jahn M, Berg G, et al. (2014) IPM strategies and their dilemmas including an introduction to www.eurowheat.org. *Journal of Integrative Agriculture* 13: 265-281.

Joseph-Horne T, Hollomon D, Manning N, Kelly SL (1996) Investigation of the sterol composition and azole resistance in field isolates of *Septoria tritici*. *Applied and Environmental Microbiology* 62: 184-190.

Kazan K, Gardiner DM, Manners JM (2012) On the trail of a cereal killer: recent advances in *Fusarium graminearum* pathogenomics and host resistance. *Molecular Plant Pathology* 13: 399-413.

Kelly SL, Lamb DC, Corran AJ, Baldwin BC, Kelly DE (1995) Mode of action and resistance to azole antifungals associated with the formation of 14α-methylergosta-8, 24 (28)-dien-3β, 6α-diol. *Biochemical and Biophysical Research Communications* 207: 910-915.

Kelly SL, Kelly DE (2013) Microbial cytochromes P450: biodiversity and biotechnology. Where do cytochromes P450 come from, what do they do and what can they do for us? *Philosophical Transactions of the Royal Society B: Biological Sciences* 368: 20120476.

Kema GHJ, Yu D, Rijkenberg FHJ, Shaw MW, Baayen RP (1996) Histology of the pathogenesis of *Mycosphaerella graminicola* in wheat. *Phytopathology* 86: 777-786.

Keon JPR, Hargreaves JA (1983) A cytological study of the net blotch disease of barley caused by *Pyrenophora teres*. *Physiological Plant Pathology* 22: 321-329.

Keon JP, White GA, Hargreaves JA (1991) Isolation, characterization and sequence of a gene conferring resistance to the systemic fungicide carboxin from the maize smut pathogen, *Ustilago maydis*. *Current Genetics* 19: 475-481.

Keon J, Antoniw J, Carzaniga R, Deller S, Ward JL, Baker JM, Beale MH, Hammond-Kosack K, Rudd JJ (2007) Transcriptional adaptation of *Mycosphaerella graminicola* to programmed cell death (PCD) of its susceptible wheat host. *Molecular Plant Microbe Interaction* 20: 178-193.

Kildea S, Dunne B, Mullins E, Cooke LR, Mercer PC, O'Sullivan E (2010) Pyraclostrobin reduces germ tube growth of QoI-resistant *Mycosphaerella graminicola* pycnidiospores and the severity of Septoria tritici blotch on winter wheat. *Plant Pathology* 59: 1091-1098.

Kim YK, Xiao CL (2011) Stability and fitness of pyraclostrobin-and boscalid-resistant phenotypes in field isolates of *Botrytis cinerea* from apple. *Phytopathology* 101: 1385-1391.

Klosowski AC, May de Mio LI, Miessner S, Rodrigues R, Stammler G (2016) Detection of the F129L mutation in the cytochrome *b* gene in *Phakopsora pachyrhizi*. *Pest Management Science* 72: 1211-1215.

Kretschmer M, Leroch M, Mosbach A, Walker AS, Fillinger S, Mernke D, Schoonbeek HJ, Pradier JM, Leroux P, De Waard MA, Hahn M (2009) Fungicide-driven evolution and molecular basis of multidrug resistance in field populations of the grey mould fungus *Botrytis cinerea*. *PLoS Pathogens* 5: e1000696.

Kretschmer M (2012) Emergence of multi-drug resistance in fungal pathogens: a potential threat to fungicide performance in agriculture. In: Thind TS (ed.) *Fungicide Resistance in Crop Protection: Risk and Management*, CABI, Wallingford, UK, pp. 251-268.

Kuck K-H, Gisi U (2006) FRAC mode of action classification and resistance risk of fungicides. In: Krämer W, Schirmer U (eds.) *Modern Crop Protection Compounds*, Wiley-VCH, Weinheim, Germany. pp. 415-432.

Kuck K-H, Russell PE (2006) FRAC: combined resistance risk assessment. *Aspects of Applied Biology* 78: 3-10.

Lalève A, Fillinger S, Walker AS (2014) Fitness measurement reveals contrasting costs in homologous recombinant mutants of *Botrytis cinerea* resistant to succinate dehydrogenase inhibitors. *Fungal Genetics and Biology* 67: 24-36.

Lehmensiek A, Bester AE, Sutherland MW, Platz G, Kriel WM, Potgieter GF, Prins R (2010) Population structure of South African and Australian *Pyrenophora teres* isolates. *Plant Pathology* 59: 504-515.

Leisova L, Kucera L, Minarikova V (2005) AFLP-based PCR markers that differentiate spot and net forms of *Pyrenophora teres*. *Plant Pathology* 54: 66-73.

Leonard KJ, Mundt CC (1984) Methods for estimating epidemiological effects of quantitative resistance to plant diseases. *Theoretical and Applied Genetics* 67: 219-230.

Leroux P, Berthier G (1988) Resistance to carboxin and fenfuram in *Ustilago nuda* (Jens.) Rostr., the causal agent of barley loose smut. *Crop Protection* 7: 16-19.

Leroux P, Fritz R, Debieu D, Albertini C, Lanen C, Bach J, Gredt M, Chapeland F (2002) Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. *Pest Management Science* 58: 876-888.

Leroux P, Albertini C, Gautier A, Gredt M, Walker AS (2007) Mutations in the *CYP51* gene correlated with changes in sensitivity to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Management Science* 63: 688-698.

Leroux P and Walker AS (2011) Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Management Science* 67: 44-59.

Lesniak KE, Proffer TJ, Beckerman JL, Sundin GW (2011) Occurrence of QoI resistance and detection of the G143A mutation in Michigan populations of *Venturia inaequalis*. *Plant Disease* 95: 927-934.

Li B, Kadura I, Fu DJ, Watson DE (2004) Genotyping with TaqMAMA. *Genomics* 83: 311-320.

Li J, Zhou M, Li H, Chen C, Wang J, Zhang Y (2006) A study on the molecular mechanism of resistance to amicarthiazol in *Xanthomonas campestris* pv. *citri*. *Pest Management Science* 62: 440-445.

Lightfoot DJ, Able AJ (2010) Growth of *Pyrenophora teres in planta* during barley net blotch disease. *Australasian Plant Pathology* 39: 499-507.

Linde CC, Zhan J, McDonald BA (2002) Population structure of *Mycosphaerella graminicola*: from lesions to continents. *Phytopathology* 92: 946-955.

Liu J, Blaylock LA, Endre G, Cho J, Town CD, van den Bosch KA, Harrison MJ (2003) Transcript profiling coupled with spatial expression analyses reveals genes involved in distinct developmental stages of an arbuscular mycorrhizal symbiosis. *The Plant Cell* 15: 2106-2123.

Liu Z, Ellwood SR, Oliver RP, Friesen TL (2011) *Pyrenophora teres*: profile of an increasingly damaging barley pathogen. *Molecular Plant Pathology* 12: 1-19.

Louw JP, Crous PW, Holz G (1996) Relative importance of the barley net blotch pathogens *Pyrenophora teres* f. *teres* (net type) and *P. teres* f. *maculata* (spot type) in South Africa. *African Plant Protection* 2: 89-95.

Loyce C, Meynard JM, Bouchard C, Roland B, Lonnet P, Bataillon P, Bernicot MH, Bonnefoy M, Charrier X, Doussinault G, et al. (2008) Interaction between cultivar and

crop management effects on winter wheat diseases, lodging and yield. *Crop Protection* 27: 1131-1142.

Lucas JA (1998) *Plant Pathology and Plant Pathogens (3rd edition)*. Blackwell Science, Oxford, UK, pp. 274.

Lucas JA, Fraaije BA (2008) QoI resistance in *Mycosphaerella graminicola*: what have we learned so far? In: Dehne HW, Deising HB, Gisi U, Kuck K-H, Russell P, Lyr H (eds.) *Modern Fungicides and Antifungal Compounds Vol. V*, Deutsche Phytomedizinische Gesellschaft (DPG), Braunschweig, Germany, pp. 71-77.

Lucas JA, Hawkins NJ, Fraaije BA (2015) Chapter two- the evolution of fungicide resistance. *Advances in Applied Microbiology* 90: 29-92.

Luo CX, Hu MJ, Jin X, Yin LF, Bryson PK, Schnabel G (2010) An intron in the cytochrome *b* gene of *Monilinia fructicola* mitigates the risk of resistance development to QoI fungicides. *Pest Management Science* 66: 1308-1315.

Mair W, Lopez-Ruiz F, Stammler G, Clark W, Burnett F, Hollomon D, Ishii H, Thind TS, Brown JKM, Oliver RP, et al. (2016) Proposal for a unified nomenclature for target-site mutations associated with resistance to fungicides. *Pest Management Science* 72: 1449-1459.

Mak C (2010) Grass genomics on the wild side. *Nature Biotechnology* 28: 242.

Maklashina E, Rothery RA, Weiner JH, Cecchini G (2001) Retention of heme in axial ligand mutants of succinate:ubiquinone oxidoreductase (complex II) from *Escherichia coli*. *Journal of Biological Chemistry* 276: 18968-18976.

Maklashina E, Rajagukguk S, McIntire WS, Cecchini G (2010) Mutation of the heme axial ligand of *Escherichia coli* succinate-quinone reductase: Implications for heme ligation in mitochondrial complex II from yeast. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1797: 747-754.

Manners JG (1993) *Principles of Plant Pathology (2nd edition)*. Cambridge University Press, Cambridge, UK, pp. 343.

Marshall R, Kombrink A, Motteram J, Loza-Reyes E, Lucas J, Hammond-Kosack KE, Thomma BPHJ, Rudd JJ (2011) Analysis of two in planta expressed LysM effector homologs from the fungus *Mycosphaerella graminicola* reveals novel functional properties and varying contributions to virulence on wheat. *Plant Physiology* 156: 756-769.

Marzani QA (2011) *Fungicide Resistance and Efficacy for Control of* Pyrenophora teres *and* Mycosphaerella graminicola *on Barley and Wheat*. Doctoral thesis, University of Nottingham, Nottingham, UK. Available online: http://eprints.nottingham.ac.uk/12633/ [accessed 23-03-2017].

Mathre DE (1997) *Compendium of Barley Diseases (2nd edition)*. Press- The American Phytopathological Society, St. Paul, US, pp. 90.

Matsson M, Ackrell BA, Cochran B, Hederstedt L (1998) Carboxin resistance in *Paracoccus denitrificans* conferred by a mutation in the membrane-anchor domain of succinate: quinone reductase (complex II). *Archives of Microbiology* 170: 27-37.

Matsson M, Hederstedt L (2001) The carboxin-binding site on *Paracoccus denitrificans* succinate: quinone reductase identified by mutations. *Journal of Bioenergetics and Biomembranes* 33: 99-105.

McLean MS, Howlett BJ, Hollaway GJ (2010) Spot form of net blotch, caused by *Pyrenophora teres* f. *maculata*, is the most prevalent foliar disease of barley in Victoria, Australia. *Australasian Plant Pathology* 39: 46-49.

Miessner S, Stammler G (2010) *Monilinia laxa*, *M. fructigena* and *M. frutticola*: Risk estimation of resistance to QoI fungicides and identification of species with cytochrome *b* gene sequences. *Journal of Plant Diseases and Protection* 117: 162-167.

Mikaberidze A, McDonald BA (2015) Fitness cost of resistance: Impact on management. In: Ishii H, Hollomon DW (eds.) *Fungicide Resistance in Plant Pathogens. Principles and a Guide to Practical Management*, Springer, Tokyo, Japan, pp. 77-89.

Minarikova V, Polisenska I (1999) Analysis of populations of *Pyrenophora teres* on barley in the Czech Republic. *Plant Protection Science* 4: 115-120.

Mirzadi Gohari A, Ware SB, Wittenberg AHJ, Mehrabi R, Ben M'Barek S, Verstappen ECP, van der Lee TAJ, Robert O, Schouten HJ, de Wit PPJGM, Kema GHJ (2015) Effector discovery in the fungal wheat pathogen *Zymoseptoria tritici*. *Molecular Plant Pathology* 16: 931-945.

Miyamoto T, Ishii H, Seko T, Tomita Y, Kobori S, Ogawara T (2008) Occurrence of boscalid-resistant isolates of cucumber Corynespora leaf spot fungus (*C. cassiicola*). *Japanese Journal of Phytopathology* 74: 37-38.

Miyamoto T, Ishii H, Stammler G, Koch A, Ogawara T, Tomita T, Fountaine J, Ushio T, Seko T, Kobori S (2010) Distribution and molecular characterization of *Corynespora cassiicola* strains resistant to boscalid. *Plant Pathology* 59: 873-881.

Morschhäuser J (2010) Regulation of multidrug resistance in pathogenic fungi. *Fungal Genetics and Biology* 47: 94-106.

Murray GM, Brennan JP (2010) Estimating disease losses to the Australian barley industry. *Australasian Plant Pathology* 39: 85-96.

Nakamura K, Yamaki M, Sarada M, Nakayama S, Vibat CRT, Gennis RB, Nakayashiki T, Inokuchi H, Kojima S, Kita K (1996) Two hydrophobic subunits are essential for the heme b ligation and functional assembly of complex II (succinate-ubiquinone oxidoreductase) from *Escherichia coli*. *Journal of Biological Chemistry* 271: 521-527.

Narayanasamy P (2002) *Microbial Plant Pathogens and Crop Disease Management*. Science Publishers, Inc., Enfield, US, pp. 553.

Neumann S, Paveley ND, Beed FD, Sylvester-Bradley R (2004) Nitrogen per unit leaf area affects the upper asymptote of *Puccinia striiformis* f. sp. *tritici* epidemics in winter wheat. *Plant Pathology* 53: 725-732.

Niemann S, Müller U (2000) Mutations in SDHC cause autosomal dominant paraganglioma, type 3. *Nature Genetics* 26: 268.

Nødvig CS, Nielsen JB, Kogle ME, Mortensen UH (2015) A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PLoS One* 10: e0133085.

Ochiai H (2015) Single-base pair genome editing in human cells by using site-specific endonucleases. *International Journal of Molecular Sciences* 16: 21128-21137.

O'Connell MR, Oakes BL, Sternberg SH, East-Seletsky A, Kaplan M, Doudna JA (2014) Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature* 516: 263-266.

Oerke EC, Dehne HW, Schunbeck F, Weber A (1994) *Crop Production and Crop Protection: Estimated Losses in Major Food and Cash Crops*. Elsevier Science, Amsterdam, Netherlands, pp. 808.

Oerke EC (2006) Crop losses to pests. *The Journal of Agricultural Science* 144: 31-43.

Olesen JE, Jørgensen LN, Petersen J, Mortensen J (2003) Effects of rate and timing of nitrogen fertilizer on disease control by fungicides in winter wheat 2. Crop growth and diseases development. *Journal of Agricultural Sciences* 140: 15-29.

Oleson BT (1994) World wheat production, utilization and trade. In: Bushuk W, Rasper VF (eds.) *Wheat: Production, Properties and Quality*, Springer, US, pp. 1-11.

Oliver RP, Hewitt HG (2014) *Fungicides in Crop Protection (2nd edition)*. CABI Publishing, Wallingford, UK, pp. 200.

Omrane S, Sghyer H, Audéon C, Lanen C, Duplaix C, Walker AS, Fillinger S (2015) Fungicide efflux and the MgMFS1 transporter contribute to the multidrug resistance phenotype in *Zymoseptoria tritici* field isolates. *Environmental Microbiology* 17: 2805-2823.

Ōmura S, Shiomi K (2007) Discovery, chemistry, and chemical biology of microbial products. *Pure and Applied Chemistry* 79: 581-591.

Ou SH (1980) Pathogen variability and host resistance in rice blast disease. *Annual Review of Phytopathology* 18: 167-187.

Oyedotun KS, Yau PF, Lemire BD (2004) Identification of the heme axial ligands in the cytochrome b562 of the *Saccharomyces cerevisiae* succinate dehydrogenase. *Journal of Biological Chemistry* 279: 9432-9439.

Oyedotun KS, Sit CS, Lemire BD (2007) The *Saccharomyces cerevisiae* succinate dehydrogenase does not require heme for ubiquinone reduction. *Biochimica et Biophysica Acta (BBA)- Bioenergetics* 1767: 1436-1445.

Özkan H, Brandolini A, Schäfer-Pregl R, Salamini F (2002) AFLP analysis of a collection of tetraploid wheats indicates the origin of emmer and hard wheat domestication in southeast Turkey. *Molecular Biology and Evolution* 19: 1797-1801.

Parker SR, Lovell DJ, Royle DJ, Paveley ND (1999) Analyzing epidemics of *Septoria tritici* for improved estimates of disease risk. In: Lucas JA, Bowyer P, Anderson HM (eds.) *Septoria on Cereals: A Study of Pathosystems*, CABI Publishing, Wallingford, UK, pp. 96-107.

Perez-Tomas R (2006) Multidrug resistance: retrospect and prospects in anti-cancer drug treatment. *Current Medicinal Chemistry* 13: 1859-1876.

Piening L (1968) Development of barley net blotch from infested straw and seed. *Canadian Journal of Plant Science* 48: 623-625.

Ponomarenko A, Goodwin SB, Kema G (2011) Septoria tritici blotch (STB). *The Plant Health Instructor*. Available online: doi:10.1094/PHI-I-2011-0407-01 [accessed 10-04-2017].

PPDB: Pesticide Properties DataBase (2017) Available online: http://sitem.herts.ac.uk/aeru/ppdb/en/ [accessed 15-01-2017].

Quaedvlieg W, Kema GHJ, Groenewald JZ, Verkley GJM, Seifbarghi S, Razavi M, Gohari A, Mirzadi, Mehrabi R, Crous PW (2011) *Zymoseptoria* gen. nov.: a new genus to accommodate *Septoria*-like species occurring on graminicolous hosts. *Persoonia-Molecular Phylogeny and Evolution of Fungi* 26: 57-69.

Ranhotra GS (1994) World wheat production, utilization and trade. In: Bushuk W, Rasper VF (eds.) *Wheat: Production, Properties and Quality*, Springer, US, pp. 12-24.

Rau D, Maier FJ, Papa R, Brown AHD, Balmas V, Saba E, Schäfer W, Attene G (2005) Isolation and characterization of the mating-type locus of the barley pathogen *Pyrenophora teres* and frequencies of mating-type idiomorphs within and among fungal populations collected from barley landraces. *Genome* 48: 855-869.

Rau D, Attene G, Brown AHD, Nanni L, Maier FJ, Balmas V, Saba E, Schäfer W, Papa R (2007) Phylogeny and evolution of mating-type genes from *Pyrenophora teres*, the causal agent of barley 'net blotch' disease. *Current Genetics* 51: 377-392.

Rehfus A, Miessner S, Achenbach J, Strobel D, Bryson R, Stammler G (2016) Emergence of succinate dehydrogenase inhibitor resistance of *Pyrenophora teres* in Europe. *Pest Management Science* 72: 1977-1988.

Rehfus A, Strobel D, Bryson B, Stammler G (2017) Sensitivity of *Pyrenophora teres* to succinate dehydrogenase inhibitors in Europe. In: Deising HB, Fraaije B, Mehl A, Oerke EC, Sierotzki H, Stammler G (eds.) *Modern Fungicides and Antifungal Compounds Vol. VIII*, Deutsche Phytomedizinische Gesellschaft (DPG), Braunschweig, Germany, pp. 175-180.

Rehfus A, Strobel D, Bryson B, Stammler G (accepted) Mutations in *sdh* genes in field isolates of *Zymoseptoria tritici* and impact on the sensitivity to various succinate dehydrogenase inhibitors. *Plant Pathology* (accepted author manuscript doi:10.1111/ppa.12715).

Royle DJ (1994) Understanding and predicting epidemics: a commentary based on selected pathosystems. *Plant Pathology* 43: 777-789.

Rudd JJ, Keon J, Hammond-Kosack KE (2008) The wheat mitogen-activated protein kinases TaMPK3 and TaMPK6 are differentially regulated at multiple levels during compatible disease interactions with *Mycosphaerella graminicola*. *Plant Physiology* 147: 802-815.

Rudd JJ, Antoniw J, Marshall R, Motteram J, Fraaije B, Hammond-Kosack K (2010) Identification and characterisation of *Mycosphaerella graminicola* secreted or surface-associated proteins with variable intragenic coding repeats. *Fungal Genetics and Biology* 47: 19-32.

Rudd JJ, Kanyuka K, Hassani-Pak K, Derbyshire M, Andongabo A, Devonshire J, Lysenko A, Saqi M, Desai NM, Courbot M, et al. (2015) Transcriptome and metabolite profiling of the infection cycle of *Zymoseptoria tritici* on wheat reveals a biphasic interaction with plant immunity involving differential pathogen chromosomal contributions and a variation on the hemibiotrophic lifestyle definition. *Plant Physiology* 167: 1158-1185.

Ruprecht J, Yankovskaya V, Maklashina E, Iwata S, Cecchini G (2009) Structure of *Escherichia coli* succinate:quinone oxidoreductase with an occupied and empty quinone-binding site. *Journal of Biological Chemistry* 284: 29836-29846.

Ryder NS, Frank I, Dupont MC (1986) Ergosterol biosynthesis inhibition by the thiocarbamate antifungal agents tolnaftate and tolciclate. *Antimicrobial Agents and Chemotherapy* 29: 858-860.

Sánchez-Vallet A, Saleem-Batcha R, Kombrink A, Hansen G, Valkenburg DJ, Thomma BP, Mesters JR (2013) Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. *Elife* 2: e00790.

Sánchez-Vallet A, McDonald MC, Solomon PS, McDonald BA (2015) Is *Zymoseptoria tritici* a hemibiotroph? *Fungal Genetics and Biology* 79: 29-32.

Samson RA, Hoekstra ES, Frisvad JC (2004) *Introduction to Food- and Airborne Fungi (7th edition)*. Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

Sanderson FR (1976) *Mycosphaerella graminicola* the ascogenous state of *Septoria tritici*. *New Zealand Journal of Botany* 14: 359-360.

Sarpeleh A, Wallwork H, Catcheside DEA, Tate ME, Able AJ (2007) Evidence of involvement of proteinaceous toxins from *Pyrenophora teres* in symptom development of net blotch of barley. *Phytopathology* 97: 907-915.

Sarpeleh A, Wallwork H, Tate ME, Catcheside DEA, Able AJ (2008) Initial characterization of phytotoxic proteins isolated from *Pyrenophora teres*. *Physiological and Molecular Plant Pathology* 72: 73-79.

Sauter H, Steglich W, Anke T (1999) Strobilurins: evolution of a new class of active substances. *Angewandte Chemie-International Edition* 38: 1329-1349.

Scalliet G, Bowler J, Luksch T, Kirchhofer-Allan L, Steinhauer D, Ward K, Niklaus M, Verras A, Csukai M, Fonné-Pfister R, et al. (2012) Mutagenesis and functional studies with succinate dehydrogenase inhibitors in the wheat pathogen *Mycosphaerella graminicola*. *PLoS One* 7: e35429.

Schaller CW (1955) Inheritance of resistance to net blotch of barley. *Phytopathology* 45: 174-176.

Schmitz H, Mehl A, Kerz-Moehlendick F (2014) Punktmutationen am Zielgen der SDHI Fungizide- Relevanz für den Getreidebau? *Julius-Kühn-Archiv* 447: 185. Available online: https://ojs.openagrar.de/index.php/JKA/article/view/3297/3415 [accessed 11-03-2017].

Schwinn FJ (1982) Socio-economic impact of fungicide resistance. In: Dekker J, Georgopoulos SG (eds.) *Fungicide Resistance in Crop Protection*, Centre for Agricultural Publishing and Documentation, Wageningen, Netherlands, pp. 16-23.

Schwinn FJ (1992) Significance of fungal pathogens in crop production. *Pesticide Outlook* 3: 18-28.

Semar M, Strobel D, Koch A, Klappach K, Stammler G (2007) Field efficacy of pyraclostrobin against populations of *Pyrenophora teres* containing the F129L mutation in the cytochrome *b* gene. *Journal of Plant Diseases and Protection* 114: 117-119.

Serenius M, Mironenko N, Manninen O (2005) Genetic variation, occurrence of mating types and different forms of *Pyrenophora teres* causing net blotch of barley in Finland. *Mycological Research* 109: 809-817.

Serivastava KD, Tewari AK (2002) Fungal diseases of wheat and barley: foliar diseases. In: Gupta VK, Paul YS (eds.) *Diseases of Field Crops*, INDUS, New Delhi, India. pp 58-78.

Sevastos A, Labrou NE, Flouri F, Malandrakis A (2016) Glutathione transferase-mediated benzimidazole-resistance in *Fusarium graminearum*. *Pesticide Biochemistry and Physiology*. Available online: http://doi.org/10.1016/j.pestbp.2016.11.002 [accessed 10-11-2016].

Shaw MW, Royle DJ (1989) Airborne inoculum as a major source of *Septoria tritici* (*Mycosphaerella graminicola*) infections in winter wheat crops in the UK. *Plant Pathology* 38: 35-43.

Shipton WA, Boyd WRJ, Rosielle AA, Shearer BI (1971) The common Septoria diseases of wheat. *The Botanical Review* 37: 231-262.

Shipton WA, Khan TN, Boyd WJR (1973) Net blotch of barley. *Review of Plant Pathology* 52: 269-290.

Shoemaker RA (1962) *Drechslera* Ito. *Canadian Journal of Botany* 40: 808-836.

Sierotzki H, Wullschleger J, Gisi U (2000) Point-mutation in cytochrome *b* gene conferring resistance to strobilurin fungicides in *Erysiphe graminis* f. sp. *tritici* field isolates. *Pesticide Biochemistry and Physiology* 68: 107-112.

Sierotzki H, Frey R, Wullschleger J, Palermo S, Karlin S, Godwin J, Gisi U (2007) Cytochrome *b* gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. *Pest Management Science* 63: 225-233.

Sierotzki H, Scalliet G (2013) A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. *Phytopathology* 103: 880-887.

Silkin Y, Oyedotun KS, Lemire BD (2007) The role of Sdh4p Tyr-89 in ubiquinone reduction by the *Saccharomyces cerevisiae* succinate dehydrogenase. *Biochimica et Biophysica Acta (BBA)- Bioenergetics* 1767: 143-150.

Simon MR, Cordo CA, Perrello AE, Struik PC (2003) Influence of nitrogen supply on the susceptibility of wheat to *Septoria tritici*. *Journal of Phytopathology* 151: 283-289.

Sivanesan A (1987) Graminicolous species of *Bipolaris, Curvularia, Drechslera, Exserohilum* and their teleomorphs. *Mycological Papers* 158: 1-261.

Skinner W, Bailey A, Renwick A, Keon J, Gurr S, Hargreaves J (1998) A single amino-acid substitution in the iron-sulphur protein subunit of succinate dehydrogenase determines resistance to carboxin in *Mycosphaerella graminicola*. *Current Genetics* 34: 393-398.

Smedegård-Petersen V (1971) *Pyrenophora teres* f. sp. *maculata* f. *nov*. and *Pyrenophora teres* f. *teres* on barley in Denmark. *Yearbook of the Royal Veterinary and Agricultural University*, Copenhagen, Denmark, pp. 124-144.

Smedegård-Petersen V (1977) Isolation of two toxins produced by *Pyrenophora teres* and their significance in disease development of net-spot blotch of barley. *Physiological Plant Pathology* 10: 203-211.

Solomon PS, Oliver RP (2001) The nitrogen content of the tomato leaf apoplast increases during infection by *Cladosporium fulvum*. *Planta* 213: 241-249.

Solomon PS, Oliver RP (2002) Evidence that c-aminobutyric acid is a major nitrogen source during *Cladosporium fulvum* infection of tomato. *Planta* 214: 414-420.

Speakman JB, Pommer E-H (1986) A simple method for producing large volumes of *Pyrenophora teres* spore suspension. *Bulletin of the British Mycological Society* 20: 129-130.

Sprague R (1950) *Diseases of Cereals and Grasses in North America*. Ronald Press, New York, US, pp. 538.

Stammler G, Brix HD, Glättli A, Semar M, Schoefl U (2007) Biological properties of the carboxamide boscalid including recent studies on its mode of action. In: *Proceedings of the BCPC XVI International Plant Protection Congress*, British Crop Protection Council (BCPC), Alton, UK, pp. 16-21.

Stammler G, Carstensen M, Koch A, Semar M, Strobel D, Schlehuber S (2008a) Frequency of different CYP51-haplotypes of *Mycosphaerella graminicola* and their impact on the epoxiconazole-sensitivity and -field efficacy. *Crop Protection* 27: 1448-1456.

Stammler G, Kern L, Semar M, Glaettli A, Schoefl U (2008b) Sensitivity of *Mycosphaerella graminicola* to DMI fungicides related to mutations in the target gene *cyp51* (14α-demethylase). In: Dehne HW, Deising HB, Gisi U, Kuck K-H, Russell P, Lyr H (eds.) *Modern Fungicides and Antifungal Compounds Vol. V*, Deutsche Phytomedizinische Gesellschaft (DPG), Braunschweig, Germany, pp. 137-142.

Stammler G (2008) Mode of action, biological performance and latest monitoring results of boscalid sensitivity. In: *18th Symposium of the Research Committee on Fungicide Resistance*, The Phytopathological Society of Japan, Shimane, Japan, pp. 30-43.

Stammler G, Glättli A, Koch A, Schlehuber S (2010) Mutations in the target protein conferring resistance to SDHI fungicides. In: Dehne HW, Deising HB, Gisi U, Kuck K-H, Russell PE, Lyr H (eds.) *Modern Fungicides and Antifungal Compounds Vol. VI*, Deutsche Phytomedizinische Gesellschaft (DPG), Braunschweig, Germany, pp. 195-198.

Stammler G, Semar M (2011) Sensitivity of *Mycosphaerella graminicola* (anamorph: *Septoria tritici*) to DMI fungicides across Europe and impact on field performance. *EPPO Bulletin* 41: 149-155.

Stammler G (2012) Resistance risk of corn pathogens to QoI fungicides. *Outlooks on Pest Management* 23: 211-214.

Stammler G, Schutte GC, Speakman J, Miessner S, Crous PW (2012) *Phyllosticta* species on citrus: risk estimation of resistance to QoI fungicides and identification of species with cytochrome *b* gene sequences. *Crop Protection* 48: 6-12.

Stammler G, Wolf A, Glättli A, Klappach K (2015) Respiration inhibitors: complex II. In: Ishii H, Hollomon DW (eds.) *Fungicide Resistance in Plant Pathogens. Principles and a Guide to Practical Management*, Springer, Tokyo, Japan, pp. 105-117.

Steffens JJ, Pell EJ, Tien M (1996) Mechanisms of fungicide resistance in phytopathogenic fungi. *Current Opinion in Biotechnology* 7: 348-355.

Steinfeld U, Sierotzki H, Parisi S, Gisi U (2002) Comparison of resistance mechanisms to strobilurin fungicides in *Venturia inaequalis*. In: Lyr H, Russell PE, Dehne H-W, Gisi U, Kuck K-H (eds.) *Modern Fungicides and Antifungal Compounds Vol. II*, 13th International Reinhardsbrunn-Symposium, Verlag Th. Mann Gelsenkirchen, Bonn, Germany, pp. 167-176.

Stukenbrock EH, Banke S, Javan-Nikkhah M, McDonald BA (2007) Origin and domestication of the fungal wheat pathogen *Mycosphaerella graminicola* via sympatric speciation. *Molecular Biology and Evolution* 24: 398-411.

Stukenbrock EH, Bataillon T, Dutheil JY, Hansen TT, Li R, Zala M, McDonald BA, Wang J, Schierup MH (2011) The making of a new pathogen: insights from comparative population genomics of the domesticated wheat pathogen *Mycosphaerella graminicola* and its wild sister species. *Genome Research* 21: 2157-2166.

Suffert F, Sache I, Lannou C (2011) Early stages of Septoria tritici blotch epidemics of winter wheat, build-up, overseasoning, and release of primary inoculum. *Plant Pathology* 60: 166-177.

Sun F, Huo X, Zhai Y, Wang A, Xu J, Su D, Bartlam M, Rhao Z (2005) Crystal structure of mitochondrial respiratory membrane protein complex II. *Cell* 121: 1043-1057.

Taylor J (2001) *Microorganisms and Biotechnology (Vol. 16)*. Nelson-Thornes Publisher, UK, pp. 168-169.

Tekauz A (1990) Characterisation and distribution of pathogenic variation in *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* from western Canada. *Canadian Journal of Plant Pathology* 12: 141-148.

Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S (2002) Agricultural sustainability and intensive production practices. *Nature* 418: 671-677.

Thomma BPHJ, Van Esse HP, Crous PW, de Wit PJ (2005) *Cladosporium fulvum* (syn. *Passalora fulva*), a highly specialized plant pathogen as a model for functional studies on plant pathogenic Mycosphaerellaceae. *Molecular Plant Pathology* 6: 379-393.

Tompkins DK, Wright AT, Fowler DB (1992) Foliar disease development in no-till winter wheat. Influence of agronomic practices on powdery mildew development. *Canadian Journal of Plant Science* 72: 965-972.

Torriani SF, Brunner PC, McDonald BA, Sierotzki H (2009) QoI resistance emerged independently at least 4 times in European populations of *Mycosphaerella graminicola*. *Pest Management Science* 65: 155-162.

Torriani SF, Melichar JP, Mills C, Pain N, Sierotzki H, Courbot M (2015) *Zymoseptoria tritici*: a major threat to wheat production, integrated approaches to control. *Fungal Genetics and Biology* 79: 8-12.

Tran QM, Rothery RA, Maklashina E, Cecchini G, Weiner JH (2006) The quinone binding site in *Escherichia coli* succinate dehydrogenase is required for electron transfer to the heme b. *Journal of Biological Chemistry* 281: 32310-32317.

Tran QM, Rothery RA, Maklashina E, Cecchini G, Weiner JH (2007) *Escherichia coli* succinate dehydrogenase variant lacking the heme b. *Proceedings of the National Academy of Sciences* 104: 18007-18012.

Trindade S, Sousa A, Xavier KB, Dionisio F, Ferreira MG, Gordo I (2009) Positive epistasis drives the acquisition of multidrug resistance. *PLoS Genetics* 5: e1000578.

Van Caeseele L, Grumbles J (1979) Ultrastructure of the interaction between *Pyrenophora teres* and a susceptible barley host. *Canadian Journal of Botany* 57: 40-47.

Van den Berg CGJ, Rossnagel BG (1990) Effects of temperature and leaf wetness period on conidium germination and infection of barley by *Pyrenophora teres*. *Canadian Journal of Plant Pathology* 12: 263-266.

Van den Berg F, van den Bosch F, Paveley ND (2013) Optimal fungicide application timings for disease control are also an effective anti-resistance strategy: a case study for *Zymoseptoria tritici* (*Mycosphaerella graminicola*) on wheat. *Phytopathology* 103: 1209-1219.

Van den Bosch F, Paveley N, Shaw M, Hobbelen P, Oliver R (2011) The dose rate debate: does the risk of fungicide resistance increase or decrease with dose? *Plant Pathology* 60: 597-606.

Van den Bosch F, Oliver R, van den Berg F, Paveley N (2014) Governing principles can guide fungicide-resistance management tactics. *Annual Review of Phytopathology* 52: 175-195.

Veloukas T, Leroch M, Hahn M, Karaoglanidis GS (2011) Detection and molecular characterization of boscalid-resistant *Botrytis cinerea* isolates from strawberry. *Plant Disease* 95: 1302-1307.

Veloukas T, Markoglou AN, Karaoglanidis GS (2013) Differential effect of *Sdh*B gene mutations on the sensitivity to SDHI fungicides in *Botrytis cinerea*. *Plant Disease* 97: 118-122.

Veloukas T, Kalogeropoulou P, Markoglou AN, Karaoglanidis GS (2014) Fitness and competitive ability of *Botrytis cinerea* field isolates with dual resistance to SDHI and QoI fungicides, associated with several *sdh* B and the *cyt* b G143A mutations. *Phytopathology* 104: 347-356.

Verreet JA, Klink H, Hoffmann GM (2000) Regional monitoring for disease prediction and optimization of plant protection measures: The IPM wheat model. *Plant Disease* 84: 816-826.

Von Schmeling B, Kulka G (1966) Systemic fungicidal activity of 1,4-oxathiin derivates. *Science* 152: 659-660.

Waalwijk C, Mendes O, Verstappen ECP, de Waard MA, Kema GHJ (2002) Isolation and characterization of the mating-type idiomorphs from the wheat septoria leaf blotch fungus *Mycosphaerella graminicola*. *Fungal Genetics and Biology* 35: 277-286.

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* 218: 1-14.

Webster J (1951) Graminicolous *Pyrenomycetes* II. The occurrence of the perfect stage of *Helminthosporium teres* in Britain. *Transactions of the British Mycological Society* 34: 309-317.

Welch RM, Graham RD (2004) Breeding for micronutrients in staple food crops from a human nutrition perspective. *Journal of Experimental Botany* 55: 353-364.

Wittenberg AHJ, van der Lee TAJ, Ben M'Barek S, Ware SB, Goodwin SB, Kilian A, Visser RGF, Kema GHJ, Schouten HK (2009) Meiosis drives extraordinary genome plasticity in the haploid fungal plant pathogen *Mycosphaerella graminicola*. *PLoS One* 4: e5863.

Wood PM, Hollomon DW (2003) A critical evaluation of the role of alternative oxidase in the performance of strobilurin and related fungicides acting at the Qo site of complex III. *Pest Management Science* 59: 499-511.

Yang F, Li W, Jørgensen HJ (2013) Transcriptional reprogramming of wheat and the hemibiotrophic pathogen *Septoria tritici* during two phases of the compatible interaction. *PLoS One* 8: e81606.

Yankovskaya V, Horsefield R, Törnroth S, Luna-Chavez C, Miyoshi H, Léger C, Byrne B, Cecchini G, Iwata S (2003) Architecture of succinate dehydrogenase and reactive oxygen species generation. *Science* 299: 700-704.

Yeh PJ, Tschumi AI, Kishony R (2006) Functional classification of drugs by properties of their pairwise interactions. *Nature Genetics* 38: 489-494.

Yeh PJ, Hegreness MJ, Aiden AP, Kishony R (2009) Drug interactions and the evolution of antibiotic resistance. *Nature Reviews Microbiology* 7: 460-466.

Yin YN, Kim YK, Xiao CL (2011) Molecular characterization of boscalid resistance in field isolates of *Botrytis cinerea* from apple. *Phytopathology* 101: 986-995.

Zhan J, Pettway RE, McDonald BA (2003) The global genetic structure of the wheat pathogen *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial diversity, regular recombination, and gene flow. *Fungal Genetics and Biology* 38: 286-297.

Zhan J, Stefanato FL, McDonald BA (2006) Selection for increased cyproconazole tolerance in *Mycosphaerella graminicola* through local adaptation and in response to host resistance. *Molecular Plant Pathology* 7: 259-268.

Zhou QJ, Zhai YJ, Liu M, Sun F (2011) Thiabendazole inhibits ubiquinone reduction activity of mitochondrial respiratory complex II via a water molecule mediated binding feature. *Protein & Cell* 2: 531-542.

7 Supplementary Material

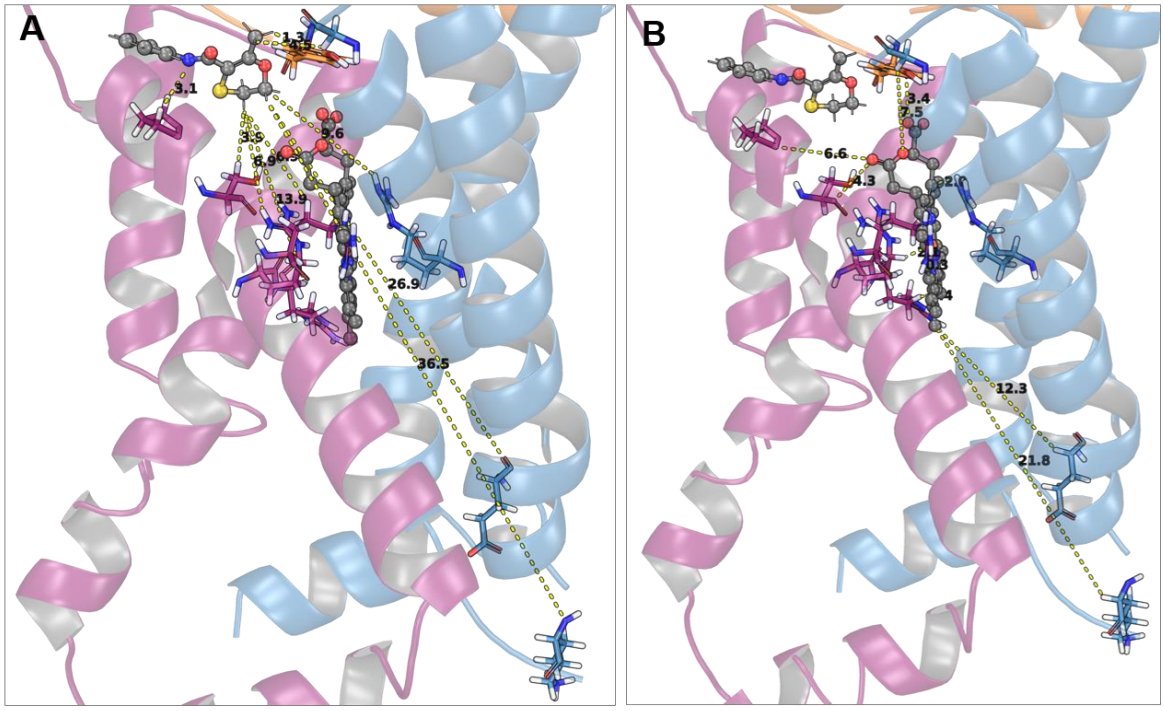


Figure 48: Length measurements (PyMol measurement tool) in homology model of *P. teres*. A: Distance of substituted amino acids to carboxin (nearest atoms). **B**: Distance of substituted amino acids to haem b group (nearest atoms).

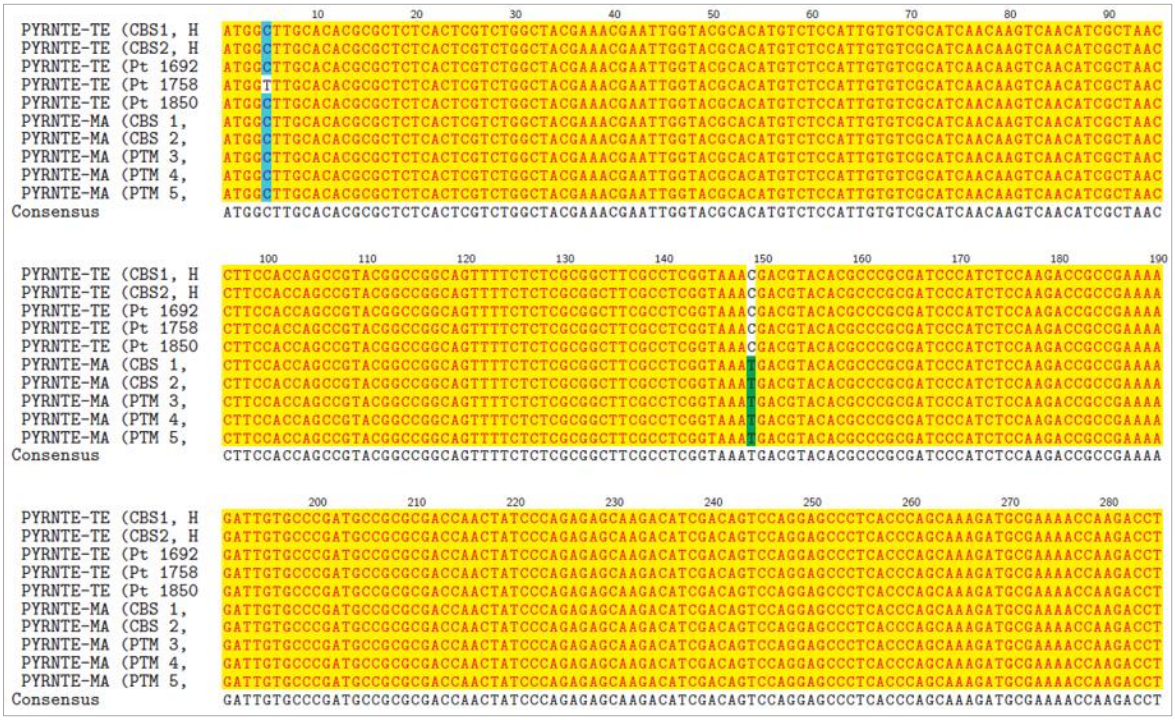


Figure 49-Part 1: *SdhB* DNA sequence alignment of PTT and PTM. Further description is given on page 170.

Figure 49-Part 2: *SdhB* DNA sequence alignment of PTT and PTM. Further description is given on page 170.

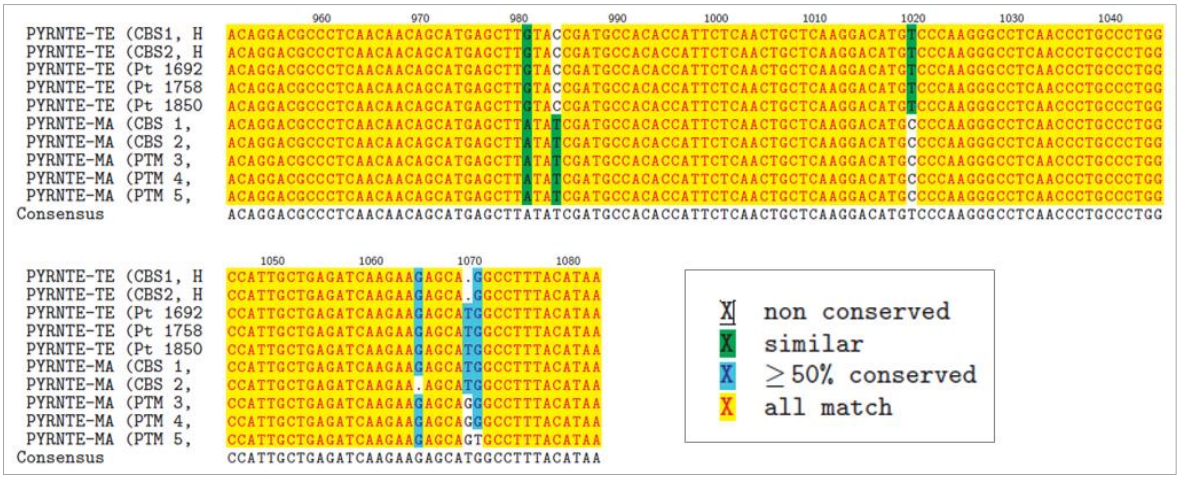


Figure 49-Part 3: *SdhB* DNA sequence alignment of PTT and PTM. *SdhB* DNA sequence alignment of five PTT and five PTM isolates is given. Two PTT and two PTM isolates were obtained from CBS fungal isolate collection (Centraalbureau voor Schimmelculturen, Utrecht, Netherlands) and all four isolates were originated from Hungary. The other PTT isolates shown are isolates from France and the UK obtained from 'Random monitoring'. The other PTM isolates were isolated during the present study from infected leaves which showed spot-type lesions. These leaves were sent from Italy (isolate PTM 3), Germany (isolate PTM 4) and Denmark (isolate PTM 5). In total, six SNPs in *SdhB* were detected in PTM compared to PTT. Primer KES 1847 (sequencing primer of B-H277Y) anneals at nucleotide positions 975-990. Here, two SNPs between PTT and PTM sequences are detectable which explains why this assay does not work for PTM isolates.

Figure 50-Part 1: *SdhC* DNA sequence alignment of PTT and PTM. Further description is given on page 171.

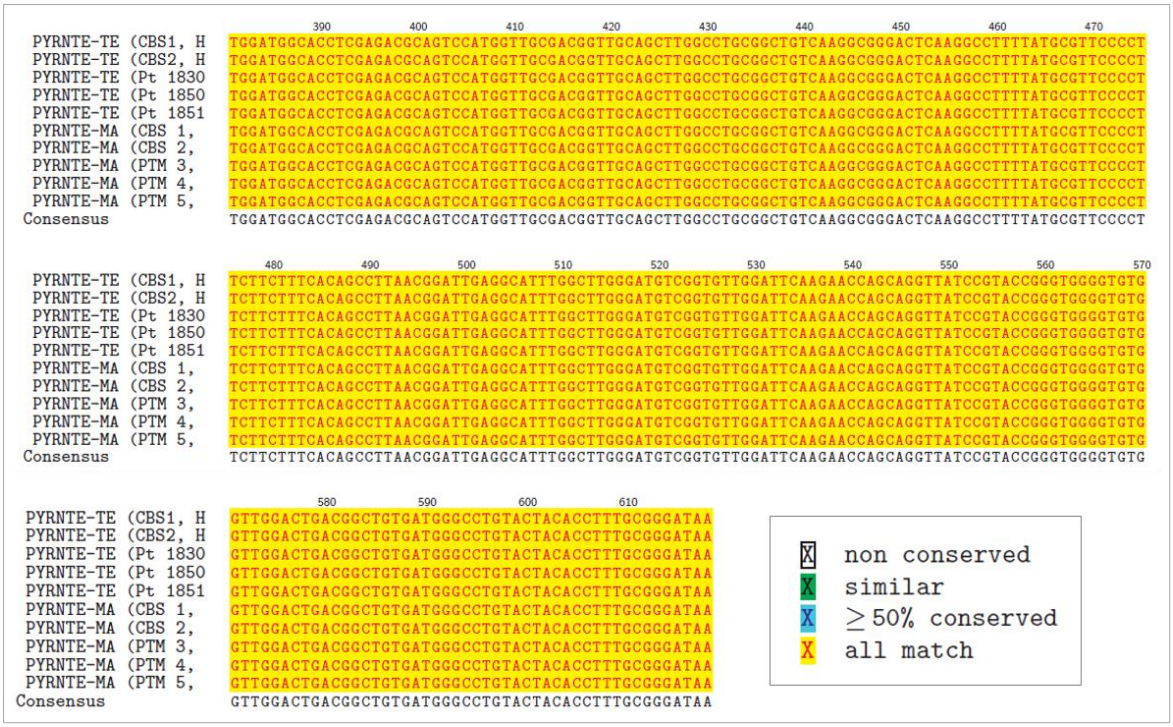


Figure 50-Part 2: *SdhC* DNA sequence alignment of PTT and PTM. *SdhC* DNA sequence alignment of five PTT and five PTM isolates is given. Two PTT and two PTM isolates were obtained from CBS and originated from Hungary. The other PTT isolates shown are isolates from France and the UK obtained from 'Random monitoring'. The other PTM isolates were isolated during the present study from infected leaves which showed spot-type lesions. These leaves were sent from Italy (isolate PTM 3), Germany (isolate PTM 4) and Denmark (isolate PTM 5). In total, six SNPs in *SdhC* were detected in PTM compared to PTT.

Table 34: Net blotch infected leaf samples of *P. teres* with spot-type lesions and their origin. Samples in the list showed spot-like lesions on barley leaves in addition to no detectable signals in assay B-H277Y (other assays resulted in 'robust' signals).

Isolate-ID	Country	Origin	Sampling Date	Comments
81/14	PL	Łany Wielkie	April 2014	spot lesions
124/14	ES	Zambrana	May 2014	spot lesions
129/14	IT	Conselice	May 2014	spot lesions
15/15	DE	Oberbohingen	April 2015	spot lesions
31+32/15	UK	Shotley/Euston	April 2015	spot lesions
37-39/15	PL	Łany Wielkie	April 2015	spot lesions
162/15	HU	Szentlörinckata	May 2015	spot lesions
176-179/15	UK	Stoke by Clare, Thornham Magna, Bildeston, Walkington	June 2015	spot lesions
182-183/15	UK	Naughton, St. Osyth	June 2015	spot lesions
248+250/15	DK	Flakkebjerg, Hinnerup	June 2015	spot lesions
13/16	IT	Voghera	May 2015	spot lesions
34/16	UK	Stoke by Clare	April 2016	spot lesions
52+53/16	UK	Cherhill	May 2016	spot lesions
91+95/16	UK	St. Osyth, Ockbrook	June 2016	spot lesions
98+99/16	UK	Bildeston	June 2016	spot lesions
101-103/16	UK	Ipswhich, Sevenhampton, Fosote	June 2016	spot lesions
144/16	SK	Maly Saris	June 2016	spot lesions
167/16	DK	Stensmark	July 2016	spot lesions

Table 35: Diseased leaf area of *Z. tritici* isolates used in glasshouse studies on the SDHI sensitivity Diseased leaf area is shown for two one-day preventative tests (P1-1 and P1-2) and two four-day curative tests (C4-1 and C4-2). Curative tests were carried out in a glasshouse chamber with optimised light conditions for *Z. tritici* which allowed higher infection rates.

Isolate-ID	SDHIres	Diseased leaf area in untreated [%]				
		P1-1	P1-2	C4-1	C4-2	Mean
1965	no	7 ± 0.8	13 ± 1.4	41 ± 3.4	70 ± 3.6	33 ± 14.4
3718	no	29 ± 2.8	13 ± 1.4	55 ± 4.3	92 ± 4.1	47 ± 17.2
6027	B-T268I	61 ± 2.9	55 ± 3.5	90 ± 3.3	99 ± 0.8	76 ± 10.8
6032	B-T268I	64 ± 1.7	58 ± 3.2	81 ± 5.2	95 ± 1.7	75 ± 8.4
5548	C-T79N	69 ± 6.7	60 ± 5.8	96 ± 1.4	100 ± 0	81 ± 9.9
6031	C-T79N	57 ± 2.4	44 ± 4.3	98 ± 0.3	100 ± 0	75 ± 14.2
5745	C-N86S	67 ± 2.5	47 ± 6.3	83 ± 2.1	97 ± 1.0	74 ± 10.7
6035	C-N86S	85 ± 3.7	53 ± 3.2	97 ± 1.2	99 ± 0.8	84 ± 10.6
6029	C-H152R	47 ± 3.3	46.5 ± 7.2	66 ± 2.7	100 ± 0	71 ± 13.4
6036	C-H152R	50 ± 4.8	32 ± 2.4	75 ± 3.8	92 ± 5.8	62 ± 13.3

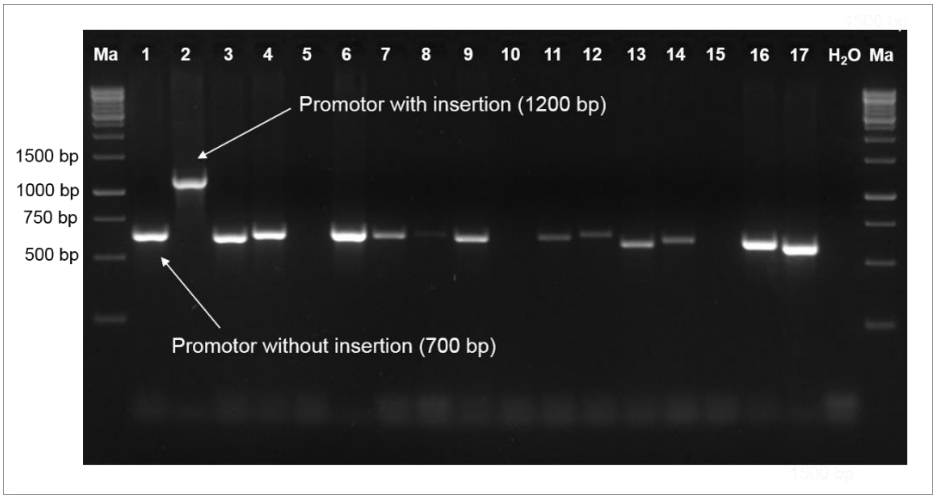


Figure 51: Verification of the absence of a promotor insertion in the *Mg*MFS1 transporter in SDHI resistant isolates of *Z. tritici* by PCR. Sequences of oligonucleotides were taken from Omrane et al. (2015) (Z4_110044_FW and Z4_110044_RV) and were used to amplify a part of *Mg*MFS1 promotor in SDHI resistant field isolates of *Z. tritici*. As shown by the authors in Omrane et al. (2015), tolnaftate-sensitive isolates showed an amplicon of 700 bp, whereas most tolnaftate-resistant isolates showed an insertion of 519 bp, which led to an amplicon size of 1200 bp. Ma: Size marker, 1: IPO323 (tolnaftate-sensitive), 2: St 5950 (tolnaftate-resistant), 3-17: SDHI resistant isolates (St 5548, 5745, 6027-6039).

Table 36: Laboratory mutants of *Z. tritici* that were generated during the present study. All clones on second selection plate were screened for known SNPs (B-H267R/Y/L, B-T268I, B-I269V, C-T79N, C-N86S (K), C-G90R and C-H152R). Some selected isolates were further sequenced.

Isolates	Applied spore number	Conc. [mg a.i. L^{-1}]	No. of colonies	No. of colonies (2. selection)	SDH-B and SDH-C variants (number of isolates)
IPO323	1.77E+09	10	7	7	C-H152R (6), unknown (1)
	1.77E+09	1	10	2	C-G90R (1), unknown (1)
St 3718	7.56E+08	10	8	1	C-H152R
	7.56E+08	1	100	2	Unknown (2)
St 2847	4.45E+08	10	3	3	C-V48G (1), B-H267Y (1), unknown (1)
	4.45E+08	1	25	0	no colonies
St 3573	9.04E+08	10	41	15	B-N225I (1), C-N86K (2), C-H152R (3), unknown (9)
	9.04E+08	1	57	2	unknown (2)
St 3955	1.10E+09	10	0	0	no colonies
	1.10E+09	1	39	0	no colonies
St 5821	7.72E+08	10	7	7	C-H145R (2), C-H152R (5)
	7.72E+08	1	4	2	unknown

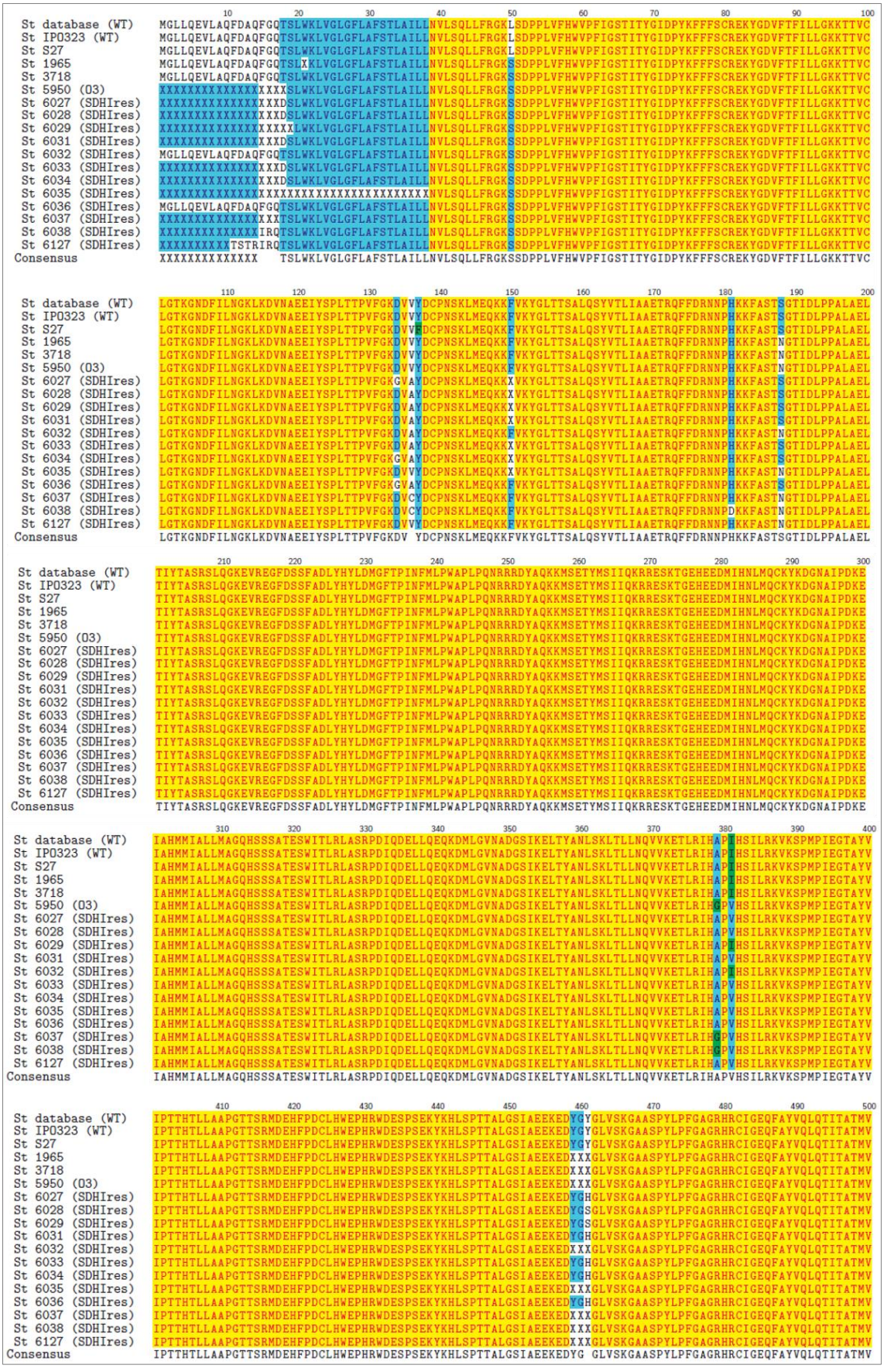


Figure 51-Part 1: Alignments of CYP51 amino acid sequence of *Z. tritici.* Further description is given on page 174.

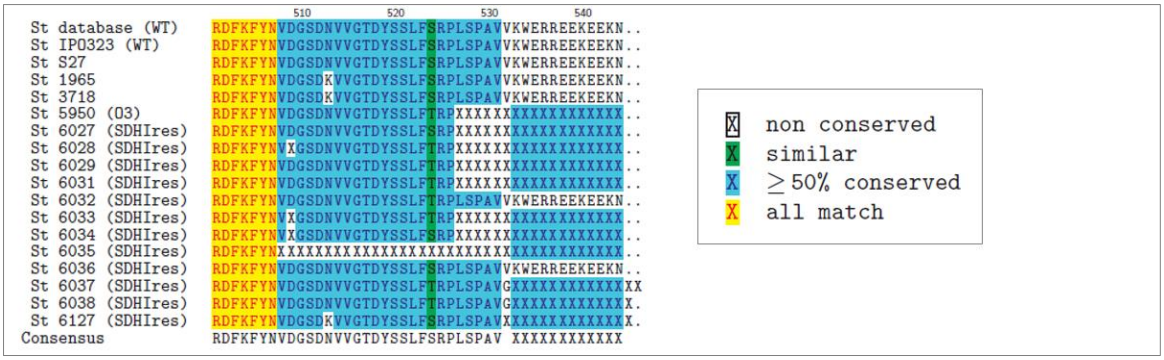


Figure 52-Part 2: Alignments of CYP51 amino acid sequence of *Z. tritici*. Isolates shown are SDHI sensitive (sequence from the database, St IPO323, St 1965, St 3718 and St 5950) and some SDHI resistant isolates that were additionally characterised for their CYP51-type (St 6027-29, St 6031-38 and St 6127). For some sequences the beginning and the end of CYP51 sequence is missing. Amino acid exchanges known to reduce the DMI sensitivity are D134G, V136A/C, A379G, I381V, deletions Y459-Y461 (isolates which have deletions show an X at these positions), Y459D/S/C, Y461S/H and S524T. These alterations can be found in different combinations in *Z. tritici* isolates (e.g. R6-R12, O3).

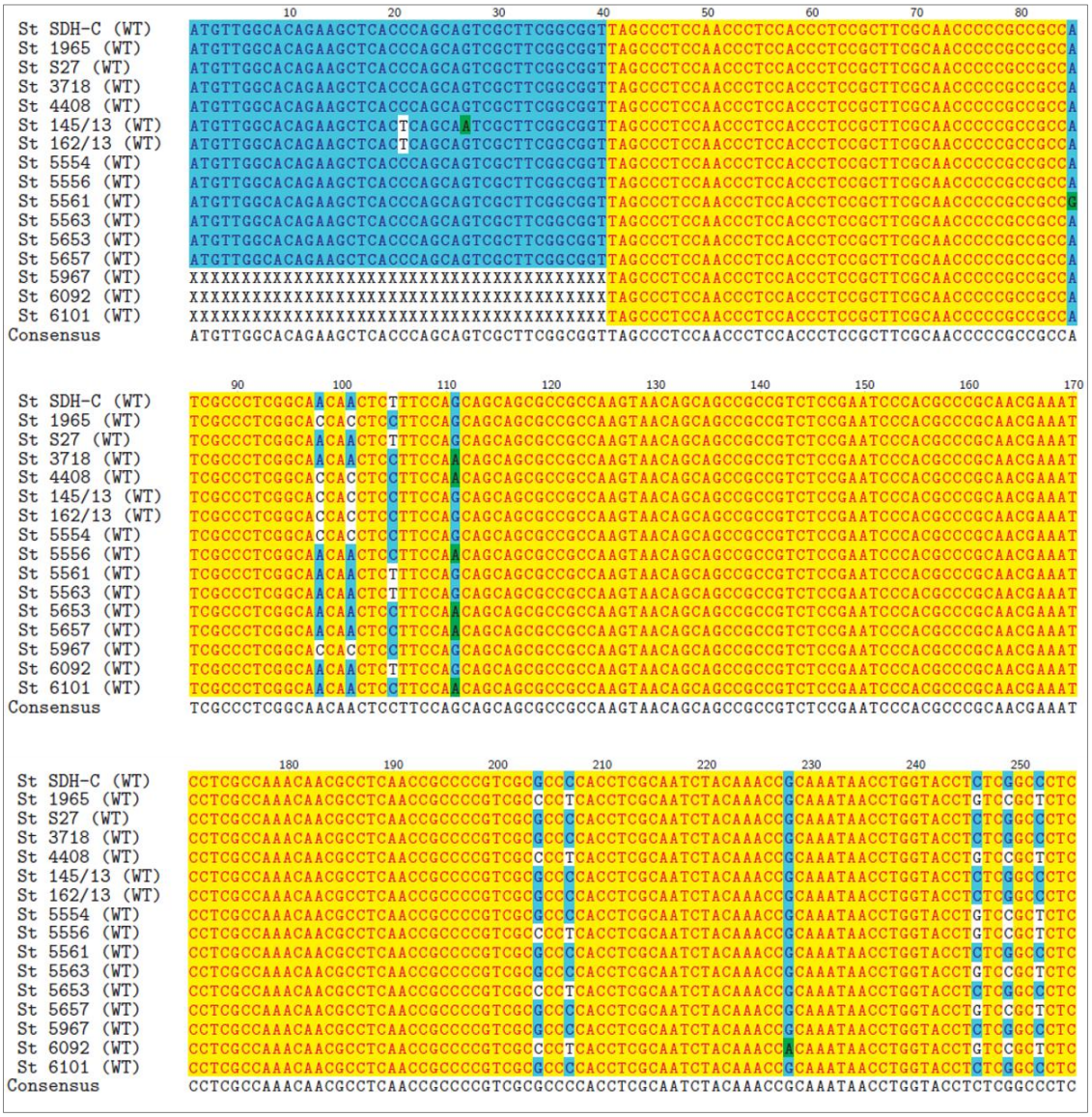


Figure 53-Part 1: *SdhC* cDNA sequence alignments of SDHI sensitive isolates of *Z. tritici*. Further description is given on page 175.

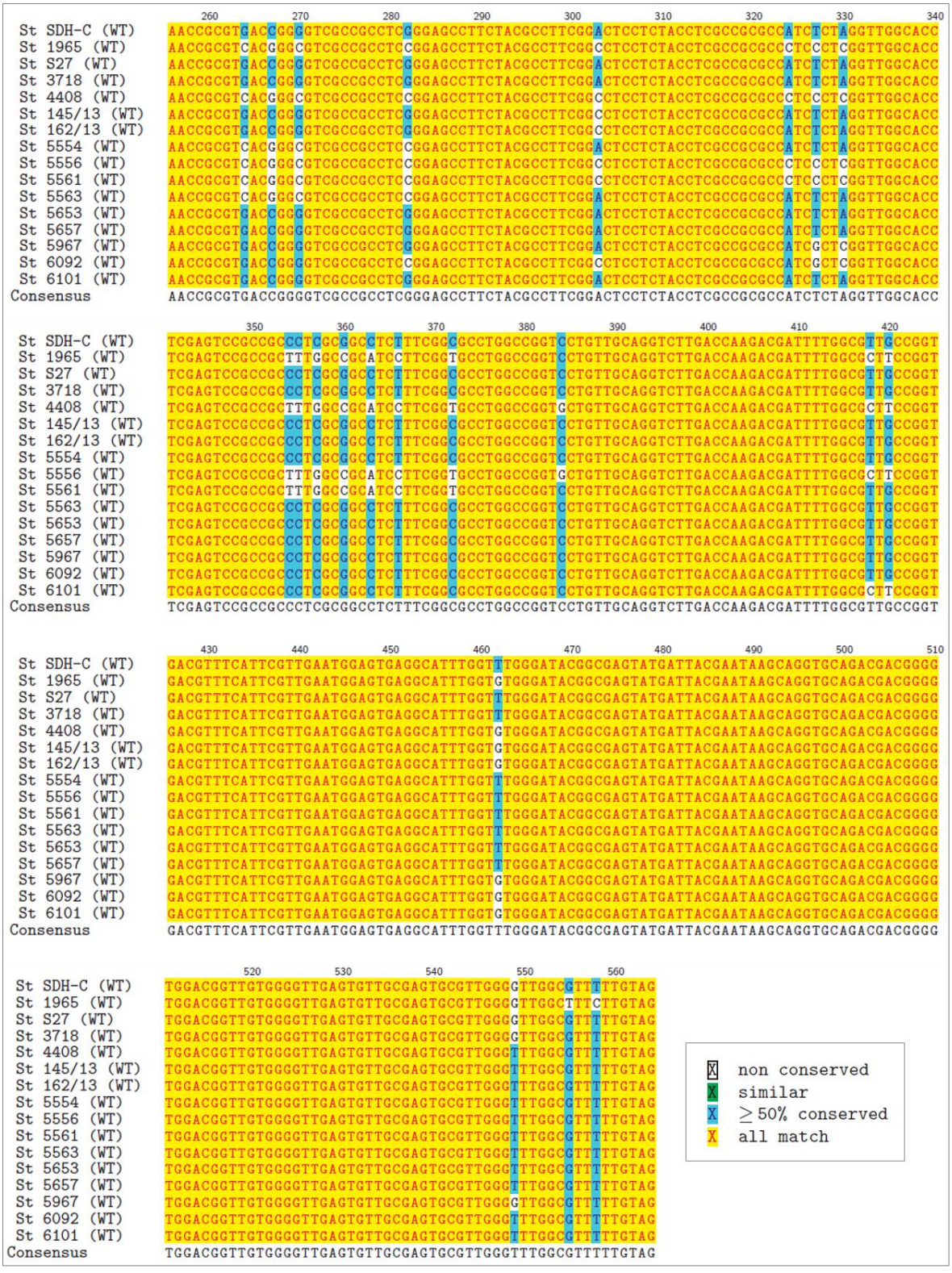


Figure 53: *SdhC* cDNA sequence alignments of SDHI sensitive isolates of *Z. tritici*. Sensitive isolates shown are taken from different countries and years. The beginning of three sequences is missing. Several SNPs can be observed in *SdhC* sequence even within sensitive isolates. Most of them are silent mutations, however, quantitative SNP detection systems (e.g. pyrosequencing and qPCR) are more challenging due to these variable positions.

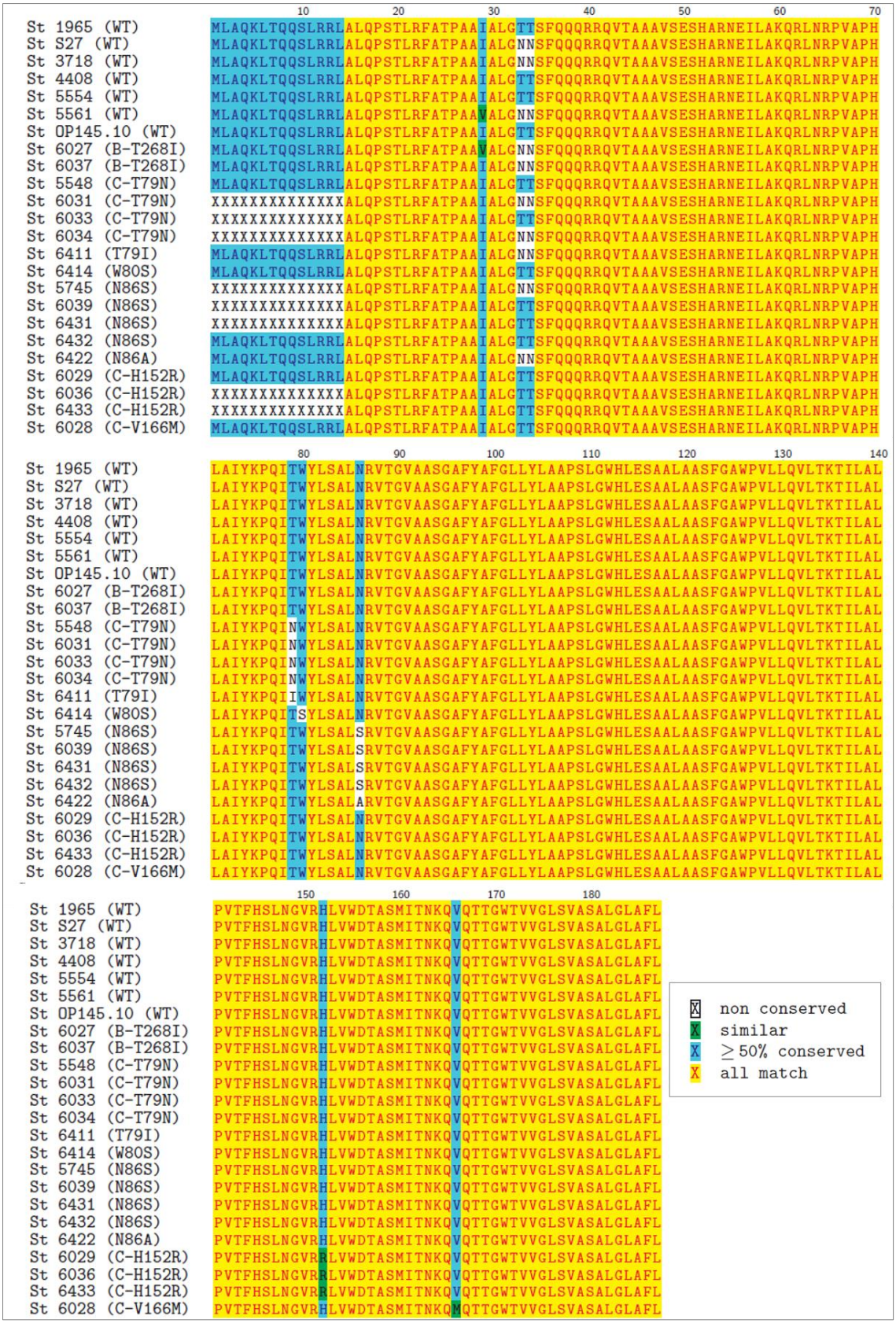


Figure 54: Alignment of SDH-C amino acid sequence in *Z. tritici*. Isolates shown are some selected SDHI sensitive (WT) and SDHI resistant strains with the amino acid exchanges B-T268I, C-T79N/I, C-W80S, C-N86S/A, C-H152R and C-V166M (beginning of SDH-C sequence is missing for some sequences). At positions 33-34, two variants of SDH-C are present in sensitive and resistant isolates of *Z. tritici* (N33, N34 or T33, T34).

8 Supplementary Tables

Table 37: Overview of 'Random Monitoring' isolates of *P. teres* in 2012-2015. Isolates were gained and preanalysed by EpiLogic company. All SDHI resistant and sensitive isolates that were analysed in the present study are listed in the column isolates analysed.

Country	Routes	Sampling Date	No. of isolates	Isolates analysed	
				SDHIres	sensitiv
IE	Dundulk-Dublin-Carrickmacross	13-07-2012	3	0	0
UK	Edinburgh-Berwick	12-07-2012	2	0	0
UK	Newcastle-Harrogate	12-07-2012	8	0	0
UK	Harrogate-Newark	12-07-2012	1	0	0
UK	East Anglia (North)	11-07-2012	10	0	0
UK	Cambridge-Dover	11-07-2012	2	0	0
BE	Brüssel-Aachen	28-06-2012	10	0	0
FR	Calais-Lille	27-06-2012	15	0	0
FR	St. Quentin-Reims	27-06-2012	10	0	0
FR	Paris-Reims	09-06-2012	10	0	0
FR	Reims-Troyers	08-06-2012	10	0	0
FR	Bourges-Nevers	08-06-2012	10	0	0
FR	Auch-Toulouse	31-05-2012	9	0	0
DK	Kolding-Nyborg	04-07-2012	5	0	0
DK	Nyborg-Kopenhagen	04-07-2012	2	0	0
DE	Flensburg-Kappeln-Eckernfeld	04-07-2012	5	0	0
DE	Oldenburg i.H.-Hamburg	15-06-2012	15	0	0
DE	Lübeck-Rostock	15-06-2012	8	2	0
DE	Hannover-Kassel	05-07-2012	10	0	0
DE	Dortmund-Warburg	14-06-2012	10	0	0
DE	Köln-Aachener Bucht	28-06-2012	10	0	0
DE	Magdeburg-Halle	15-06-2012	10	0	0
			Σ175	2	0
UK	Edinburgh-Berwick	23-07-2013	6	2	0
UK	Newcastle-Harrogate	23-07-2013	8	2	1
UK	Harrogate-Newark	23-07-2013	2	1	0
UK	East Anglia (North)	22-07-2013	6	1	0
UK	Cambridge-Dover	22-07-2013	6	3	1
BE	Brüssel-Aachen	01-07-2013	10	2	2
FR	Calais-Lille	30-06-2013	10	2	1
FR	St. Quentin-Reims	30-06-2013	10	3	0
FR	Paris-Reims	24-06-2013	10	3	0
FR	Reims-Troyes	23-06-2013	10	1	0
FR	Bourges-Nevers	23-06-2013	10	1	0
SE	Malmö-Hörby	16-07-2013	15	0	0
DK	Kolding-Nyborg	15-07-2013	10	2	0
DK	Nyborg-Kopenhagen	15-07-2013	15	3	1
DE	Eckernfeld-Kiel-Oldenburg i.H	07-07-2013	10	4	0
DE	Oldenburg i.H.-Hamburg	07-07-2013	10	4	0
DE	Lübeck-Rostock	08-07-2013	10	9	0
DE	Rostock-Greifswald	08-07-2013	10	1	1
DE	Greifswald-Neubrandenburg	08-07-2013	10	7	0
DE	Hannover-Kassel	26-06-2013	10	4	2
DE	Dortmund-Warburg	07-07-2013	10	4	0
DE	Magdeburg-Halle	28-06-2013	10	3	0
PL	Görlitz-Breslau	05-07-2013	10	0	1

CZ	Velke-Brünn-Mikulor	03-07-2013	10	0	0
HU	Nickelsdorf-Györ-Sopron	03-07-2013	10	0	0
IT	Brescia-Verona- Alessandria	06-06-2013	10	0	0
			Σ 248	62	10
IE	Dunulk-Dublin-Carrickmacross	13-07-2014	10	0	5
UK	Edinburgh-Berwick	14-07-2014	10	0	4
UK	Newcastle-Harrogate	13-07-2014	10	0	3
UK	Harrogate-Newark	14-07-2014	10	0	2
UK	East Anglia (North)	14-07-2014	10	0	4
UK	Cambridge-Dover	14-07-2014	10	0	2
NL	Groningen-Appungedam-Winsch.	02-07-2014	10	0	1
BE	Brüssel-Aachen	22-06-2014	5	3	2
FR	Calais-Lille	21-06-2014	15	5	2
FR	St. Quentin-Reims	21-06-2014	10	8	1
FR	Paris-Reims	01-06-2014	9	8	1
FR	Reims-Troyes	31-05-2014	15	15	0
FR	Bourges-Nevers	31-05-2014	4	3	1
FR	Auch-Toulouse	17-05-2014	3	0	3
SE	Malmö-Hörby	03-07-2014	10	1	1
DK	Nyborg-Kopenhagen	03-07-2014	10	0	1
DE	Oldenburg i.H.-Hamburg	03-07-2014	10	5	1
DE	Lübeck-Rostock	25-06-2014	10	7	0
DE	Hannover-Kassel	17-06-2014	5	2	2
DE	Dortmund-Warburg	02-07-2014	15	11	2
DE	Magdeburg-Halle	17-06-2014	14	5	1
DE	Sinsheim-Crailsheim	24-05-2014	3	0	1
DE	Schweinfurt-Rothenburg	05-06-2014	9	1	1
PL	Görlitz-Breslau	27-06-2014	10	0	2
PL	Oppeln-Gleiwitz	27-06-2014	10	0	0
CZ	Velke-Brünn-Mikulor	13-06-2014	10	0	1
			Σ 247	74	44
UK	East Anglia (North)	12-07-2015	6	3	2
UK	Cambridge-Dover	12-07-2015	2	2	0
FR	Calais-Lille	28-06-2015	20	16	3
FR	St. Quentin-Reims	29-06-2015	10	6	2
FR	Paris-Reims	03-06-2015	20	17	2
FR	Reims-Troyes	02-06-2015	10	5	2
FR	St. Menehould-Metz-Saarbrücken	29-06-2015	10	8	0
FR	Bourges-Nevers	02-06-2015	20	15	1
SE	Malmö-Hörby	06-07-2015	10	1	3
DK	Nyborg-Kopenhagen	06-07-2015	15	1	2
DE	Oldenburg i.H.-Hamburg	20-06-2015	15	15	0
DE	Lübeck-Rostock	19-06-2015	10	9	1
DE	Hannover-Kassel	16-06-2015	15	12	1
DE	Magdeburg-Halle	16-06-2015	15	3	2
DE	Sinsheim-Crailsheim	28-06-2015	15	0	0
DE	Schweinfurt-Rothenburg	12-06-2015	15	0	0
DE	Niederbayern	04-07-2015	10	0	0
PL	Görlitz-Breslau	24-06-2015	15	0	2
HU	Nickelsdorf-Györ-Sopron	10-06-2015	5	0	0
IT	Brescia-Verona	16-05-2015	15	0	2
			Σ 253	113	25

Table 38: SDHI resistant *Z. tritici* isolates collected in the 'Random monitoring' and on trial sites in the years 2012-2016 in Europe. Internal- and external-ID (in most cases EpiLogic-ID), sampling country (region), date and information about the monitoring is given. Isolates were either obtained from trial sites of BASF SE (n=10) or Teagasc Institute (n=1) or from 'Random SDHI monitoring' (n=29) or 'Random DMI monitoring' (n=3).

Internal-ID	External-ID	Country	Region	Sampling Date	Monitoring
St 5548	St 12-150/4	FR	Nord-Pas de Calais	2012	Trial
St 5745	St 13-018/4	DE	Grevenbroich	2013	Trial
St 6027	St 15-082/3	FR	Picardie	2015	Random SDHI
St 6028	St 15-229/1	UK	Scotland	2015	Random SDHI
St 6029	St 15-336/2	IE	Leinster	2015	Trial
St 6030	St 15-338/1	IE	Leinster	2015	Trial
St 6031	St 15-338/2	IE	Leinster	2015	Trial
St 6032	St 15-339/1	IE	Leinster	2015	Trial
St 6033	St 15-339/2	IE	Leinster	2015	Trial
St 6034	St 15-339/3	IE	Leinster	2015	Trial
St 6035	St 15-345/1	IE	Munster	2015	Random SDHI
St 6036*	OP15.13	IE	Oak Parc	2015	Trial-Teagasc
St 6037	St 15-263/1	UK	South West	2015	Random SDHI
St 6038	St 15-328/10	IE	Leinster	2015	Trial
St 6039	St 15-394/2	IE	Northern Ireland	2015	Trial
St 6127	St 15-151/9	UK	East Midlands	2015	Random DMI
St 6149	St 15-330/3	IE	Leinster	2015	Random DMI
St 6156	St 15-331/8	IE	Leinster	2015	Random DMI
St 6407	St 16-005/2	UK	East Midlands	2016	Random SDHI
St 6409	St 16-096/1	UK	West Midlands	2016	Random SDHI
St 6410	St 16-167/1	IE	Leinster	2016	Random SDHI
St 6411	St 16-167/2	IE	Leinster	2016	Random SDHI
St 6412	St 16-167/3	IE	Leinster	2016	Random SDHI
St 6413	St 16-168/3	IE	Leinster	2016	Random SDHI
St 6414	St 16-174/1	IE	Leinster	2016	Random SDHI
St 6415	St 16-178/1	IE	Leinster	2016	Random SDHI
St 6416	St 16-179/2	IE	Leinster	2016	Random SDHI
St 6417	St 16-186/1	IE	Munster	2016	Random SDHI
St 6418	St 16-188/3	IE	Munster	2016	Random SDHI
St 6419	St 16-190/1	IE	Munster	2016	Random SDHI
St 6420	St 16-190/2	IE	Munster	2016	Random SDHI
St 6421	St 16-190/3	IE	Munster	2016	Random SDHI
St 6422	St 16-343/1	NL	Groningen	2016	Random SDHI
St 6423	St 16-345/1	NL	Groningen	2016	Random SDHI
St 6424	St 16-345/3	NL	Groningen	2016	Random SDHI
St 6425	St 16-347/2	NL	Flevoland	2016	Random SDHI
St 6426	St 16-347/3	NL	Flevoland	2016	Random SDHI
St 6427	St 16-348/1	NL	Groningen	2016	Random SDHI
St 6428	St 16-349/1	NL	Groningen	2016	Random SDHI
St 6429	St 16-349/2	NL	Groningen	2016	Random SDHI
St 6431	St 16-360/3	UK	East Midlands	2016	Random SDHI
St 6432	St 16-361/1	UK	South West	2016	Random SDHI
St 6433	St 16-361/2	UK	South West	2016	Random SDHI

***** St 6036 was kindly provided by Dr. S. Kildea (Teagasc Institute, Ireland)

Table 39: *P. teres* leaf samples collected in various European countries in the years 2014-2016.

Year	Country	No. of locations/ samples	Locations	Sampling date (no. of samples)
2014	BE	2/2	Bolinne, Mignault	June (2)
	BG	1/1	Letnitsa	July (1)
	CZ	3/3	Kromeriz, Rokytnice, Vestec by Chrudim	May (2) June (1)
	DE	27/56	Aurich, Bedburg, Beetzendorf, Bernburg, Borwere, Bühren, Buxheim, Dedelow, Dunau, Erding, Erlangen-Hüttendorf, Feistenaich, Gadegast, Grucking, Günzburg, Hamerstorf, Höckelheim, Köfering, Lippetal-Hüttinghausen, Mallersdorf, Manker, Oberboihingen, Ohrensen, Ostenfeld, Sachsen, Steinkimmen, Walkendorf	Apr (36) May (14) June (4) Nov (2)
	DK	14/21	Akirkeby, Allingabro, Dubgardvej, Flakkebjerg, Forsøgsmarken, Herning, Hobro, Middelfart, Nørager, Nr. Aby, Rønde, Sejet, Tølløse, Vivild	May (3) June (11) July (7)
	ES	3/3	Altorricón, Villamartin, Zambrana	May (3)
	FR	32/82	Amiens, Arc sur Tille, Beugnâtre, Bignan, Bucy Saint Liphard, Chavagnes, Chazeuil, Chouday, Coudres, Coulommes, Douchy les Ayette, Ecquetot, Freshnes l' Archeveque, Gibourne, La Bouexière, La Croix-en-Brie, La Veuve, Labergement-lès-Seurre, Loyat, Ludes, Magnicourt-en-Comte, Marchélepot, Montans, Neuville-au-Cornet, Patay, Saint-Léger-aux-Bois, Sery, Souchez, Trouhans, Vatan, Vouillé, Warmeriville	Apr (32) May (16) June (34)
	FI	9/20	Hanho, Jokioinen, Lammi, Lieto, Pernaja, Ruukki, Sarvilahti, Sotkamo, Ylistaro	July (20)
	IE	3/3	Blarney, Kildalton, Oak Park	June (2) July (1)
	IT	2/2	Conselice, Ozzano Dell' Emilia	Apr (1) May (1)
	LV	10/13	Auce, Jelgavas, Kurstsu, Lielauce, Penkule, Priekule, Priekuli, Sesava, Striki, Zebrene	June (9) July (4)
	NO	1/1	Meldal	Aug (1)
	PL	3/10	Łany Wielkie, Pagów, Sosnicowice	Apr (2) May (2) June (5) July (1)
	RO	1/2	Timis	Apr (2)
	SE	11/12	Evertsholm, Färjestaden, Forsa, Gotland, Hardeberga, Hassleholm, Högby, Kalmar, Kävlinge, Mörbylanga, Skälsund	June (3) July (9)
	UA	4/6	Korobochkino Tchugyev, Snigurivka, Terezene, Uzhnoukrainsk	May (1) June (5)
	UK	6/6	Bildeston, Dereham, Grantham, Oulton, Retford, St. Osyth	Mar (1) May (5)
2015	BE	5/5	Alleur, Graux, Lonzee, Mignault, Perwez	June (5)
	BG	2/8	Dobrovnitsa, Ognyanovo	June (8)
	CZ	4/4	Krasne Udoli, Oskorinek, Osoblaha, Rokytnice	May (2) June (1) July (1)
	DE	9/14	Bedburg, Bothkamp, Bühren, Gronai, Hingste, Klipphausen, Oberbohingen, Waldsee, Walkendorf	Apr (3) May (3) June (7)

				July (1)
	DK	7/16	Boris, Flakkebjerg, Haderslev, Hinnerup, Holstebro, Midtfalster, St. Heddinge	June (15) July (1)
	ES	5/5	Castilleja del Campo, Cerrado, Coronil, Vencillon, Villamartin	Jan (2) Apr (2) June (1)
	FI	5/10	Hauho, Inkoo, Jokioinen, Ruukki, Ylistaro	July (3) Aug (7)
	FR	41/115	Arc sur Tille, Arcay, Aussonce, Baudrieres, Bignan, Breal sous Montfort, Castelnau Destretefond, Chalons en Champagne, Chambley, Chazeuil, Chenay, Cherance, Chouday, Coinces, Eton, Flacey, Fontaine-Francaise, Framecourt, Frevin capelle, La Croix-en-Brie, La Veuve, Lagrave, Le Vieil Evreux, Leudeville, Mandeville, Marchélepot, Marliens, Milly-la-Foret, Montans, Montharville, Neuville Saint Vaast, Paudy, Pontfaverger, Reneve, Saint-Léger-aux-Bois, Saint Martin du Fouilloux, Saint Ouen D'Attez, Trouhans, Vouillé, Warmeriville	Apr (24) May (40) June (51)
	HU	2/3	Szekszard, Szentlörinckata	May (1) June (2)
	IE	3/5	Blarney, Oakpark, Wicklow	June (1) July (4)
	IT	5/5	Caluso, Castelnuovo Scrivia, Conselice, Lovolo di Albettone, Ozzano Dell' Emilia	May (4) June (1)
	LV	3/3	Auce, Eleja, Kursisi	May (1) June (1) July (1)
	PL	2/6	Lany Wielkie, Pagów	Apr (3) May (2) June (1)
	RO	2/5	Fantana, Sânpetru de Câmpie	June (5)
	SE	11/17	Astorp, Bjerby Öland, Borrby, Fötegården, Grimskullen, Hemmesdynge, Kastlösa Öland, Marstad, Öberga, Svalöv, Vickleby Öland	July (17)
	SK	1/1	Spisska Bela	June (1)
	UA	17/19	BezzabotovkaChernechaSloboda, Chornokintci, Dalnik, Danilivka, Korobochkino, lviv, Mazky, Miheya, Terezyne, Razdolnoe, Rivne, Sarata, Sharhorod, Snigurivka, Stryzhivka, Velikii	May (3) June (16)
	UK	15/16	Blagg, Bildeston, Bourton on the water, Bridgwater, Dereham, Euston, Great Barton, Little Weighton, Shotley, Stoke by Clare, St. Osyth, Thornham Magna, Naughton, Upleadon, Walkington	Apr (2) May (3) June (11)
2016	BE	2/2	Mignault, Perwez	May (2)
	BG	2/9	Kalugerovo, Letnitsa	Mar (1) Apr (8)
	CZ	1/1	Prerov	June (1)
	DE	8/20	Bedburg, Gronau, Hoya, Krauchenwies, Möglingen, Oberbohingen, Waldsee, Walkendorf	Apr (2) May (5) June (13)
	DK	18/20	Åbenrå, Bogense, Brønderslev, Flakkebjerg, Flynder, Hobro, Horsens, Kolding, Nykøbing F., Regstrup, Ringsted, Romo, Rønnede, Stensmark Grenå, Viborg, Vissenbjerg, Vojens	June (13) July (7)
	EE	1/1	Jogeva	Aug (1)

	ES	3/3	Monzon, Utrera, Villamartin	May (29) June (1)
	FI	6/10	Hanho, Inkoo, Jokioinen, Lieto, Ruukki, Ylistaro	Sept (10)
	FR	30/41	Allouagne, Baccon, Banteux, Blagny-sur-Vingeanne, Bligny les Beaune, Chouday, Coinces, Conde, Cuperly, Freshnes l' Archeveque, Grandville, La Croix-en-Brie, Louvigny, Marchélepot, Meuilley, Mignaloux-Beauvoir, Montans, Neuville-au-Cornet, Notre Dame d'Allencon, Quetigny, Réalcamp, Reneve, Saint Gregoire, Saint-Palais, St. Maclou de Folleville, Vatan, Vaudemange, Villettes, Yevres	April (3) May (27) June (11)
	IE	5/5	Carrigtwohill, Fermoy, Glanmire, Ovens, Scartbarry	June (4) July (1)
	IT	3/3	Conselice, Ozzano dell' Emilia, Voghera	May (3)
	LT	5/5	Akademija, Baisiogala, Kaunas, Radviliskis, Siauliai	Aug (5)
	LV	2/2	Peterlauki, Satiki	June (1) July (1)
	PL	7/10	Bydgoszcz, Grodkow, Jaroslawiec, Lobez, Sosnicowice, Sroda Wielkopolska	Apr (1) June (9)
	RO	1/3	Fantana	June (3)
	SE	8/10	Borrby, Engköping, Falköping, Grästorp, Hallstahammar, Saleby, Uppsala, Västerås	July (2) Aug (8)
	SK	4/4	Detva, Krupina, Maly Saris, Rimavska Sobota	May (1) June (2) July (1)
	UA	10/10	Busk, Dalnik, Dolsk, Hajvoron, Hrystynivka, Hybalivka, Korobochkino, Lyubar, Nastashka, Terezine	Apr (1) May (9)
	UK	16/21	Beverley, Bildeston, Bury St. Edmunds, Caythorpe Heath, Cherhill, Dereham, Fosote, Ipswhich, Kings Bromley, Newark-on-Trent, Ockbrook, Sevenhampton, Stoke by Clare, St. Osyth, Watton, Willingale	April (1) May (4) June (14) July (2)

Table 40: *Z. tritici* leaf samples collected in Ireland and the United Kingdom from commercial sites in the year 2015.

Country	No. of locations/ samples	Origin	Sampling Date
IE	21/24	Ballagh Hse, Blarney, Cahir, Carrigtohill, Castletownroche, Cruicetown, Daver, Delvin, Donerale, Martinstown, Middleton, Morganstown, Muchgrange, New Inn, Parsonstown, Portaferry, Reynoldstown, Riverstick, Salterstown, Scartbarry, Watergrasshill	Mar (24)
UK	11/13	Chudleigh, Dunsford, East Allington, Hurcott, Ideford, Isham, Ottery St. Mary, Payhembury, Powderham, Spaxton, Temple Bruer	Mar (1) Apr (12)

Table 41: SDHI sensitive and resistant isolates of *P. teres* used in sensitivity tests. All isolates were analysed in microtiter tests, underlined isolates were additionally used in sensitivity tests *in planta*.

Isolate-ID	Country	Region/Route	Year	SDHIres	QoIres	Origin
Pt 1020	UK	North Lophans	1998	no	no	Trial site
Pt 1022	UK	Swanessey	1998	no	no	Trial site
Pt 1522	UK	Harrogate-Newark	2004	no	no	Random QoI
Pt 1530	FR	Calais-Abbeville	2004	no	no	Random QoI
Pt 1682	DE	Hannover-Kassel	2012	no	F129L	Random QoI
Pt 1685	DE	Lübeck-Rostock	2012	no	F129L	Random QoI
Pt 1719	PL	Görlitz-Breslau	2013	no	no	Random SDHI
Pt 1720	UK	Cambridge-Dover	2013	no	no	Random SDHI
Pt 1722	UK	Newcastle-Harrogate	2013	no	no	Random SDHI
Pt 1724	DE	Hannover-Kassel	2013	no	no	Random SDHI
Pt 1725	DE	Hannover-Kassel	2013	no	no	Random SDHI
Pt 1727	BE	Brüssel-Aachen	2013	no	F129L	Random SDHI
Pt 1728	BE	Brüssel-Aachen	2013	no	no	Random SDHI
Pt 1732	FR	Calais-Lille	2013	no	F129L	Random SDHI
Pt 1735	DE	Rostock-Greifswald	2013	no	no	Random SDHI
Pt 1758	UK	East Anglia (North)	2014	no	no	Random SDHI
Pt 1830	DE	Dortmund-Warburg	2014	no	no	Random SDHI
Pt 1850	FR	Calais-Lille	2014	no	no	Random SDHI
Ptm 1	HU	unknown	unknown	no	no	CBS
Ptm 2	HU	unknown	unknown	no	no	CBS
Pt 1683	DE	unknown	2013	B-H277Y	na	Trial site
Pt 1686	DE	unknown	2013	B-H277Y	na	Trial site
Pt 1687	DE	unknown	2013	B-H277Y	F129L	Trial site
Pt 1710	FR	La Veuve	2013	B-H277Y	no	Trial site
Pt 1773	FR	Calais-Lille	2014	B-H277Y	F129L	Random SDHI
Pt 1852	FR	Paris-Reims	2014	B-H277Y	no	Random SDHI
Pt 1688	FR	La Veuve	2013	C-N75S	no	Trial site
Pt 1696	FR	La Veuve	2013	C-N75S	no	Trial site
Pt 1697	FR	La Veuve	2013	C-N75S	no	Trial site
Pt 1698	FR	La Veuve	2013	C-N75S	no	Trial site
Pt 1699	FR	La Veuve	2013	C-N75S	no	Trial site
Pt 1703	FR	Bouges-le-Chateau	2013	C-N75S	F129L	Trial site
Pt 1666	DE	unknown	2013	C-G79R	no	Trial site
Pt 1667	DE	unknown	2013	C-G79R	no	Trial site
Pt 1669	DE	unknown	2013	C-G79R	F129L	Trial site
Pt 1670	DE	unknown	2013	C-G79R	no	Trial site
Pt 1671	DE	unknown	2013	C-G79R	no	Trial site
Pt 1674	DE	unknown	2013	C-G79R	F129L	Trial site
Pt 1675	DE	unknown	2013	C-G79R	no	Trial site
Pt 1678	DE	unknown	2013	C-G79R	no	Trial site
Pt 1680	DE	unknown	2013	C-G79R	no	Trial site
Pt 1681	DE	unknown	2013	C-G79R	no	Trial site
Pt 1706	FR	Bouges-le-Chateau	2013	C-G79R	no	Trial site
Pt 1723	DE	Hannover-Kassel	2013	C-G79R	no	Random SDHI
Pt 1726	BE	Brüssel-Aachen	2013	C-G79R	no	Random SDHI
Pt 1734	DE	Lübeck-Rostock	2013	C-G79R	no	Random SDHI
Pt 1737	DE	Greifswald-Neubranden.	2013	C-G79R	no	Random SDHI
Pt 1762	BE	Brüssel-Aachen	2014	C-G79R	no	Random SDHI
Pt 1672	DE	unknown	2013	C-H134R	F129L	Trial site
Pt 1707	FR	Bouges-le-Chateau	2013	C-H134R	no	Trial site
Pt 1708	FR	Bouges-le-Chateau	2013	C-H134R	no	Trial site
Pt 1689	FR	Bouges-le-Chateau	2013	C-S135R	no	Trial site
Pt 1690	FR	Bouges-le-Chateau	2013	C-S135R	no	Trial site
Pt 1691	FR	Bouges-le-Chateau	2013	C-S135R	no	Trial site
Pt 1692	FR	Bouges-le-Chateau	2013	C-S135R	no	Trial site
Pt 1693	FR	Bouges-le-Chateau	2013	C-S135R	no	Trial site
Pt 1694	DE	Soest	2013	C-S135R	no	Trial site
Pt 1695	FR	La Veuve	2013	C-S135R	no	Trial site

Pt 1700	FR	Bouges-le-Chateau	2013	C-S135R	no	Trial site
Pt 1701	FR	Bouges-le-Chateau	2013	C-S135R	no	Trial site
Pt 1702	FR	Bouges-le-Chateau	2013	C-S135R	no	Trial site
Pt 1704	FR	Bouges-le-Chateau	2013	C-S135R	no	Trial site
Pt 1705	FR	Bouges-le-Chateau	2013	C-S135R	F129L	Trial site
Pt 1718	FR	Marchélepot	2013	C-S135R	F129L	Trial site
Pt 1731	FR	St. Quentin-Reims	2013	C-S135R	F129L	Random SDHI
Pt 1831	DE	Dortmund-Warburg	2014	C-S135R	F129L	Random SDHI
Pt 1721	UK	Harrogate-Newark	2013	D-D124E	F129L	Random SDHI
Pt 1730	FR	Bourges-Nevers	2013	D-D124N	F129L	Random SDHI
Pt 1738	DE	Greifswald-Neubranden.	2013	D-H134R	no	Random SDHI
Pt 1739	DE	Greifswald-Neubrandenburg	2013	D-H134R	no	Random SDHI
Pt 1766	BE	Brüssel-Aachen	2014	D-H134R	no	Random SDHI
Pt 1823	DE	Hannover-Kassel	2014	D-H134R	no	Random SDHI
Pt 1733	DE	Dortmund-Warburg	2013	D-D145G	no	Random SDHI
Pt 1736	DE	Rostock-Greifswald	2013	D-D145G	no	Random SDHI
Pt 1827	DE	Dortmund-Warburg	2014	D-D145G	no	Random SDHI
Pt 1835	DE	Dortmund-Warburg	2014	D-D145G	no	Random SDHI
Pt 1838	DE	Magdeburg-Halle	2014	D-D145G	no	Random SDHI
Pt 1801	FR	Bourges-Nevers	2014	D-E178K	F129L	Random SDHI
Pt 1802	FR	Bourges-Nevers	2014	D-E178K	F129L	Random SDHI

Table 42: Pairs of isolates used in competition studies on *P. teres* Isolates were mixed according to collection year (only isolates 2014 were included) and their sampling origin.

Isolate-ID	Country	Origin	Date	Resistance	Mixture	Fitness test
Pt 1819	DE	Lübeck-Rostock	2014	C-G79R	Mix 1	C-G79R against sensitive isolates
Pt 1844	DE	Sinsheim-Crailsheim	2014	no		
Pt 1787	FR	Paris-Reims	2014	C-G79R	Mix 2	
Pt 1785	FR	Paris-Reims	2014	no		
Pt 1765	BE	Brüssel-Aachen	2014	C-G79R	Mix 3	
Pt 1763	BE	Brüssel-Aachen	2014	no		
Pt 1819	DE	Lübeck-Rostock	2014	C-G79R	Mix 4	
Pt 1830	DE	Dortmund-Warburg	2014	no		
Pt 1853	FR	Paris-Reims	2014	C-G79R	Mix 5	
Pt 1747	UK	Edinburgh-Berwick	2014	no		
Pt 1742	IE	Dundulk-Dublin-Carrick.	2014	F129L	Mix 1	F129L against sensitive isolates
Pt 1746	UK	Edinburgh-Berwick	2014	no		
Pt 1771	FR	Calais-Lille	2014	F129L	Mix 2	
Pt 1804	FR	Auch-Toulouse	2014	no		
Pt 1749	UK	Edinburgh-Berwick	2014	F129L	Mix 3	
Pt 1755	UK	East Anglia (North)	2014	no		
Pt 1803	FR	Bourges-Nevers	2014	F129L	Mix 4	
Pt 1810	DE	Oldenburg i.H.-Hamburg	2014	no		
Pt 1748	UK	Edinburgh-Berwick	2014	F129L	Mix 5	
Pt 1830	DE	Dortmund-Warburg	2014	no		
Pt 1784	FR	Paris-Reims	2014	C-G79R+F129L	Mix 1	Double mutants against F129L
Pt 1803	FR	Bourges-Nevers	2014	F129L		
Pt 1790	FR	Reims-Troyes	2014	C-G79R+F129L	Mix 2	
Pt 1776	FR	St.Quentin-Reims	2014	F129L		
Pt 1784	FR	Paris-Reims	2014	C-G79R+F129L	Mix 3	
Pt 1748	UK	Edinburgh-Berwick	2014	F129L		
Pt 1782	FR	St.Quentin-Reims	2014	C-G79R+F129L	Mix 1	Double mutants against C-G79R
Pt 1853	FR	Paris-Reims	2014	C-G79R		
Pt 1770	FR	Calais-Lille	2014	C-G79R+F129L	Mix 2	
Pt 1765	BE	Brüssel-Aachen	2014	C-G79R		
Pt 1772	FR	Calais-Lille	2014	C-G79R+F129L	Mix 3	

Pt 1787	FR	Paris-Reims	2014	C-G79R		
Pt 1782	FR	St.Quentin-Reims	2014	C-G79R+F129L	Mix 1	Double mutants against sensitive isolates
Pt 1804	FR	Auch-Toulouse	2014	no		
Pt 1770	FR	Calais-Lille	2014	C-G79R+F129L	Mix 2	
Pt 1785	FR	Paris-Reims	2014	no		
Pt 1772	FR	Calais-Lille	2014	C-G79R+F129L	Mix 3	
Pt 1810	DE	Oldenburg i.H.-Hamburg	2014	no		
Pt 1790	FR	Reims-Troyes	2014	C-G79R+F129L	Mix 4	
Pt 1755	UK	East Anglia (North)	2014	no		

Table 43: SDHI sensitive and resistant isolates of *Z. tritici* used in sensitivity tests. All isolates were analysed in microtiter tests, underlined isolates were additionally used in sensitivity tests *in planta*.

Isolate-ID	Country	Region/Route	Year	SDHIres	QoIres	Origin
IPO323	NL	unknown	1981	no	no	reference
S27	UK	unknown	1994	no	no	reference
St 1965		unknown		no	no	
St 3573	IE	Crowleys Mallow	2003	no	G143A	Trial site
St 3718	DE	Böhl	2004	no	no	Trial site
St 4408	SE	unknown	2008	no	no	unknown
St 5821	IE	unknown	2011	no	F129L	unknown
St 6027	FR	Picardie	2015	B-T268I	G143A	Random
St 6032	IE	Leinster	2015	B-T268I	G143A	Trial site
St 6037	UK	South West	2015	B-T268I	G143A	Random
St 5548	FR	Agnieres	2012	C-T79N	G143A	Trial site
St 6030	IE	Leinster	2015	C-T79N	G143A	Trial site
St 6031	IE	Leinster	2015	C-T79N	G143A	Trial site
St 6033	IE	Leinster	2015	C-T79N	G143A	Trial site
St 6034	IE	Leinster	2015	C-T79N	G143A	Trial site
St 6038	IE	Leinster	2015	C-T79N	G143A	Trial site
St 5745	DE	Grevenbroich	2013	C-N86S	G143A	Trial site
St 6035	IE	Munster	2015	C-N86S	G143A	Random
St 6029	IE	Leinster	2015	C-H152R	G143A	Trial site
St 6036	IE	Oak Park	2015	C-H152R	G143A	Trial site
St 6028	UK	Scotland	2015	C-V166M	G143A	Random
Is1-55-3	parental isolate St 1965		2008	B-H267L	no	Lab mutants 2008 (previous studies)
B1E-7-4			2008	B-H267Y	no	
Fp-1-55-1			2008	B-I269V	no	
Mt-1-55-1			2008	C-N86K	no	
A0-13-5	parental isolate IPO323		2008	C-N86K	no	
A0-13-7			2008	C-G90R	no	
B3-6-18	parental isolate St 3718		2008	C-H152R	no	
B0-3-7			2008	C-H152R	no	
14-X10-3718-4	parental isolate St 3718		2014	C-H152R	no	Lab mutants 2014 (present study)
14-X10-323-7	parental isolate IPO323		2014	C-H152R	no	
14-X10-323-9			2014	C-H152R	no	
14-X10-3573-2	parental isolate St 3573		2014	C-H152R	G143A	
14-X10-3573-5			2014	C-H152R	G143A	
14-X10-5821-1	parental isolate St 5821		2014	C-H152R	F129L	
14-X10-5821-3			2014	C-H152R	F129L	

Table 44: Isolates and pairs of isolates used in competition studies on *Z. tritici* Isolates were mixed according to collection year, their sampling origin and resistance status towards QoIs and DMIs (field isolates).

Isolate-ID	Country	SDHIres	QoIres	CYP51 type	Mixture
St 5497	IE	no	G143A	R11	A
St 6027	FR	B-T268I	G143A	R11	
St 6059	UK	no	G143A	new 2015	B
St 6032	IE	B-T268I	G143A	new 2015	
St 5314	IE	no	no	R9	C
St 6029	IE	C-H152R	G143A	R9	
St 5497	IE	no	G143A	R11	D
St 6036	IE	C-H152R	G143A	R11	
St 5995	IE	no	G143A	R12	E
St 6033	IE	C-T79N	G143A	R12	
St 5497	IE	no	G143A	R11	F
St 6034	IE	C-T79N	G143A	R11	

Zusammenfassung

Phytopathogene Pilze wie *Pyrenophora teres* und *Zymoseptoria tritici* verursachen ertragsrelevante Krankheiten an Gerste und Weizen und sind in allen Getreide-anbaugebieten weltweit verbreitet. Die Bekämpfung der Netzfleckenkrankheit an Gerste, ausgelöst durch den Erreger *P. teres*, und der Septoria-Blattdürre an Weizen, ausgelöst durch den Erreger *Z. tritici*, wird größtenteils durch den Einsatz von Fungiziden gewährleistet. Dabei finden die drei „single-site"-Fungizid-Klassen der „Quinone-outside"-Inhibitoren (QoIs), der Demethylase-Inhibitoren (DMIs) und der Succinat-Dehydrogenase-Inhibitoren (SDHIs) den größten Einsatz. Durch die rasche Entwicklung der G143A-Substitution im Cytochrom bc_1-Komplex (CYTB) in der europäischen Population von *Z. tritici* haben QoIs bereits stark an Wirkung verloren. In der *P. teres* Population wurde die G143A bisher noch nicht entdeckt, dafür wurde der F129L-Austausch im CYTB nachgewiesen. Die F129L-Substitution löst ebenfalls eine QoI-Resistenz aus, jedoch in geringerem Maße als die G143A-Substitution. Zudem hat sich die Population von *Z. tritici* über viele Jahre hinweg gegenüber den DMI-Fungiziden angepasst, wodurch auch hier eine verringerte Feldwirkung dieser Fungizide in Westeuropa zu beobachten ist. Die SDHIs repräsentieren die neuste der drei Fungizid-Klassen und inhibieren die pilzliche Succinat-Dehydrogenase (SDH), die ein wichtiger Bestandteil der Atmungskette und des Citrat-Zyklus in Lebewesen darstellt. Die Entstehung von Fungizid-Resistenzen in den Pflanzenpathogenen *P. teres* und *Z. tritici* gegenüber der Klasse der SDHIs und deren Charakterisierung war Bestandteil der vorliegenden Arbeit. Isolate beider Pathogene wurden in großangelegten „Monitoring"-Studien gesammelt und umfassten alle wichtigen Gersten- und Weizenanbaugebiete Europas.

SDHI-resistente Isolate zeigten Punktmutationen in den Genen *SdhB*, *SdhC* und *SdhD*, die zu Aminosäure-Substitutionen in den SDH-B, SDH-C und SDH-D-Untereinheiten des SDH-Komplexes führen. Die ersten resistenten Isolate wurden im Jahr 2012 gesammelt und führten zu der Aminosäure-Substitution, Histidin zu Tyrosin an Position 277 der SDH-B Untereinheit in *P. teres* (B-H277Y) und Threonin zu Asparagin an Position 79 der SDH-C Untereinheit in *Z. tritici* (C-T79N). Im Falle von *P. teres* wurde ein starker Anstieg der resistenten Isolate in den folgenden Jahren hauptsächlich in Deutschland und in Frankreich festgestellt. Die detektierten Punktmutationen führten zu den Aminosäure-Substitutionen B-H277Y, C-S73P, C-N75S, C-G79R, C-H134R, C-S135R, D-D124N/E, D-H134R, D-G138V, D-D145G und D-E178K. Die Sequenzierung der resistenten Isolate zeigte, dass jedes Isolat nur einen Austausch in der SDH aufwies, nie jedoch zwei oder mehr Substitutionen in einem Isolat aufzufinden waren. *In vitro*- und *in planta*-Sensitivitätsstudien wurden durchgeführt und zeigten, dass jede Substitution einen spezifischen Einfluss auf

die Sensitivität der SDHIs hatte. Verschiedene SDHIs, die auf dem Markt erhältlich sind, wurden verglichen und es zeigte sich, dass sich alle SDHIs in Bezug auf die Resistenzstärke der einzelnen Mutationen ähnlich verhielten. Die meisten SDHI-resistenten Isolate von *P. teres* hatten den C-G79R-Austausch. Diese Substitution führte zu einem der stärksten Wirkungsverluste aller SDH-Varianten. Substitutionen, die einen schwächerem Wirkungsverlust der SDHIs aufwiesen, wie z.B. die C-N75S- und D-D145G-Substitution, wurden zusätzlich zur C-G79R häufig in der Feldpopulation gefunden. Die durchgeführten Gewächshausstudien deuten darauf hin, dass SDHI-Fungizide immer noch zu einer effektiven Bekämpfung der Netzfleckenkrankheit eingesetzt werden können, auch wenn der Anteil der resistenten Isolate in der Population hoch ist. Dies gilt vor allem für einen präventiven Fungizid-Einsatz und für die „schwach" und „moderat" angepassten Isolate, die Substitutionen wie z.B. B-H277Y, C-N75S, D-D124N und D-D145G aufweisen. Die SDHI-resistenten Isolate wurden auf das Vorkommen multipler Resistenzen zu QoI Fungiziden untersucht. Es zeigte sich, dass die C-G79R-Mutanten aus dem Jahr 2013 keine F129L-Substitution im CYTB aufwiesen, wohingegen die Anzahl der doppelt resistenten Isolate über die Beobachtungsjahre zunahm.

Im Vergleich zu dem raschen Aufkommen von SDHI-resistenten Isolaten von *P. teres* in Ländern, wie z.B. Deutschland und Frankreich, scheint die Entwicklung von SDHI-Resistenzen bei *Z. tritici* langsamer vonstattenzugehen. Bisher wurden nur wenige SDHI-resistente *Z. tritici* Isolate überhaupt gesammelt (42 resistente von 3431 untersuchten Isolaten, 1,2%). In Ländern wie Irland, Großbritannien und in den Niederlanden wurde auch bei *Z. tritici* ein Anstieg der resistenten Isolate beobachtet, jedoch nur in geringem Ausmaß. Die Aminosäure-Substitutionen B-N225I, B-T268I/A, C-N86S/A, C-T79N/I, C-W80S, C-H152R und C-V166M wurden in den resistenten Isolaten aus diesen und weiteren Ländern, wie Frankreich und Deutschland, über die Jahre gefunden. Vier Isolate wiesen sogar zwei Mutationen in zwei unterschiedlichen *Sdh*-Genen in einem Isolat auf. Die Punktmutationen führten hier zu den Aminosäure-Substitutionen B-R240L+C-T79N, B-T268I+C-I29V, B-T268A+C-F23S und C-H152R+D-R47W. Der höchste Wirkungsverlust aller SDHIs wurde bei Isolaten mit der C-H152R-Substitution beobachtet. Die am häufigsten gefundenen SDH-Varianten, C-T79N und C-N86S, wiesen dagegen deutlich geringere Wirkungsverluste, verglichen mit der C-H152R-Substitution, auf.

Vergleichende Pathogenitätsstudien mit einigen SDHI-resistenten und sensitiven Isolaten von *P. teres* und *Z. tritici* wurden im Gewächshaus durchgeführt um potenzielle Fitnessnachteile der SDHI-resistenten Mutanten zu untersuchen. Quantitative Detektionssysteme der SNPs, die zur SDHI-Resistenz führen, wurden entwickelt und genutzt, um die Zu- oder Abnahme der resistenten Allel-Varianten in Mischungen von resistenten und sensitiven Isolaten zu beobachten. Dabei wurden Gersten- und Weizen-Sämlinge mit den Isolat-Mischungen inokuliert und über mehrere Vermehrungszyklen ohne Fungizid-Einsatz propagiert. Feldisolate von *P. teres* wiesen eine hohe Variabilität der

„Fitness“ unabhängig von ihrem Resistenz-Status auf. Trotz der hohen Schwankungen war im Mittel aller Mischungen eine Abnahme der resistenten Allele zu beobachten. Bei *Z. tritici* wurden die Feldisolate auf das Vorkommen von multiplen Resistenzen hin untersucht und entsprechend mit SDHI-sensitiven Isolaten mit gleichem Resistenzhintergrund (mit Ausnahme der SDHI Resistenz) gemischt. Ein signifikanter Abfall der resistenten Allel-Varianten war im Falle der B-T268I- und der C-H152R-Substitution zu beobachten. Im Gegensatz dazu zeigten C-T79N-Mutanten eine hohe Pathogenität und es wurde keine Abnahme der Resistenz-Allele in diesen Mischungen detektiert. Die Ergebnisse deuten darauf hin, dass die Fitnessnachteile, ausgelöst durch verschiedene Mutationen, unterschiedlich stark sein können.

Es wurde gezeigt, dass beide phytopathogene Pilzarten eine hohe Anzahl an verschiedenen „Target-site“-Mutationen im Falle der SDHI-Resistenz entwickeln können und diese sich stark zwischen den beiden Pathogenen unterscheiden (mit der Ausnahme der C-N75S in *P. teres* und der homologen Substitution C-N86S in *Z. tritici*). Das kann mit der spezies-abhängigen Variation des SDH-Enzyms zusammenhängen, aber auch in der unterschiedlichen Biologie der Pathogene (z.B. Wirtpflanze, geographisches Vorkommen der Krankheiten) und einem unterschiedlichen Fungizid-Einsatz (z.B. Intensität in Gerste und Weizen) begründet liegen. Die Abwesenheit von einer dominanten „Target-site“-Mutation in beiden Pathogenen, wie z.B. im Falle der QoI-Resistenz durch G143A in *Z. tritici*, deutet darauf hin, dass SDHIs in der Zukunft immer noch effektiv zur Kontrolle beider Pflanzenkrankheiten eingesetzt werden können. Trotzdem ist ein Anti-Resistenz-Management für den Einsatz von SDHIs essentiell und sollte nicht nur auf Mischungen und Alternierung von Fungiziden beruhen, sondern auch integrierte Bekämpfungsstrategien stärker mit einbinden.

Danksagung

Ganz besonders danken möchte ich Ralf (Prof. Dr. Vögele) für die Unterstützung und die Möglichkeit zu promovieren und den vielen hilfreichen Gesprächen zu meinem Thema. Ich möchte dir auch dafür danken, dass du mich während meiner Diplomarbeit so freundlich in deine Arbeitsgruppe aufgenommen hast. Ich habe alle Phytomediziner der Universität Hohenheim (vor allem die abendlichen Grillfeste unserer Gruppe) oft vermisst in den letzten drei Jahren.

Mein herzlicher Dank gilt auch Gerd (Dr. Stammler) für die Möglichkeit, an interessanten und praxisrelevanten Projekten innerhalb der BASF mitzuarbeiten und für die intensive Betreuung während meiner Doktorarbeit. Vielen Dank auch für die herzliche Aufnahme in eine so nette, fleißige und hilfsbereite Arbeitsgruppe.

Des Weiteren möchte ich mich ganz herzlich bei den Europäischen Kollegen in der BASF bedanken, allen voran natürlich Rosie (Dr. Bryson) und Dieter (Herr Strobel) für die wichtigen Gespräche zu meinem Thema und die interessanten Einblicke in den Firmenalltag.

Vielen Dank auch an Herrn Prof. Dr. Philip Lane, Herrn Dr. Ulf Groeger, Herrn Dr. Horst-Dieter Brix und Herrn Dr. Karl-Heinz Lorenz für die Möglichkeit an zahlreichen Meetings und Tagungen teilnehmen zu können und dort auch meine Forschungsergebnisse präsentieren zu dürfen.

Entscheidend zum Gelingen dieser Arbeit beigetragen hat die hilfsbereite Atmosphäre in dem netten Labor-Team. Vielen Dank an Birgit, Simone, Angi, Gabi und Marcel für die tolle Zeit mit euch und dass ich mich auf euch immer verlassen konnte. Danke auch an Sarah, meine liebe Mit-Doktorandin, für die gemeinsame Zeit (inklusive gemeinsamem Bibbern vor Vorträgen) und dass du mir über den Alltag hinaus so eine gute Freundin geworden bist. Liebe Anna, danke auch dir für die gemeinsame Zeit, die zahlreichen Gespräche und deine geteilte Leidenschaft zur Forschung. Ich habe dich sehr gerne betreut während deiner Masterarbeit und wünsche dir für deine Doktorarbeit viel Kraft und gutes Gelingen. Bedanken möchte ich mich auch beim ganzen Labor-Team Koch, insbesondere bei Andreas (für hilfreiche Tipps bei den kniffligen Assays), Margit und Dagmar und dem Mykologen-Team Marina und Max für eure Hilfe bei allen möglichen Fragen. Vielen Dank, John (Dr. Speakman), auch für deine gründliche Korrektur meiner englischen Arbeit. Weiterhin möchte ich mich bei dem fleißigen Gewächshaus-Team für die Bereitstellung meiner Getreidepflanzen bedanken (Danke vor allem an Thomas und Paul) und bei Lissy (Frau Brill) für die Bereitstellung der Standard-Nährmedien. Vielen Dank auch an Iain (Dr.

Proctor) für die hilfreichen Tipps für die statistische Auswertung. Dank euch war die Arbeit so viel einfacher!

Zu guter Letzt gilt mein Dank meiner Familie und meinen Freunden, die mich jederzeit unterstützt und bekräftigt haben. Insbesondere danke ich meinen Eltern, Ingrid und Bernd, für den fortwährenden Glauben an mich und der finanziellen Unterstützung während meines Studiums und meinem Freund Matthias, der mich durch seinen Rückhalt und sein Verständnis in allen Abschnitten der Dissertation begleitet hat. Ohne euch wäre das alles nicht möglich gewesen! Danke auch an meine 89-jährige Oma, die immer an mich gedacht hat und oft nachgefragt hat wie die Doktorarbeit vorangeht und an Bea für ihre bedingungslose Freundschaft. Vielen Dank auch an meine kleinen Patenkinder, Johanna und Gabriel, und ihrer Mama und ihren Großeltern, Christina, Christa und Werner für die gemeinsame Zeit (auch wenn diese bisher zu kurz gekommen ist). Das ändern wir in Zukunft aber!

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig und ausschließlich unter Verwendung der angegebenen Quellen und Hilfsmittel verfasst habe.

Diese Arbeit wurde in dieser oder ähnlicher Form noch keiner Prüfungsbehörde vorgelegt.

Ort, Datum	Unterschrift

Lebenslauf

Zur Person

Name:	Rehfus
Vorname:	Alexandra
Geburtsdatum:	21.12.1985
Familienstand:	ledig

Schulausbildung

1992-1996:	Grundschule St. Bernhardt, Esslingen am Neckar
1996-2005:	Schelztor-Gymnasium, Esslingen am Neckar, Abschluss: allgemeine Hochschulreife (Note 2,5)

Akademische Ausbildung

10/2005-03/2012:	Studium der Agrarbiologie an der Universität Hohenheim, Stuttgart, Fachrichtung: Agrarbiotechnologie, Abschluss: Diplom Agrarbiologin (Note 1,4)
	Berufspraktikum bei der IdentXX GmbH in Stuttgart (09-11/2009)
	Berufspraktikum am Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) in Gatersleben (05-08/2011)
	Diplomarbeit am Institut für Phytomedizin, Universität Hohenheim, Thema: „Untersuchungen zur Pathogenese von *Erwinia amylovora* (Feuerbrand) an Apfel mittels quantitativer PCR“ (Note 1,0)
04/2012-12/2013:	Wissenschaftliche Mitarbeiterin am Institut für Phytomedizin, Universität Hohenheim, Thematik: „Transformation der obligat biotrophen Rostpilze *Uromyces fabae* und *Phakopsora pachyrhizi*“
02/2014-05/2017:	Doktorarbeit bei der BASF SE in Limburgerhof, Thema: „Analysis of the emerging situation of resistance to succinate dehydrogenase inhibitors in *Pyrenophora teres* and *Zymoseptoria tritici* in Europe”

Publikationen

Joos M, Rehfus A, Rex A, Buck S, Voegele RT (2012) Cultivar testing, pathogenesis and quantitative distinction of live and dead cells of *E. amylovora*. In: *Proceedings of the 15th International Conference on Organic Fruit-Growing*. Foerdergemeinschaft Oekologischer Obstbau e.V. (FOEKO), pp. 153-159.

Stammler G, Rehfus A, Prochnow J, Bryson R, Strobel D (2014) New findings on the development of insensitive isolates of *Pyrenophora teres* towards SDHI fungicides. *Julius-Kühn-Archiv* 447: 568.

Rehfus A, Miessner S, Achenbach J, Strobel D, Bryson R, Stammler G (2016) Emergence of succinate dehydrogenase inhibitor resistance of *Pyrenophora teres* in Europe. *Pest Management Science* 72: 1977-1988.

Rehfus A, Strobel D, Bryson B, Stammler G (2017) Sensitivity of *Pyrenophora teres* to succinate dehydrogenase inhibitors in Europe. In: Deising HB, Fraaije B, Mehl A, Oerke EC, Sierotzki H, Stammler G (eds.) *Modern Fungicides and Antifungal Compounds Vol. VIII*, Deutsche Phytomedizinische Gesellschaft (DPG), Braunschweig, Germany, pp. 175-180.

Rehfus A, Strobel D, Bryson B, Stammler G (accepted 2017) Mutations in *sdh* genes in field isolates of *Zymoseptoria tritici* and impact on the sensitivity to various succinate dehydrogenase inhibitors. *Plant Pathology* (accepted author manuscript doi:10.1111/ppa.12715).

Tagungen während der Dissertation:

03/2014:	Teilnahme an der Jahrestagung der DPG-Arbeitskreise Mykologie und Wirt-Parasit-Interaktion in Aachen mit Vortrag: „Impact of mutations in the *sdh* gene on the sensitivity to different SDH-inhibiting fungicides“
09/2014:	Teilnahme an der 59. Deutschen Pflanzenschutztagung in Freiburg, Posterbeitrag: „New findings on the development of insensitive isolates of *Pyrenophora teres* towards SDHI fungicides“
09/2015:	Teilnahme an der internationalen Resistance 2015-Tagung am Rothamsted Research in Harpenden (England) mit Posterbeitrag: „Adaption of *Pyrenophora teres* to SDHI fungicides in Europe“
04/2016:	Teilnahme am 18. Internationalen Reinhardsbrunn-Symposium in Friedrichroda mit Vortrag: „Sensitivity of *Pyrenophora teres* to succinate dehydrogenase inhibitors in Europe”

09/2016: Teilnahme an der 60. Deutschen Pflanzenschutztagung in Halle mit Vortrag: „Sensitivitätssituation von Getreidepathogenen in Europa gegenüber Succinat-Dehydrogenase Inhibitoren“

Ort, Datum, Unterschrift