Characterisation of metrafenone and succinate dehydrogenase inhibitor resistant isolates of the grapevine powdery mildew *Erysiphe necator*

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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.

a.i.	active ingredient
amp	ampicillin
bp	base pair
Cyp51	sterole 14α-demethylase cytochrome P450 gene
Cyt *b*	cytochrome *b* gene
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
ha	hectare
kb	kilo base pair
MDR	multidrug resistance
MFN	metrafenone
n	number
NTC	no-template control
PCR	polymerase chain reaction
QoI	uinone 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
TAE	tris-acetate-EDTA

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1. Introduction

1.1 The host grapevine

Cultivated grapevine, *Vitis vinifera* L. subsp. *vinifera* (syn: *Vitis vinifera* subsp. *sativa*) is a flowering, perennial plant, which was domesticated from wild populations of *Vitis vinivera* L. subsp. *sylvestris* (Levadoux 1956; Arroyo-Garcia *et al.* 2006). It has been cultivated over more than 6000 years and it is likely that it has been spread by humans from the geographical region of the Middle East to Central Europe. Only few plant species have such a long cultivation history and are additionally linked with religious and cultural activities (Terral *et al.* 2010). Nowadays, with around 7 million ha harvested area world-wide, and producing yearly a gross profit of 48 billion Euro (http://www.fao.org/faostat/en/#data; 2014), grapevine is the most widely cultivated and important fruit crop (Vivier and Pretorius 2002; Bouby *et al.* 2013). Almost half of the total area is located in Europe with over 3.2 million ha (http://www.fao.org/faostat/en/#data; 2014). Grapes can either be eaten as fresh table grapes, dried as raisins or can be processed to secondary products such as juice and vinegar or fermented to wines and after distillation to brandy (Bouby *et al.* 2013). The fruit is harvested once a year and the size as well as the organoleptic properties of the fruit is variable, strongly dependent on the cultivar and the purpose of the production. The species *V. vinifera* includes about 5000 cultivars (Vivier and Pretorius 2002), but the wine industry, that represents the most important production sector, use only relatively few, well established cultivars. The major part of these centuries-old elite varieties is maintained by a strict managed system of vegetative propagation (Bowers *et al.* 1999; Myles *et al.* 2011). Classical breeding approaches play so far a minor role for the improvement of elite cultivars because of a long life cycle, negative inbreeding traits and the maintenance of enological qualities (Gray and Dhekney 2014). Viticulture relies on the use of grafted plants, in which the rootstock is built by another species or interspecific hybrid resistant to soil-borne pests and pathogens and negative abiotic conditions. Successful cultivars of *V. vinifera*, which are often highly susceptible to the majority of pathogens are typically used as scion (Mullins *et al*. 1992; Vivier and Pretorius 2002). As such, this sets the stages for plant health problems during the vegetative period, since several pests can cause damages on nearly every green tissue, especially, leaves, flowers and fruits. Diseases on grapevine can be caused by fungal pathogens, bacteria, viruses, nematodes and insects. The most problematic and important fungal diseases in viticultural regions are powdery mildew (*Erysiphe necator*), downy mildew (*Plasmopara viticola*), bunch rot (*Botrytis cinerea*) and black rot (*Guignardia bidwellii*). The development of the different diseases is strongly dependent on the weather conditions and is in general endemic. Long periods of wet weather and mild temperatures favour bunch rot and downy mildew infection, whereas powdery mildew

infection is favoured by dry, warm and windy conditions. In regions favourable for any particular disease, epidemics can occur with losses of up to 80%, depending on the fungal pathogen (Pearson and Goheen 1988). Therefore, modern grapevine production relies on integrated crop protection practices.

1.2 The pathogen *Erysiphe necator*

The Ascomycetes family of the Erysiphaceae is commonly known as powdery mildew fungi. They share an obligate biotrophic life style that is characterised by the penetration of living epidermal cells of the host plant and the formation of haustoria to absorb nutrients (Glawe 2008; Gadoury *et al.* 2012a). The superficial mycelium produces profuse numbers of conidia, that leads to the characteristic whitish-grey, dusty or powdery appearance of the diseased plants (Figure 1) and explains the common name that is usually given to this group of plant pathogens (Pearson and Goheen 1988; Glawe 2008).

Figure 1: Representative symptoms and signs of different powdery mildew fungi on their host. A: *Blumeria graminis* f.sp. *tritici* on wheat B: *Podosphaera leucotricha* on apple leaves C: *Podosphaera xanthii* on cucurbit (source: BASF picture pool).

They can infect a wide range of agricultural crops. In addition to pome fruits, cereals and many ornamentals, grapevine is an economically important host plant of powdery mildews.

1.2.1 Biology and ecological relevance

The causal agent of grape powdery mildew, *Erysiphe necator* Schw. (syn. *Uncinula necator* (Schw.) Burill; anamorph *Oidium tuckeri* Berk.) is a wide spread plant pathogen in viticultural areas world-wide. The pathogen was introduced from North-East America into Europe and the western part of the United States (Gadoury *et al.* 2012a). The first occurrence of *E. necator* in Europe was reported in England in 1845, but the disease was first noted two years later in France where it caused devastating losses (Pearson and Goheen 1988). The host range of the pathogen is restricted to genera within the Vitaceae, although grapevine (*Vitis vinifera*) is the most economically important host. The importance of the crop, its susceptibility to the

disease and the infection of all green tissues, makes this disease and its effective management a main objective in all grape growing areas (Leinhos *et al.* 1997; Gadoury *et al.* 2012a).

Depending on the climatic region, there are two different overwintering structures. The major overwintering form is the chasmothecium (formerly cleistothecium), which results from sexual reproduction in the autumn (Figure 2). Originating from the fusion of hyphae of opposite mating types, chasmothecia can be formed on the surface of all infected parts of the host (Pearson and Goheen 1988). Because of the accumulation of a pigmented lipid, the chasmothecia change from yellow to dark brown in the maturation process and form appendages with characteristic crooks. During rain events, mature chasmothecia are dispersed to the bark or on the soil in late summer and overwinter in bark crevices (Gadoury and Pearson 1988; Gadoury *et al.* 2012a, b). In spring, between bud break and bloom, the release of sexual ascospores from chasmothecia is induced by rain, irrigation or fog (Gadoury and Pearson 1990a). In contrast to the discharge of the ascospores, the requirements for germination and infection are not dependent on high wetness (Gadoury and Pearson 1990 a, b). Initial infections are generally found on the lower surface of young leaves in close proximity to the bark (Pearson and Gadoury 1987).

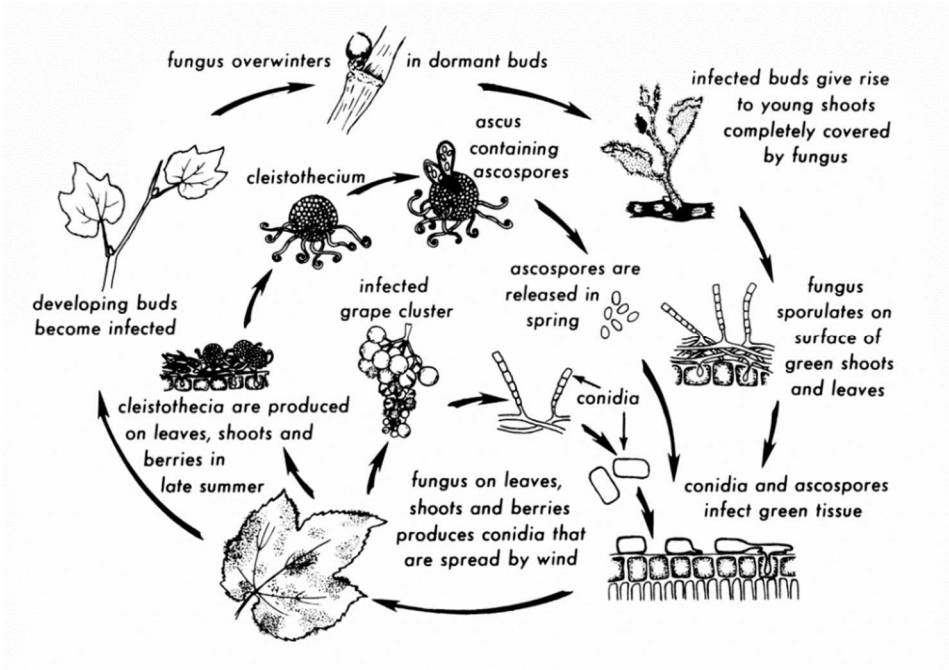


Figure 2: Disease cycle of grape powdery mildew *E. necator*. (Source: Pearson and Goheen 1988).

In areas with mild winters, like California, South Australia or South Europe, the development of the epidemic is more complex. Ascocarps can also survive within leaf litter on the vineyards floor and it is known that ascospore release can occur several weeks before bud break, leading to an elongated disease cycle (Cortesi *et al.* 1997; Magaray *et al.* 1997). Furthermore, dormant infected lateral buds can serve as additional overwintering source, which give rise to young

shoots completely covered with the fungus in the following spring (so called flag shoots, Hill 1990; Sall and Wyrsinski 1982). In these regions, the primary inoculum is based on ascospores and intense conidial production from flag shoots. The germination process of ascospores as well as conidia is initiated by the formation of a single germ tube, which terminates in a multilobed primary appressorium. From its lower surface, a hypha penetrates the cuticle and the epidermal cell wall and forms a globose haustorium that absorbs nutrients from the host (Pearson and Goheen 1988; Gadoury *et al.* 2012 a, b). After branching of hyaline, superficial hyphae and the formation of hyphal appressoria in regular intervals, multiseptate conidiophores are formed (Figure 3A). Depending on the weather conditions, they are formed usually five days after haustoria formation and arise from vegetative hyphae perpendicular to the host epidermis (Leinhos *et al.* 1997; Glawe 2008). Each conidiophore produces a chain of single cylindo-ovoid conidia with the oldest at the distal end. However, long conidial chains are rarely seen on infected plant material in the field due to release of mature conidiospores (Leinhos *et al.* 1997; Gadoury *et al.* 2012a). Resulting mildew colonies are capable to produce vast numbers of asexual conidia on a daily basis. Conidial infection is possible at a broad temperature range throughout the season, leading to multiple infection cycles relatively independent of moisture events. The occurrence of free water on plant surfaces is detrimental for conidial germination and may lead to bursting of conidia. Favourable conditions for strong powdery mildew infections are moderate temperatures, limited direct sunlight and high humidity without wetness (Pearson and Goheen 1988; Gadoury *et al.* 2012b).

Figure 3: *E. necator* infections on grapevine. A: Conidiophores with mature conidia 7 days after inoculation B: Infected grapevine leaves (variety Blaufränkisch) C: Strongly infected fruits with the typical berry split (variety Blaufränkisch).

Young vine leaves are most susceptible to *E. necator* infection, and although the sensitivity of the leaves decreases with age, mature leaves never become completely immune and can also become heavily infected (Figure 3B, Doster and Schnathorst 1985; Develey-Rivière and Galiana 2007). The highest susceptibility phase for powdery mildew infection is the period of bud break, bloom and fructification. For highly susceptible *V. vinifera* cultivars berries can be

easily infected 1-2 weeks after they have set, which is also dependent on the sugar content of the berries (Pearson and Goheen 1988; Gadoury *et al.* 2003; Ficke *et al.* 2004). The typical berry split is caused by the infection of berries in early stages leading to stunted berry epidermal cell growth while the pulp simultaneously continues to expand and forces the splitting (Figure 3C). Beyond yield losses that can be caused by strong infections of fruit clusters, even berries with small infection quantities can make the harvest unmarketable. Especially in the production of wine, the high enological demands can be changed by small infections rates. Even a disease level of 3 - 5% can lead to an off-flavour and are categorised as useless for wine production (Pearson and Goheen 1988; The Australian Wine Research Institute 2010).

1.2.2 Control of *E. necator*

Grapevine powdery mildew control can be achieved by using resistant varieties and proper vineyard management, but mainly relies on the protection of the plant with fungicides. Protection programmes that combine these techniques are usually defined as integrated disease management programmes (Leinhos *et al.* 1997; Gadoury *et al.* 2012a). During the dormant season, the level of primary ascospore infection in the following spring can be reduced by removing infected leaves from the vineyard ground. Prophylactic techniques to alter the position or amount of leaves, shoots and fruits are commonly defined as canopy management. It is used to improve wine quality, harvesting and to reduce disease pressure (Pearson and Goheen 1988). Temperature, humidity, wind and evaporation alter the significance of fungal diseases. An open canopy enables good air circulation, sun exposure and prevents shade maintaining a microclimate less favourable for powdery mildew infection (Travis *et al.* 1987; Smart *et al.* 1990). In addition, open canopies also contribute to more efficient applications of fungicides by enabling a more precise wetting and distribution of the substances on the plant tissue (Pearson and Goheen 1988; Smart *et al.* 1990; Basu 2014).

Less than 10 years after the first occurrence of *E. necator* in Europe, in 1854, sulphur was the first chemical control agent used for grape powdery mildew control. Because of its efficacy and low cost, sulphur is still frequently applied as a dust or wettable powder in various formulations (Pearson and Goheen 1988). Especially in organic viticulture, where the number of available solutions is low, it is an essential element for grape powdery mildew control. With the development of synthetic chemistry based fungicides, the number of active ingredients (a.i.), which allow the control of *E. necator* has dramatically increased. The classification is made by the Fungicide Resistance Action Committee (FRAC), an expert consortium, made up of representatives of the manufacturers. The classification of modern fungicides is done with respect to their biochemical target or mode of action in the biosynthetic pathways of plant

pathogens (FRAC 2017; www.frac.info). The groupings are made according to inhibited metabolic processes, host plant defence inducers and molecules with an unknown mode of action. The major fungicide groups include inhibitors of mitochondrial respiration chain, sterol biosynthesis, signal transduction pathways or act as multisite inhibitors. Some fungicides, like the aryl-phenyl ketones and quinoxyfen, have a more narrow activity spectrum restricted to powdery mildews. The most important fungicides used for powdery mildew control in grapevine are shown in Table 1.

Table 1: Overview of important fungicide groups used for powdery mildew control in grapevine (Source: FRAC 2017, www.frac.info).

Mode of Action	Group name	Common name	Target
Fungal respiration	SDHIs (succinate dehydrogenase inhibitors)	Boscalid Fluopyram Fluxapyroxad	Block the mitochondrial respiration chain and tricarboxylic cycle by inhibition the complex II (succinate dehydrogenase)
	QoIs (quinone outside inhibitors)	Kresoxim-methyl Trifloxystrobin Pyraclostrobin	Inhibit the complex III / bc1 complex at the Quinone outside site (cytochrome *b*) and therefore block the mitochondrial respiration
SBI (sterol biosynthesis inhibitors)	Class I (DMIs; demethylation inhibitors)	Difenoconazol Tebuconazol Myclobutanil Penconazol Tetraconazole	Prevent C14 demethylation step in fungal sterol biosynthesis by inhibiting C14 demethylase (erg11 / cyp51)
Signal transduction	Azanaphthalenes	Quinoxyfen	Mechanism unknown
Unknown	Aryl-phenyl-ketones	Metrafenone Pyriofenone	Actin disruption (proposed)
	Phenyl-acetamides	Cyflufenamid	Mechanism unknown
Multisite	inorganic	Sulphur	Multiple targets in the mitochondrial respiration chain

Since the early 2000s, promising attempts to protect plants from powdery mildews with biological organisms have been made. The activity of these organisms is mainly based on competition for space and nutrients as well as production of natural mycotoxic metabolites. Several organisms, such as *Pseudozyma aphidis* (Gafni *et al.* 2015), *Bacillus subtilis* (Maachia

et al. 2015) or *B. amyloliquefaciens* (Li *et al.* 2014) are used. Nevertheless, their use in practice remains limited because of their lower activity compared to classical fungicides. Therefore, the control of powdery mildew is still generally based on the use of chemical substances.

Because of the susceptibility of leaves and berries, fungicide applications targeting the control of grapevine powdery mildew are performed during nearly the whole vegetative period. The number of applications varies greatly between grape growing regions and it also depends on the history of the vineyard, the seasonal disease pressure and on the strength and persistence of the fungicides used. For example, in Germany is a region with a moderate powdery mildew disease pressure, protection with fungicides is recommended to start as early as the development stages BBCH 13-15 (Lorenz *et al.* 1995) depending on initial disease pressure. Subsequent applications are generally made with 10 to 14 day spray intervals, depending on the weather conditions. The average number of applications is around eight and typically ends at the beginning of ripening or BBCH 81. Organic growers typically use mainly sulphur at short spray intervals of 6-8 days leading to 12-15 applications on average per season (Personal communication: Lydia Ludwig, Siegfried Doerr, BASF SE Germany). In contrast to Germany, the optimal weather conditions for powdery mildew infection in Hungary regularly results in high disease pressure. Protection with fungicides starts at BBCH 14 - 18 and applications are made on a 10 to 14 day spray interval with an average of eight applications per season. The application period for powdery mildew control in grapevine ends generally between BBCH 81 and 83. In organic vineyards, mainly sulphur is used at short spray intervals (Personal communication: Peter Hoffmann, BASF SE Hungary). The number of applications and the disease pressure are highly variable between different growing regions. In conclusion, various climatic conditions and crop protection practices lead to a diverse intensity in the use of fungicides in Europe.

1.3 Fungicide resistance

Like all organisms, fungal populations underlie the principles of natural selection and therefore are constantly evolving in response to the changes of their environment (Hollomon 2015). The application with a fungicide, is a major change in the environment of the fungus and imposes a selection pressure on the population. As fungal genomes are plastic and may contain vast numbers of polymorphisms (Cuomo *et al.* 2007), spontaneous, neutral or even disadvantageous genotypical changes in single individuals could provide a benefit compared to the initial population, when fungicides are applied. These individuals possess a heritable reduction in the sensitivity to a specific anti-fungal agent, termed as fungicide resistance (Brent and Hollomon 2007a; Hollomon 2015). The risk for development of fungicide resistance is accelerated if the organism has a short life cycle and produces large numbers of progeny

(Brent and Hollomon 2007a). Powdery mildews have a high reproductive frequency with multiple life cycles per season, each producing vast numbers of conidia, which leads to a classification as moderate to high risk pathogens regarding the development of fungicide resistance (FRAC 2017, www.frac.info). The important need to control fungal diseases to prevent yield losses, leads to a frequent use of these compounds. The risk for the development of fungicide resistance is not only dependent on the intrinsic risk of the pathogen but also the fungicide used (Brent and Hollomon 2007a). Fungicide associated risk is dependent on the chemical class to which the fungicide belongs. Resistance towards fungicides with a multisite mode of action is very unlikely to occur. This is because multisite fungicides inhibit various biochemical steps in the fungal cell. For example, fungicides based on sulphur, where resistance has not been observed, are classified to have a very low risk for the development of fungicide resistance. In contrast, most modern fungicides have one biochemical target and act as single site inhibitors, where a single mutation in the target gene can easily affect the sensitivity to a specific compound and cause high levels of resistance. Therefore, most of them are considered to have a medium to high risk for the development of resistance (Brent and Hollomon 2007b). As all compounds of one fungicide group share the same biochemical target, a specific resistance mechanism emerging for one member, normally leads to a reduced sensitivity towards all members of this fungicide class but not to other mode of actions. This phenomenon is termed as cross-resistance and the knowledge about the cross-resistance pattern between members of one fungicide class is an essential part of fungicide resistance management (Brent and Hollomon 2007a; Hollomon 2015). For most single site fungicide classes used in practice, resistance has already emerged or the intrinsic risk for the development of resistance is high. Knowledge about the sensitivity situation of a fungal population is important to ensure a sustainable use of crop protection compounds. Knowledge is created in sensitivity monitoring programs where samples from different countries are analysed and classified regarding their sensitivity towards a specific fungicide. In Europe, after ratification of the last directive on registration and use of plant protection products (Regulation 1107/2009), the monitoring of the sensitivity of plant protection products became a mandatory requirement for crop protection companies (EPPO 2017; www.eppo.int).

1.3.1 Molecular mechanisms leading fungicide resistance

Different molecular mechanisms can lead to a reduced sensitivity towards fungicides. Besides an overexpression of the target itself and an enhanced efflux of the compound, an altered target site, which reduces the binding of the fungicide, can cause resistance. The overexpression of the target gene 14a-demethylase gene (*cyp51*), the target of DMI fungicides, is described for several pathogens (Cools *et al.* 2012; Ma and Michailides 2005). A reduced sensitivity is shown for the enhanced efflux of several unrelated chemical compounds, which

is caused by the overexpression of membrane efflux transporter genes in *B. cinerea* and *Zymoseptoria tritici* (Kretschmer *et al.* 2009; Leroch *et al.* 2011; Walker *et al.* 2012). Nevertheless, most cases of fungicide resistance are due to mutations in the target gene of the fungicide. For example, high resistance levels towards compounds targeting the complex III of the mitochondrial respiratory chain, also known as QoIs, can be caused by an amino acid exchange from glycine to alanine at position 143 (G143A) in the cytochrome *b* gene (Fraaije *et al.* 2002; Gisi *et al.* 2002). Besides this common mutation, which is widely spread and known for many plant pathogenic fungi, two other mutations (F129L and G137R) are known and cause low to moderate resistance (Sierotzki 2015). In general, depending on the fungal pathogen and the fungicide group, various single mutations can emerge in the same target gene at different positions. Accordingly, not only one specific mutation in the target gene can cause a reduced sensitivity to a fungicide class. Compounds with the same mode of action may interact with a certain mutation to slightly different degrees. In other words, a specific change in the target site may have a different impact on different members of one fungicide class that, in turn, lead to a more complex cross-resistance pattern. This is known for DMIs, where a single mutation or a combination of different target site mutations leads to different cross resistance patterns depending on the involved azole (Stammler *et al*. 2008; Cools *et al.* 2011; Leroux and Walker 2011).

In general, two scenarios have been described as to how fungicide resistance develops in a fungal population. A qualitative (monogenic or discrete) resistance development is in most cases the result of a single target site mutation. After fungicide application, initial resistant isolates are selected and become a predominant part of the population (Hobbelen *et al.* 2014). Sensitive and resistant individuals differ strongly in their response to the active ingredient, which leads to a distinct split in the population and a sudden loss of the effectiveness of the respective fungicide (Brent and Hollomon 2007a). In contrast, quantitative (polygenic or multistep) fungicide resistance development leads to a gradual shift or loss of sensitivity, which is in most cases caused by an accumulation of mutations in multiple genes (Angelini *et al.* 2015). The gradual loss of sensitivity in a fungal population is thereby characterised by a stepwise decrease of the efficacy of an active ingredient (Brent and Hollomon 2007a).

1.4 Metrafenone

The fungicide metrafenone (3-bromo-2´,3´,4´,6-tetramethoxy-2,6´-dimethyl-benzophenone), belongs to the chemical class of the aryl-phenyl-ketones (Köhle *et al.* 2004). The exact biochemical mode of action and the molecular target of these compounds is not yet fully elucidated (Köhle *et al.* 2004; Stammler *et al*. 2014). With metrafenone released in 2004 for cereals followed by pyriofenone in 2010, only two members of this fungicide class are

available. As benzophenone derived compounds, they share a basic benzophenone skeleton based on two benzene rings (Wu *et al.* 2014). Metrafenone is registered for the control of powdery mildews in several crops, such as cereals, grapevine and cucurbits, eye spot (*Oculimacula* spp.) in cereals and also for the control of *Dactylium dendroides* in mushroom production. The unique active ingredient had not been used previously in chemical plant protection and seems to differ from currently known mechanisms. No cross resistance could be observed to other commercial fungicide classes (Köhle *et al.* 2004; Opalski *et al.* 2006). Many efforts were made to identify the target site of metrafenone by investigating morphological changes induced by the active ingredient using microscopical methods (Köhle *et al.* 2004; Schmitt *et al.* 2006; Opalski *et al.* 2006). Morphological changes caused by fungicide treatment, such as altered growth or abnormal infection structures, were investigated to give evidence about the molecular site of action. The effect of metrafenone on the development of wheat (*Blumeria graminis* f.sp. *tritici*) and barley (*B. graminis* f.sp. *hordei*) powdery mildew during the infection was assessed (Opalski *et al.* 2006). The results of studies on the powdery mildews *E. necator* on grape and *E. pisi* on pea showed very similar morphological changes after metrafenone treatment (Schmitt *et al.* 2006). Multiple effects on asexual sporulation, vegetative growth and on the differentiation of infection structures were caused by metrafenone treatment. Morphogenetic abnormalities like malformed and multilobed appressoria, aberrant conidiophores and bursting hyphal tips led to the suggestion, that the potential target is involved in hyphal morphogenesis, polarised hyphal growth and the establishment and maintenance of cell polarity (Opalski *et al.* 2006). Hence, up to now detailed and precise information about the molecular mode of action of metrafenone is still missing.

First isolates of *B. graminis* f.sp. *tritici* with a reduced sensitivity towards metrafenone were observed in 2009 during BASF monitoring studies. These isolates were assigned into two phenotypic groups: moderately adapted and resistant. The moderately adapted isolates had a reduced sensitivity but could be controlled with registered rates of metrafenone. Isolates assigned as resistant were observed at low frequencies in the population and were not fully inhibited even with the full field rate of metrafenone (Felsenstein *et al.* 2010). Recently, first resistances were observed in field populations of *E. necator* on grapevine (Stammler *et al*. 2014; Kunova *et al.* 2016). The effectiveness of both, metrafenone and pyriofenone is similarly affected by resistant isolates, confirming that they are cross resistant (Kunova *et al.* 2016).

1.5 Succinate dehydrogenase inhibitors (SDHIs)

Although the first SDHIs were launched almost 50 years ago, with carboxin, oxycarboxin and several other compounds, the activity of these substances was limited to Basidiomycetes (Stammler *et al.* 2015). The first SDHI with a broader activity spectrum, which included important Ascomycetes, was boscalid. In addition to pathogens of arable crops, boscalid has an activity against various pathogens of fruits, vegetables and grapes (Stammler *et al.* 2007). With boscalid as starting point, SDHIs became one of the fastest growing fungicide classes due to their unique mode and site of action, effectiveness at low rates and broad activity spectrum (Sierotzki and Scalliet 2013; Stammler *et al.* 2015). Many new compounds, such as bixafen, fluopyram, fluxapyroxad and penthiopyrad, have now been developed and classified as new generation SDHI fungicides. Three different active ingredients, which target the succinate dehydrogenase, are currently registered for the control of powdery mildew in grapevine in Europe: boscalid, fluopyram and fluxapyroxad. Boscalid was introduced in 2003, fluopyram in 2011 and fluxapyroxad was registered in grapes in 2017.

The target of these molecules is the succinate dehydrogenase enzyme (SDH, synonym succinate ubiquinone oxidoreductase (SQR) or complex II). As an essential part of the mitochondrial respiration chain and tricarboxylic acid cycle, the SDH mediates the oxidation of succinate to fumarate by the reduction of ubiquinone to ubiquinol. The SDH consists of four nuclear encoded subunits: the flavoprotein SDH-A, which mediates the oxidation from succinate to fumarate, SDH-B whose iron-sulphur clusters transfer electrons to ubiquinone, and SDH-C and SDH-D with a heme b group between two antiparallel helices. SDH-A and SDH-B are located in the mitochondrial matrix, whereas SDH-C and D anchor the enzyme in the membrane (Ömura and Shiomi 2007; Glättli *et al.* 2011). The binding site for ubiquinone (Q-site) is formed by amino acid residues of the subunits SDH-B, -C and -D, which are highly conserved over many bacterial as well as eukaryotic species (Yankovskaya *et al.* 2003; Sun *et al.* 2005; Horsefield *et al.* 2006). By inhibiting the Q-site, SDHI-fungicides directly interrupt the electron transfer from the iron sulphur cluster to ubiquinone (Keon *et al*, 1991; Hägerhäll 1997; Horsefield *et al*, 2006).

The broader activity spectrum and the multitude of new SDHIs was accompanied by an increase of the structural complexity. All SDHIs share two common structural features, which are essential for fungicidal activity: The central amide group and the aromatic ring in the aniline part of the molecule. The first part ensures interactions via hydrogen-bonds whereas the latter mediates hydrophobic contacts or π – π interactions with the ubiquinone binding site of the SDH. Structure alignments as well as docking experiments indicate an identical binding mechanism for the different SDHIs and show a deeper binding compared to ubiquinone (Glättli *et al.* 2009; Glättli *et al.* 2011). Amino acids involved in the binding of ubiquinone, are predicted

to be in direct interaction with the different SDHIs. The amino acid residues tryptophan at position 224 in subunit B (*Z. tritici* numbering), serine at position 83 in subunit C and tyrosine at position 130 in subunit D are highly conserved between bacteria and eukaryotes (Horsefield *et al.* 2006; Sun *et al.* 2005; Yankovskaya *et al.* 2003). This also applies to arginine at position 87 in subunit C, which interacts with the aromatic ring of SDHIs, and the histidine 267, which may interact via an H-bond (Glättli et al. 2009; Sierotzki and Scalliet 2013).

Resistance towards SDHIs is known since the 1970s (Abiko *et al.* 1977; Ben-Yephet *et al.* 1975), which was caused by single-site mutations in the *sdh*-genes (Matsson *et al.* 1998; Skinner *et al.* 1998). Many different mutations at various positions in the SDH subunits B, C and D of plant pathogenic fungi from different crops have been identified. The mutations were found in field isolates of *B. cinerea* (Stammler *et al.* 2007; Veloukas *et al.* 2011), *Corynespora cassiicola* (Miyamoto *et al.* 2010a), *Alternaria alternata* (Avenot und Michailides, 2007), *A. solani* (Miles *et al.* 2014), *Pyrenophora teres* (Rehfus *et al.* 2016) and *Podosphaera xanthii* (Miyamoto *et al.* 2010b). Different mutations can occur at one position, such as the most common amino acid exchange from histidine to arginine, phenylalanine, tyrosine or leucine in subunit B (Glättli *et al*. 2009; Stammler *et al*. 2011; Sierotzki and Scalliet 2013). The amino acid sequence of the different SDH subunits varies depending on the pathogen analysed, which leads to a different numbering of these mutations even if they are homologous (Stammler *et al.* 2015; Mair *et al*. 2016). Up to now, more than 23 amino acid exchanges are known to cause resistance in various pathogens (FRAC 2017; Sierotzki and Scalliet 2013). In general, cross resistance can be observed between all SDHIs, meaning a particular mutation reduces the efficacy of all SDHIs. However, the resulting effect on the sensitivity seems to be dependent on the chemical compound, the mutation itself, its location and the pathogen affected, resulting in a more complex cross-resistance pattern for this fungicide group. For instance, the exchange of proline at position 225 in SDH-B in *B. cinerea* confer high resistance to boscalid, benodanil, bixafen, carboxin, fenfuram, fluxapyroxad, fluopyram and isopyrazam. Other mutations in SDH-B (H272Y/R) confer lower resistance factors or even no sensitivity loss towards fluopyram, but higher resistance levels towards boscalid (Veloukas *et al.* 2013; Hahn 2014). Interestingly, if a homologous mutation (H267L) is analysed in laboratory mutants of *Z. tritici*, complete cross resistance can be observed for all SDHIs (Scalliet *et al.* 2012).

1.6 Fitness of fungicide resistant isolates

The speed and spread of a resistant population is not only correlated with the intensity of use of the fungicide, meaning the frequency and the intensity of the selection (Hobbelen *et al.* 2014), but it is also linked to the fungicide used and the mutation itself (Ishii 2015). The emergence and evolution of fungal resistance to fungicides and the ability of resistant isolates

to grow and reproduce compared to the wildtype population is an important topic for plant pathologists (Pringle and Taylor 2002). Differences between resistant and wildtype isolates can be determined by fitness measures. Fitness is described as the survival and reproductive success of an allele, individual or group (Pringle and Taylor 2002). Several studies have shown that resistant isolates have a lower fitness than sensitive isolates and were not able to compete with the wild type population. Mutations that confer resistance towards a fungicide may be associated with an observed lower fitness condition. A restricted use of this fungicide may then allow a reintroduction in the spray program (Fernández-Ortuño *et al.* 2008). However, if there is no fitness cost associated with the resistant allele, isolates carrying these mutations persist in the field even without selection pressure (Ishii 2015). Fungal pathogens can have a complex life cycle and the choice must be made, which single aspect or single measure of fitness should be in focus in the investigations (Pringle and Taylor 2002). Different components of fitness such as mycelial growth, germination rate, spore production and the competitive ability of isolates can be assessed.

Several aspects of the fitness of resistant pathogens and the stability of resistance are still not fully understood. Fitness costs and the stability of resistance can be species specific and dependent on the fungicide itself. In fact, in some investigations it could be shown that resistant isolates were as competitive as the wildtype and persisted in the population. For example, QoI resistance was stable in *A. alternata* isolates and QoI resistant *E. necator* isolates showed even higher competitiveness compared to sensitive isolates (Vega and Dewdney 2014; Rallos *et al.* 2014). In contrast, consecutive transfers of QoI resistant *Plasmopara viticola* or *P. xanthii* populations without selection pressure, resulted in a decline or even a fully recovered sensitivity (Genet *et al.* 2006; Ishii *et al.* 2007).

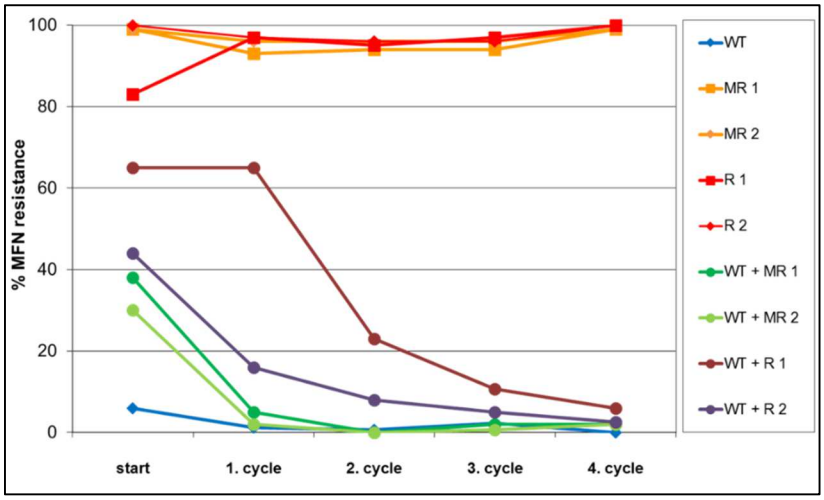


Figure 4: Competitive growth of sensitive, moderately adapted and resistant *B. graminis* f.sp. *tritici* isolates on untreated wheat leaves under laboratory conditions. Moderately adapted and resistant isolates contained the G143A mutation, which was used as marker for metrafenone resistance and detected by qPCR. WT: sensitive isolate without G143A mutation, MR: moderately adapted isolate and R: resistant isolate (Source: Stammler *et al*. 2014).

Recently, fitness studies were performed for *B. graminis* f.sp. *tritici* isolates with a reduced sensitivity towards metrafenone (Figure 4). The competitiveness of sensitive, moderately adapted and resistant *B. graminis* f.sp. *tritici* isolates was analysed on untreated leaves. The isolates were analysed individually and in mixtures, whereby moderately adapted or resistant isolates were combined with sensitive isolates. The mixtures were grown for several growth cycles without selection pressure, and this resulted in a decline of the moderately adapted and resistant isolates (Stammler *et al*. 2014). As most of these analyses are set under optimal laboratory conditions, which exclude abiotic and biotic environmental factors, they should be considered as trends, which cannot be directly converted to the development in the field.

1.7 Objectives

There were two main objectives of the present studies. First, the characterisation of metrafenone and SDHI resistant *E. necator* isolates obtained from several grape growing regions in Europe. Secondly, the investigation of the molecular mechanisms conferring a reduced sensitivity. The results of the European sensitivity monitoring and the resistance evolution of *E. necator* populations towards metrafenone and SDHIs were analysed. Isolates with reduced sensitivity were maintained as living strains for phenotypic analysis. Investigations on *E. necator* are limited by the obligate biotrophic life style of the fungus. *E. necator* cannot be cultivated on artificial media and therefore subsequent cultivation was done on detached leaves of the host plant. *In vivo* leaf disc tests and germination tests were performed to determine the level of resistance.

Resistance towards metrafenone is already known for European populations of *B. graminis* f.sp. *tritici*. The development of metrafenone resistance and the biological effects of metrafenone treatment on the infection process of *E. necator* were compared with those of *B. graminis* f.sp. *tritici*. Furthermore, studies on the specific mode of action of metrafenone were performed using whole genome sequencing. Comparative genome sequencing approaches were performed to identify genes connected to metrafenone resistance and to elucidate the resistance mechanism. As a model organism for obligate biotrophic fungi, *B. graminis* f. sp *tritici* isolates resistant towards metrafenone were sequenced. Additionally, metrafenone had a strong effect on the sporulation of *Aspergillus nidulans*, which allowed the generation of adapted isolates and the comparative genome analysis of the parental strain and its progeny.

A reduced sensitivity towards SDHIs in *E. necator* has not been described so far. In 2014, less sensitive isolates were identified for the first time and a main target of these studies was to identify the mechanisms for the sensitivity loss towards SDHIs. Target gene sequencing was used to identify the responsible molecular mechanisms. The impact on the efficacy of different SDHIs, relevant for the control of powdery mildew in grapevine, and the cross-resistance relationships were investigated. Correlations with other SDHI resistant plant pathogens were examined and a molecular genetic sensitivity monitoring method was developed. In 2016, all isolates with a reduced sensitivity were analysed and the distribution and frequency of these *sdh*-mutations was determined, respectively.

The knowledge about fitness costs associated with fungicide resistance is important to identify suitable anti-resistance strategies. Therefore, fitness studies were conducted with mixtures of sensitive and resistant *E. necator* isolates using growth competition assays without selection pressure to predict the stability and speed of fungicide resistance development.

2. Materials and methods

2.1 Equipment

Technical equipment and electric devices used during this study are listed in the following (Table 2).

Table 2: Technical equipment and electric devices used.

Name	Manufacturer
Airbrush (size 0.3), SATAminijet® 3000 B HVLP	SATA GmbH & Co. KG, Kornwestheim, Germany
Analytical balance, MC 410S	Sartorius AG, Göttingen, 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
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
Thermal cycler, peqSTAR 96X Universal Gradient	VWR International GmbH, Darmstadt, Germany
Pyrosequencing preparation, PyroMark Q96 work station	Qiagen, Hilden, Germany
Pyrosequencer, PSQ 96MA	Qiagen, Hilden, Germany
Inoculation tower	BASF SE, Ludwigshafen, 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

2.2 Chemicals and consumables

Chemicals and consumables used and the companies from which they are purchased are listed in table 3.

Table 3: Consumables and chemicals used.

Name	Manufacturer
Consumables	
Biozym LE agarose	Biozym Biotech Trading GmbH, Wien, Austria
Ethidiumbromid, 10 mg/ml	Life Technologies GmbH, Darmstadt, Deutschland
GelGreen Nucleic Acid Stain, 10.000x	Biotium Inc., Hayward, USA
6x Orange DNA loading dye	Fermentas GmbH, St. Leon-Rot, Germany
96well microtiter plate	VWR International GmbH, Darmstadt, Germany
96well PCR plate	4titude Ltd., Wotton, UK
Combitips plus	Eppendorf AG, Hamburg, Germany
Gauze	Lohmann & Rauscher GmbH & Co. KG, Neuwied, Germany
Petri dish (ø 92 mm)	Greiner Bio-One International GmbH, Frickenhausen, Germany
Petri dish (ø 92 mm) with ventilation cams	Greiner Bio-One International GmbH, Frickenhausen, 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
Chemicals	
Ampicillin, sodium salt	AppliChem GmbH, Darmstadt, Germany
D-(+)-glucose	Sigma Aldrich, St. Louis, US
DEPC-water	Ambion Inc., Austin, US
Difco™ Agar	Becton, Dickinson and Company, Franklin Lakes, US
Difco™ Potato Dextrose Agar	Becton, Dickinson and Company, Franklin Lakes, US
Difco™ LB Broth, Miller	Becton, Dickinson and Company, Franklin Lakes, US
EDTA	Calbiochem, Merck KGaA, Darmstadt, Germany
Ethanol	Sigma Aldrich, St. Louis, US
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

Name	Manufacturer
Magnesium sulphate	Merck KGaA, Darmstadt, Germany
Malt extract	VWR International GmbH, Darmstadt, Germany
Potassium chloride	Merck KGaA, Darmstadt, Germany
Potassium sulphate	Merck KGaA, Darmstadt, Germany
Sodium chloride	Sigma Aldrich, St. Louis, US
Sodium hydroxide	Merck KGaA, Darmstadt, Germany
Sodium hypochlorite (10.5 g chlorine/l)	Bernd Kraft, Maxdorf; Germany
Streptomycin sulphate	Sigma Aldrich, St. Louis, US
Tris Base, Molecular biology grade	Merck KGaA, Darmstadt, Germany
Tween20	Sigma Aldrich, St. Louis, US
Uvitex®2B	Ciba, Basel; Switzerland
Lutensol AP 6	BASF SE, Ludwigshafen, Germany

2.3 Growth media and reaction buffers

The cultivation of filamentous fungi and bacteria was done with the following liquid and agar media as listed in Table 4. Reaction buffers used in this study are also indicated.

Table 4: Cultivation media and reaction buffers.

Name	Composition	Notes
Cultivation of *Escherichia coli*		
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	pH 7 autoclaved
LB - medium (- agar)	2.5% [w/v] LB broth powder after Miller (2% [w/v] agar-agar)	autoclaved and cooled to 60°C before addition of 100 mg/l ampicillin
Cultivation of *B. graminis* f.sp *tritici* and *E. necator*		
1% water agar	1% [w/v] agar-agar 0.4% [w/v] benzimidazole 0.3% [w/v] streptomycin	autoclaved
0.4% water agar	0.4% [w/v] agar-agar 0.4% [w/v] benzimidazole 0.3% [w/v] streptomycin	autoclaved
2% millipore agar	2% [w/v] agar-agar	autoclaved
Cultivation of *Oculimacula* spp. and *A. nidulans*		
PDA - agar	3.9% [w/v] potato dextrose agar 1% [w/v] agar-agar	autoclaved
Nutrient I	1 % [w/v] glucose 0.2 % [w/v] yeast extract 8 mM [w/v] K_2HPO_4	autoclaved

Name	Composition	Notes
	1.5 mM [w/v] KH_2PO_4 4 mM [w/v] $(NH_4)_2SO_4$ 1% [w/v] agar-agar Based on nutrient medium Leroux *et al*. 2013	
Agarose gel electrophoresis		
TAE buffer (50x)	2 M Tris base 1 M acetic acid 5 mM EDTA	Dilution to 1x working solution with H_2O_{MQ}
Tris-HCl buffer (10x)	0.5 M TRIS-base	Dilution to 1x working solution with $H_2O_{MQ;}$ pH: 8, autoclaved
Pyrosequencing		
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

2.4 Enzymes, reaction kits and bacterial strain

The reaction kits, used for DNA extraction, restriction enzymes and the bacterial strain used for transformation are indicated in table 5.

Table 5: Restriction enzymes, reaction kits, solutions and bacterial strain.

Name	Manufacturer
Restriction enzymes	
BglII, FastDigest	Fermentas GmbH, St. Leon-Rot, Germany
*Eco*RI, FastDigest	Fermentas GmbH, St. Leon-Rot, Germany
Reaction kits	
CloneJET PCR Cloning Kit	Fermentas GmbH, St. Leon-Rot, Germany
NucleoSpin® DNA Plant II Kit	Macherey-Nagel GmbH & Co. KG, Düren, Germany
NucleoSpin® 8 / 96 Plant II	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
DNA extraction for whole genome sequencing	
Buffer QBT	Qiagen, Hilden, Deutschland
Buffer QC	Qiagen, Hilden, Deutschland
Buffer QF	Qiagen, Hilden, Deutschland
Proteinase K	Qiagen, Hilden, Deutschland
RNaseA	Macherey-Nagel GmbH & Co. KG, Düren, Germany

Name	Manufacturer
Polymerases	
Maxima Hot Start PCR Master Mix (2x)	Fermentas GmbH, St. Leon-Rot, Germany
Phusion Hot Start, High-Fidelity DNA Polymerase Mastermix	Finnzymes OY, Espoo, Finnland
Bacterial strain	
XL-1 Blue Competent Cells	Agilent Technologies, Santa Clara, USA

2.5 Oligonucleotides

The oligonucleotides used as primers for PCR-reactions and as sequencing primers for pyrosequencing are listed in table 6.

Table 6: Used oligonucleotides. The target gene, the purpose and the fungal organism is indicated.

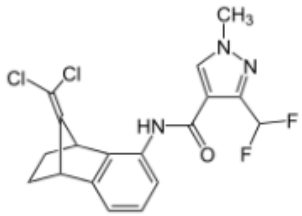
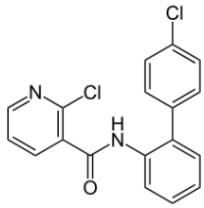
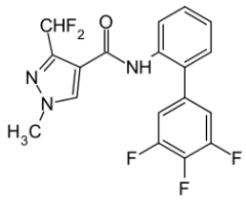
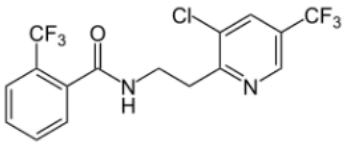
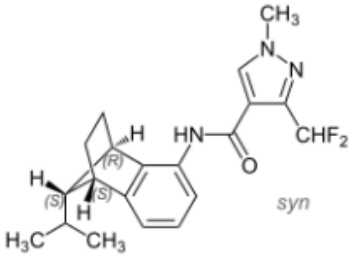
Name	Sequence (5´->3´)	Purpose
KES 2070	TCCTAAAGAAATTCCCGCTCCT	*sdh*B gene kindly provided by Anja Grabke
KES 2071	AAGCTGGAGAAAAACGCCT	
KES 2072	TCACCCACCCACGTTTGTAT	*sdh*C gene kindly provided by Anja Grabke
KES 2073	TGATTCTTAGAGATGAAGTCTGCGA	
KES 2075	TTAGCCCCGCATTGAAGCTC	*sdh*D gene kindly provided by Anja Grabke
KES 2076	GCTTTTAAGGTGAGGCGGTTC	
KES 2110	GATGGGTTATATGAGTGCATTTTG	PCR for pyrosequencingassay *sdhB*-H242R
KES 2111	5´Biotin-TTAGGACAAGTCCGAGAACAATTT	
KES 2112	GTATGAGTCTCTATCGATGT	Sequencing primer *sdhB*-H242R
KES 2113	5´Biotin-ACATGGGAAAGGCTTTTACAAAT	PCR for pyrosequencingassay *sdhC*-G169D
KES 2114	ACCAAAGCTACCAAAGCTAATGC	
KES 2115	ATCCAACTACCATCCAG	Sequencing primer *sdhC*-G169D
KES 788	GGGGTTCCTAACCAAAACAAG	PCR for pyrosequencingassay *cytb* G143A
KES 789	5´Biotin-TGATATTTTTCAACCGCTATTACC	
KES 493	GCAGATGAGCCTATGG	Sequencing primer *cytb* G143A
KES 2125	GAATCATCATGCCAAAGAATGCTACC	o-acyltransferase gene *B. graminis* f.sp. *tritici*
KES 2126	CATCTCTACTTCCATTCGCTCGATCT	
KES 2129	CTAGTAAGTTCCAATTCTCCTCTTCTACTG	o-acyltransferase gene *E. necator*
KES 2131	CTATCACTTCGATTCGACTAGCTAGC	
KES 2201	GTCATCATCGCAATACTTCTACC	Rsc complex subunit *B. graminis* f.sp. *tritici* (whole region)
KES 2212	TAGACTGAAATTATGTTGCGAC	

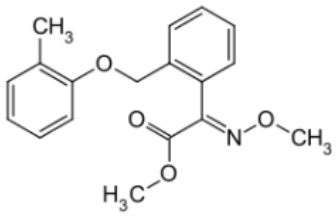
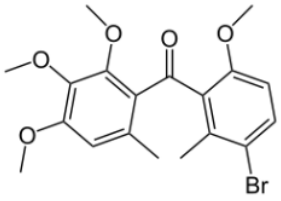
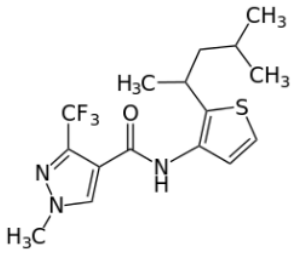
KES 2202	ATGGTTAAACGCAGTCG	Rsc complex subunit *B. graminis* f.sp. *tritici* (partial region)
KES 2203	TACGCCGTAGTCTGTTTCGTTCA	
KES 2204	CCCATTCGTTTTTGACGAGGTAG	
KES 2205	TACGATCGAGAAGTTCGAATTTC	
KES 2206	TATATTAGTTGGTGGAACTTTGA	
KES 2207	TCTCATCCGCAAGAATACCGTTA	
KES 2208	CCTTTCATCGAAGATAAAT	
KES 2209	CCCTGAGTTTTTCGGTATCG	
KES 2210	CTTTATTTTTCATTGGCACCACC	
KES 2211	ACGATTGATGAGGATTCGACTCG	
KES 2182	ACACAACAGCAGCTTTTTGGCTCT	Rsc complex subunit *E. necator* (whole region)
KES 2183	GCTCCCATAGGGATTTGTGAGCCC	
KES 2195	GTTGTTGTTTCCGTGCCA	Rsc complex subunit *E. necator* (partial regions)
KES 2196	CAACCTGACATATTAGTT	
KES 2197	TTAGACCAAGACCACCAG	
KES 2198	CAAATGACAGCTATCATG	
KES 2199	GTAGTAGTCATAGAAGTC	
KES 2200	GAGTCGTGAAAGTAGTGA	

2.6 Fungicides

Commercial available formulations or active ingredients (a.i.) were used for sensitivity characterisations of fungal isolates. The different compounds are shown in Table 7.

Table 7: Fungicides used. Active ingredients and formulations are represented.

Active ingredient / trade name	Company / market launch	Chemical structure
Benzovindiflupyr (a.i.)	Syngenta Agro GmbH, Basel, Schweiz / not launched in grapevine	
Boscalid / Cantus®	BASF SE, Ludwigshafen, Deutschland / 2003	
Fluxapyroxad / Sercadis®	BASF SE, Ludwigshafen, Deutschland / 2017	
Fluopyram / Luna Privilege®	Bayer Crop Science AG, Monheim, Deutschland / 2014	
Isopyrazam (a.i.)	Syngenta Agro GmbH, Basel, Schweiz / not launched in grapevine	

Active ingredient / trade name	Company / market launch	Chemical structure
Kresoxim-methyl / Stroby®	BASF SE, Ludwigshafen, Deutschland / 1997	
Metrafenone / Vivando® (grapevine) Flexity® (cereals)	BASF SE, Ludwigshafen, Deutschland / 2006 (2003)	
Penthiopyrad (a.i.)	Du pont, Wilmington, Delaware, United States / not launched in grapevine	
Sulphur / Kumulus®	BASF SE, Ludwigshafen, Deutschland / 1973	S

2.7 Fungal microorganisms

2.7.1 Reference strains

The *E. necator*, *B. graminis* f.sp. *tritici* and *A. nidulans* reference strains used in this study are listed below (Table 8). The remaining isolates, which were used for analysis during this thesis are listed in a table in the supplementary material (Table 27).

Table 8: Fungal organisms used.

Strain	Characteristics	Origin
***E. necator* isolates**		
Hst	Sensitive reference isolate	Field isolate; Limburgerhof, Germany
1111	Sensitive reference isolate	EpiLogic; European airborne monitoring 2014 (IT)
1012	G143A reference isolate	EpiLogic; European airborne monitoring 2011
1038	MFN resistant reference isolate	EpiLogic; European airborne monitoring 2013 (IT)
1104	B-H242R reference isolate	EpiLogic; European field monitoring 2014 (HU)
1113	C-G169D reference isolate	EpiLogic; European airborne monitoring 2014 (IT)
1131	C-G169S reference isolate	EpiLogic; European field monitoring 2015 (SK)
1232	B-I244V reference isolate	EpiLogic; European airborne monitoring 2016 (DE)
***B. graminis* f.sp. *tritici* isolates used for whole genome sequencing**		
2588	Reference genome	EpiLogic; German airborne monitoring 2014
2591	Metrafenone sensitive	EpiLogic; German airborne monitoring 2014
EP3	Metrafenone sensitive	Limburgerhof
2596	Moderately adapted towards metrafenone	EpiLogic; German airborne monitoring 2014
2593	Moderately adapted towards metrafenone	EpiLogic; German airborne monitoring 2014
2608	Metrafenone resistant	EpiLogic; German airborne monitoring 2014
2605	Metrafenone resistant	EpiLogic; German airborne monitoring 2014
***A. nidulans* isolate**		
1035	Wildtype reference strain used for the generation of metrafenone adapted isolates	Internal strain culture

2.7.2 Isolation and cultivation of *E. necator*

Isolates of *E. necator* were obtained from EpiLogic (Freising-Weihenstephan, Germany) or isolated from infected plant material. In the latter case, infected grapevine leaves or berries were incubated for one day in a box with slightly increased humidity at room temperature. With the help of a binocular microscope, single conidial chains from sporulating colonies were transferred with an ethanol sterilised single-hair brush onto surface sterilized grapevine leaves. For this, leaves were immerged in a sodium hypochlorite solution (ratio 1:5 with water) for 30 s, followed by three times wasting in sterile distilled water and placed with the abaxial side on petri dishes, which contained 1% water-agar. Leaves were dried under the laminar flow and to avoid high humidity in the plates, petri dishes with ventilation cams were used. Incubation was done in a cultivation chamber with a 12 h light and 12 h dark period at 22°C. After 9 to 10 d incubation, single chains of resulting mildew colonies were transferred onto new grapevine leaves.

2.7.3 Cultivation of *B. graminis f.sp. tritici*

Isolates of *B. graminis* f.sp*. tritici* were provided by EpiLogic (Freising-Weihenstephan, Germany). Single chains of conidia from sporulating mildew colonies were transferred with an ethanol sterilised hook formed lancet onto new leaves every 10-14 d. The culture of these isolates was maintained on cut wheat leaves (variety: Monopol) in petri dishes, which contained 0.4 % water-agar. To avoid high humidity in the plates, lids with small holes were used. Incubation was done in a cultivation chamber with a 12 h light and 12 h dark period at 22°C. Every 10 d, single chains of conidia were transferred to new wheat leaves.

2.7.4 Cultivation of *Aspergillus nidulans*

The *A. nidulans* reference strain 1035 and the generated metrafenone adapted isolates were cultivated on PDA plates at 37°C with a 12 h light and 12 h dark period in a light chamber. They were transferred every 5 d with a sterile lancet needle to new PDA plates to maintain the isolates.

2.7.5 Cultivation of *Oculimacula* spp*.*

Oculimacula spp. isolates were cultivated on PDA plates at 14°C, 12 h light and 12 h dark in a light chamber. Every 14 d isolates were transferred with a sterile lancet to new PDA plates.

2.8 Sensitivity tests

The sensitivity of fungal organisms towards different fungicides was tested and classified in different sensitivity tests, as described in the following chapters.

2.8.1 Leaf disc assay

For the leaf disc assay, six to ten day old leaves from vine plants (Riesling) were surface sterilized as described above (0). Leaf discs were cut using a cork borer (1 cm) and were transferred on 1% water agar plates. The fungicides metrafenone (for grapevine), boscalid, fluxapyroxad and fluopyram were used as formulations and diluted in H_2O Millipore. For the SDHIs isopyrazam, benzovindiflupyr and penthiopyrad no formulations are available for grapevine. Therefore, active ingredients were dissolved in DMSO and diluted in Millipore water. The following dilutions were used for all tested fungicides: 0, 0.3, 1, 3, 10 and 30 mg/l. The discs were treated with the appropriate fungicide dilution with an airbrush by hand application just before run off and afterwards air dried under the laminar flow. One day after treatment, four leaf discs treated with the same dilution were placed onto 0,4 % water agar plates. Inoculation material was prepared 10 d before on detached grapevine leaves. For the inoculation, conidia of each *E. necator* isolate were transferred with a pipette using the inoculation tower (Figure 5).

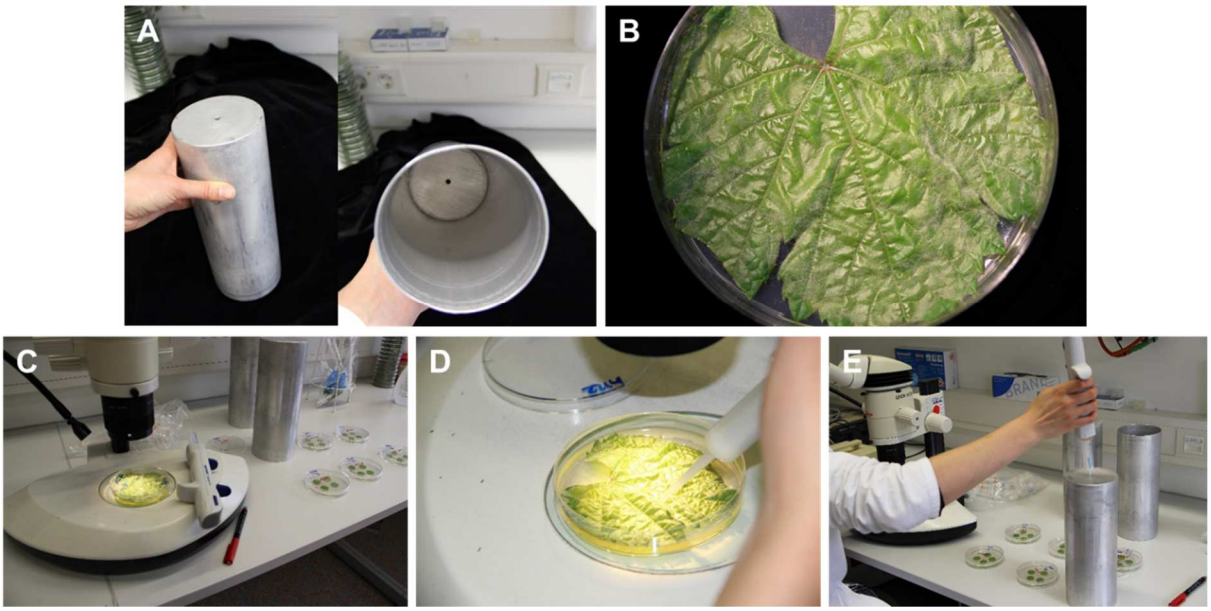


Figure 5: Inoculation method with a pipette and an inoculation tower. A: inoculation tower side view and inner side; B: Inoculation material (10 d old grapevine leaf, infected with *E. necator*; C-E: schematic workflow of the inoculation with the inoculation tower.

Thereby a multistep pipette was used to suck up conidia chains from infected 10 d old cultures with the help of a binocular microscope. A metal tube, one side open and the other with a small whole (inoculation tower Figure 5A) was positioned over the petri dish with leaf discs and the

conidia were distributed with pressure by emptying the pipette. The distribution and number of inoculated conidia was checked under the microscope to ensure similar infection conditions. All resistant isolates were preliminary grown on fungicide treated leaves in order to maintain their resistance level. The inoculated plates with the leaf discs were incubated for 10 d in a cultivation chamber, 12 h light and 12 h dark, at 22°C. After incubation, the percentage of diseased leaf area was assessed by eye with the help of a microscope. EC_{50} values (concentration with a fungal growth inhibition of 50% relative to the untreated control) were determined by interpolation with the RESLAB software from BASF SE.

2.8.2 Germination test

Fungicides were directly applied to melted and 60°C pre-cooled 2% Millipore water agar. The same fungicide concentrations were used as described for the leaf disc assay. These plates were inoculated with a pipette and an inoculation tower as described above. After 24 h incubation in a cultivation chamber 12 h light and 12 h dark at 22°C, 100 conidia were counted and the percentage of germinated conidia was calculated. A conidium was considered to be germinated, when the germ tube was twice as long as the conidium itself.

2.8.3 Detached leaf test *B. graminis* f.sp *tritici*

The sensitivity of preliminary characterised wheat powdery mildew isolates (EpiLogic) was reassessed with the help of a detached leaf test. Metrafenone was used as the formulation for cereals and diluted in H_2O Millipore to 3, 10 and 30 mg/l. Fungicides were applied in a spray chamber to wheat seedlings (variety: Monopol). 24 h after application, detached leaves were placed onto 0,4 % water agar plates and inoculated with the help of an inoculation tower as described above. Inoculation material was prepared 10 d before on untreated detached leaves. The inoculated plates with the detached leaves were incubated for 10 d in a cultivation chamber 12 h light and 12 h dark at 22°C. After incubation, the diseased leaf area was assessed by eye with the help of a microscope.

2.8.4 Other sensitivity tests

Microtiter test with *Oculimacula* spp.:

Spores were harvested with 6 ml H_2O and a sterile Drigalski spatula from 14 d-old *Oculimacula* strains grown on PDA plates. All suspensions were filtered through double-layered cheesecloth. The spore concentration was determined via light microscopy and a bright-line Thoma chamber. The final spore concentration of $3x10^4$ spores/ml was adjusted in double concentrated nutrient I medium and stored at 4°C until used. A 10,000 mg/l metrafenone stock solution was prepared and diluted to 0.03, 0.01, 0.3, 1, 3, 10 and 30 mg/l. All concentrations

were prepared in double concentrated medium and sterile water served as the negative control. 50 µl of the double concentrated fungicide dilution was mixed with 50 µl of the spore suspension. The inoculated microtiter plates were wrapped in plastic bags and incubated at 18°C for 7 d. Growth was measured with a photometer at 405 nm using the Magellan™ software. The average of the four replicates was calculated and the blank was subtracted. Growth inhibition and the EC_{50} values were calculated with the RESLAB software from BASF SE.

Agar plate test with *A. nidulans*:

The sensitivity of the generated and adapted *A. nidulans* isolates was determined in an agar plate test. *A. nidulans* spores were harvested with 4 ml sterile H_2O with the aid of a sterile Drigalski spatula from four-d -old isolates grown on PDA plates and suspended in water (0.003% Tween 20). All suspensions were filtered through double-layered cheesecloth and the spore concentration was determined via light microscopy and a bright-line Thoma chamber. A final spore concentration of $2x10^3$ spores/ml was adjusted and stored at 4°C until used. Fungicides were directly diluted in molten and to 60°C pre-cooled PDA agar. A 10,000 mg/l metrafenone stock solution was prepared in sterile H_2O and diluted to 3, 10, 30 and 100 mg/l. After cooling and drying of the plates, 100 µl of the spore suspension was used as inoculum, which was evenly spread over the agar surface with a sterile Drigalski spatula. The plates were incubated for 4 d at 37°C in a cultivation chamber with 12 h light and 12 h dark. After incubation, spores were harvested from the plates and the concentration at each fungicide concentration was determined via light microscopy and a bright-line Thoma chamber.

2.8.5 Generation of metrafenone adapted *A. nidulans* isolates

The generation of metrafenone adapted *A. nidulans* isolates was done based on spontaneous mutagenesis under metrafenone treatment. Spores of the reference strain 1035 were harvested with 6 ml H_2O and a sterile Drigalski spatula from several 4 d-old PDA plates and solved in water (0.003% tween 20). The solution was filtered through 2x gauze and the spore concentration was determined via light microscopy and a bright-line Thoma chamber by counting spores in a dilution (1:1,000 dilution). A final spore concentration of $2x10^8$ spores/ml was adjusted and stored at 4°C until used. Metrafenone was directly diluted in molten and to 60°C precooled PDA agar. A 10,000 mg/l metrafenone stock solution was prepared in sterile H_2O and diluted to 30 and 100 mg/l. After cooling and drying of the plates, 200 µl of the spore suspension was used as inoculum, which was evenly spread over the agar surface with a sterile Drigalski spatula. The plates were incubated for 4 d at 37°C in a cultivation chamber 12 h light and 12 h dark. In the following 10 d, the plates were stored at room temperature to avoid high humidity and condensation. The plates were checked every day for conidia development

and resulting conidia chains were transferred to 30 mg/l metrafenone agar plates. After successful growth and sporulation, the resulting isolates were cultivated for another growth cycle on PDA plates without metrafenone. For verification of a stable mutagenesis, the resulting isolates were again transferred to metrafenone containing PDA plates (10 mg/l).

2.9 Phenotypical analysis of *E. necator* isolates

The method used was based mainly on Leinhos *et al*., (1997).

Detached leaves were inoculated using the inoculation tower method as described above. Metrafenone was suspended in sterile distilled water and diluted to 10 mg/l. The fungicide suspension was hand-sprayed onto the adaxial surface with an airbrush just before run off, 24 h before (preventive) or 2 d or 5 d post (curative) inoculation. After application, leaves were dried under the laminar flow.

Staining solution:

0.2% (w/v) diethanol (Uvitex 2B)
dissolved in 0.1 M Tris-HCl-buffer (pH 8.5)
and supplemented with 0.2% (v/v) Lutensol AP 6

Just before staining, leaf discs (1 cm) were cut using a cork borer. Leaf discs were put onto a microscope slide and 4 – 5 droplets of the above-mentioned staining solution were placed directly onto the leaf discs and covered with a cover slide. After an incubation time of 30 min in the dark, the samples were evaluated by epifluorescence microscopy using a standard filter set (DAPI).

2.10 Standard molecular techniques

Standard molecular techniques, such as DNA extraction, PCR, restriction digestion, and agarose gel electrophoresis, were performed according to established protocols or following the manufacturers´ instructions.

2.10.1 Isolation of genomic DNA

Extraction of fungal genomic DNA for nucleic acid downstream procedures

Fungal genomic DNA was isolated from infected leaf samples (*E. necator* and *B. graminis* f.sp. *tritici*). The samples were frozen on dry ice for 10 min and homogenized by adding a metal bead and disrupting the sample with an oscillating mill (1 min at 20 Hz). The extraction was performed using NucleoSpin DNA Plant II kit (Macherey-Nagel, Düren, Germany) or NucleoSpin 8 Plant II (48-well scale; Macherey-Nagel) according to

manufacturer's instructions following the PL1 lysis buffer protocol for the extraction from plant material.

Extraction of genomic DNA from fungal spores (*B. graminis* f.sp. *tritici* and *A. nidulans*) for whole genome sequencing

Genomic DNA was extracted from conidia (~200 mg), which had been dropped into liquid nitrogen and ground to fine powder in a pre-cooled mortar and pestle. The powder was aliquoted to four 2 ml reaction tubes, mixed with 1 ml QBT-buffer, 25 µl RNAse A and 10 µl proteinase K and incubated at 50°C for 30 min. The mixtures were centrifuged for 5 min (14000 rpm, 4°C) and the supernatant was transferred to an equilibrated anion-exchange tip (Genomic-tip 100/G). After two washing steps with 8 ml QC-buffer, the DNA was eluted with 4 ml QF-buffer. The precipitation was done by using the 0.8-fold volume of isopropanol and centrifugation for 30 min (5000 rpm, 4°C). After a washing step with 70% ethanol and centrifugation for another 30 min, the pellet was air-dried and dissolved in 80 µl TE-buffer. Before sequencing, the quality of the DNA was checked with the help of a restriction digestion (*EcoR*I and *Bgl*II), gel electrophoresis and measurement with a spectrophotometer (NanoDrop 2000).

2.10.2 Polymerase chain reaction (PCR)

PCR was performed for the amplification of target genes. The pathogen specific oligonucleotides are listed in table 6 and reaction mixtures were prepared as shown in table 9. For every PCR reaction, a sample with water instead of DNA was used as negative control. Before the reaction, PCR tubes or plates were vortexed and centrifuged.

Table 9: Components for a PCR reaction.

Compounds	Volume for a 25 µl reaction (µl)
2 x Mastermix (Maxima / Phusion)	12.50
Oligonucleotides forward (10 pmol/µl)	1.25
Oligonucleotides reverse (10 pmol/µl)	1.25
DEPC-H_2O	7.50
DNA template	2.50

A schematic PCR procedure is shown in Table 10. The annealing temperature depended on the primer pair used and was adjusted for each PCR reaction. Depending on the downstream procedures, different polymerases were used. The Maxima Hot Start *Taq* polymerase was taken to amplify target DNA sequences for a subsequent pyrosequencing procedure. For cloning and sequencing of target genes, Phusion® High-Fidelity DNA polymerase with a

proofreading activity was used. The elongation time was adjusted and was dependent on the polymerase used and the fragment size.

Table 10: Schematic procedure of a PCR reaction program. Maxima polymerase or Phusion polymerase were used for amplification. The annealing temperature is dependent on polymerase and primer pair used.

Function	Temperature [°C] (Maxima / Phusion)	Time [min] (Maxima or Phusion)	Number of cycles
Initial denaturation	95	4:00 or 0:30	1
Denaturation	95	0:30 or 0:10	35
Annealing	52-72	0:30 or 0:05	
Elongation	72 / 68	1:00 / kb or 0:15 / kb	
Final elongation	72 / 68	2:00	1

Amplified fragments were used for downstream procedures such as sequencing or SNP detection via pyrosequencing. Agarose gel electrophoresis was used to separate the amplified DNA fragments. After the clean-up of the excised products with the NucleoSpin Gel and PCR Clean-up Kit, fragments were sent for sequencing.

2.10.3 Gel electrophoresis

Agarose gel electrophoresis was used for the separation and clean-up of DNA fragments. The samples were mixed with loading dye or with loading dye, which contained 20x GelGreen, if the gel was not enriched with ethidium bromide. A 1% Agarose gel (1x TAE-buffer) was run at 80 V for 60 - 90 min in 1x TAE-buffer. The DNA fragments were visualized and analysed in the EasyDoc gel documentary chamber under UV or blue light (430 – 490 nm).

2.10.4 Cloning of PCR products

For some sequencing cases, a high concentration of the target sequence was needed and the PCR products were cloned and multiplied in *E. coli.*

Ligation:

The PCR products obtained were ligated into the pJet 1.2/blunt cloning vector in accordance with the CloneJET PCR Cloning Kit (Thermo Scientific). Depending on the polymerase used the blunt-end (Phusion) or the sticky-end (Maxima) cloning protocol was used.

Transformation in *Escherichia coli*

The ligation reaction was transformed into competent cells of *E. coli* (XL1-Blue). The suspension was then aliquoted (100 µl) on ice into precooled 1.5 ml reaction tubes. The cells were incubated with 1.7 µl β-mercaptoethanol for 10 min on ice. After adding 1 µl of the ligation reaction, the cells were incubated for 30 min on ice. For the heat shock the suspension was

heated to 42°C in a water bath for 45 s and then cooled on ice for 2 min. Afterwards 900 µl preheated SOC medium (42°C) were then added. The transformation reaction was incubated at 37°C for 1 h at 250 rpm on a thermo block. 100 µl and 200 µl of the cell suspension were plated onto LB-agar plates, which contained 100 µg/ml ampicillin. The plates were incubated overnight at 37°C.

Plasmid-preparation

Colonies of transformed cells were picked with a sterile pipet tip and grown at 37°C overnight in 2 ml liquid LB medium with 100 µg/ml ampicillin in 14 ml Greiner-tubes on a shaker with 250 rpm. For plasmid isolation, the NucleoSpin Plasmid Kit was used and the manufacturer's protocol was followed.

2.10.5 Restriction analysis

Restriction digestion was used as a quality check for genomic DNA or to test if the correct insert had been incorporated into the plasmid via gel electrophoresis. For this, the target DNA or plasmid was mixed and incubated with a restriction enzyme for 30 min at 37°C. The composition of the restriction reaction mixture is shown in Table 11. 1 % agarose gel was used for visualization of the restriction digestion.

Table 11: Components of a restriction digestion.

Compounds	Volume for a 20 µl reaction (µl)
FastDigest®-buffer (10x)	2
FastDigest®Enzyme (*Eco*RI or *Bgl*II)	1
Water	15
Target DNA	2

2.10.6 Sequencing

Sequencing of plasmids and other PCR-products was conducted via Sanger sequencing in an internal department of BASF SE. For larger DNA-fragments, primer walking sequencing was used. For this, plasmids with the target gene were sent to the company SeqLab (Göttingen, Germany) for sequencing.

2.10.7 Pyrosequencing

Pyrosequencing assays were developed using Pyrosequencing Assay Design software (Qiagen, Hilden, Germany) for quantification of single nucleotide polymorphisms (SNPs),

which led to resistant phenotypes. DNA fragments were amplified using Maxima Hot Start PCR Master Mix and primer pairs with biotinylated oligonucleotides. Standard PCR was done according to section 2.10.2 and every DNA template was analysed twice. In addition, a no-template-control was prepared for every PCR reaction. The PCR products were immobilised on 3 µl streptavidin sepharose beads mixed with 37 µl binding buffer and incubated for 15 min at 1400 rpm. The Vacuum Prep Worktable (Qiagen) was used to fix the beads. After adding 40 µl binding buffer and another fixation step, 70% ethanol was used for an initial clean-up step for 10 s. This was followed by a denaturation for 10 s in 0.2 M NaOH for single-strand preparation and a washing step for 10 s in 1x pyrosequencing washing buffer. After transfer of the single stranded samples to 38.75 µl annealing buffer, which contained 1.25 µl of the corresponding sequencing primer, the samples were heated at 80°C for 2 min. Following the manufacturer's instructions, the samples were pyrosequenced using PyroMarkGoldQ96 reagents on a PSQ 96MA machine (Qiagen). Validation of the developed assays was done using dilutions of the reference DNA used and standard mixtures 10/90%, 25/75%, 50/50%, 75/25% and 90/10% mixtures of wildtype and mutated isolate, as well as 100% wild type and mutated isolate. Furthermore, the specificity was tested by using DNA from other related or with grapevine associated fungal pathogens.

2.11 Fitness tests

Competition assays were performed for the evaluation of possible fitness penalties connected with metrafenone resistance or due to amino acid substitutions in the SDH. To mix equal amounts of the different isolates, spore suspensions were used instead of dry inoculation. If spore suspensions are used as inoculum, a fast work flow is necessary to prevent bursting of the conidia. *E. necator* infected leaves were placed in a 50 ml falcon tube with 10 ml millipore water (0.003% Tween 20) and shaken by hand. Spore density was adjusted to 10^5 spores/ml and the suspensions of strains with reduced metrafenone / SDHI fungicide sensitivity (mutants) were mixed with sensitive partners (metrafenone / SDH wild types). In this study, three different approaches were used to determine potential fitness cost.

2.11.1 Competitive ability of population mixtures

In this approach, spore suspensions of five metrafenone sensitive and five metrafenone resistant *E. necator* strains were mixed in equal ratios (sensitive / resistant-mixture). A sensitive-resistant-mixture, were all used isolates (sensitive and resistant) were mixed in same portions, was used to observe the development of metrafenone resistance over time. Each mixture (500 µl/leaf) was sprayed with an air brush (nozzle size 0.3 mm) on three surface sterilized (0) untreated detached leaves on 1% water agar. Additionally, for the first, the fourth and the ninth cycle three metrafenone treated leaves, were inoculated with the same spore

suspension. The application was done one day before inoculation and a concentration of 10 mg/l metrafenone was used. Inoculated leaves were incubated in a cultivation chamber 12 h light and 12 h dark at 22°C. After 10 d, the percentage of the diseased leaf area was assessed by eye with the help of a dissecting microscope (cycle 0). Spores were harvested and used to prepare spore suspensions for the next infection cycle. Inoculated leaves were placed in a 50 ml falcon tube with 4 ml millipore water (0.003% Tween 20) and shaken by hand. Following this principle, a total of nine infection cycles were conducted and the development of metrafenone resistance was calculated relative to the untreated control. A schematic work flow is shown in Figure 6**Fehler! Verweisquelle konnte nicht gefunden werden.**.

Another approach was done with the multisite fungicide sulphur (Kumulus®), to add an additional stress factor (Figure 6). The preparation of the spore suspensions and the workflow was done as described above. Hwever, instead of untreated grapevine leaves, each mixture was sprayed with an air brush (500 µl/leaf) on three surface sterilized and sulphur treated detached leaves. A sulphur concentration of 50 mg/l was chosen as suitable and application was done one day before inoculation.

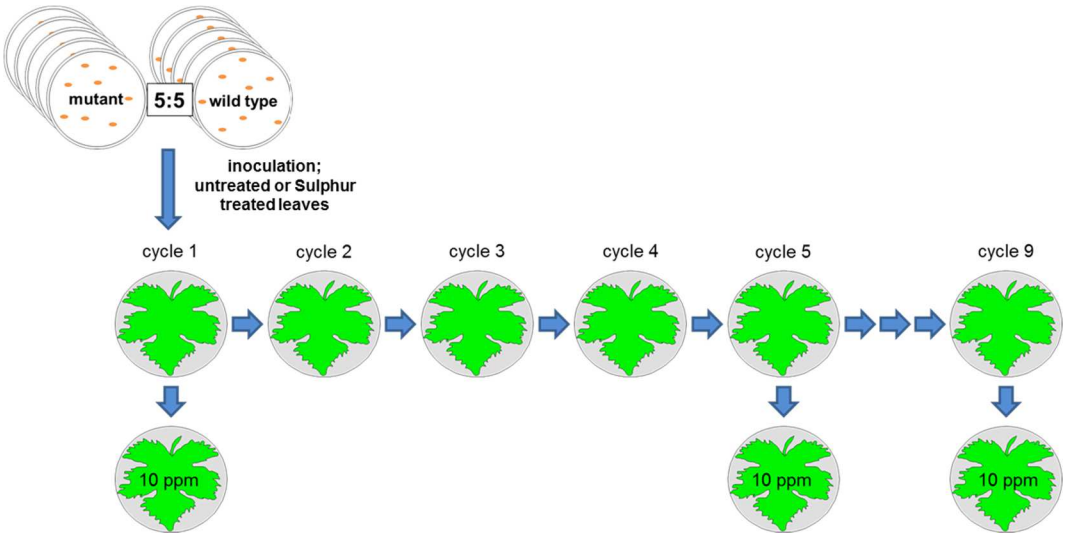


Figure 6: Schematic work flow of the fitness tests with artificial population mixtures. The inoculation was done on untreated (competitive ability of population mixtures) or sulphur treated (competitive ability of population mixtures under sulphur treatment) grapevine leaves.

2.11.2 Growth competition of paired mixtures

For this fitness test, spore suspension mixtures of single strains were combined in a 1:1 ratio of wild type isolates and isolates with a resistant phenotype. Suspensions of the single isolates served as control. Each mixture was sprayed with an air brush on two untreated detached leaves on 1% water agar (500 µl/leaf). The remaining spore suspension was used for DNA extraction and detection of resistance frequency by pyrosequencing (2.10.7) (cycle 0). Inoculated leaves were incubated in a cultivation chamber with 12 h light and 12 h dark at

22°C. After 10 d, the infected leaves were used to prepare the inoculum for the next infection cycle, the remaining suspension was used for DNA extraction and frequency detection of resistant genotypes. A total of six infection cycles were conducted. The work flow of the molecular genetic fitness test is shown in Figure 7.

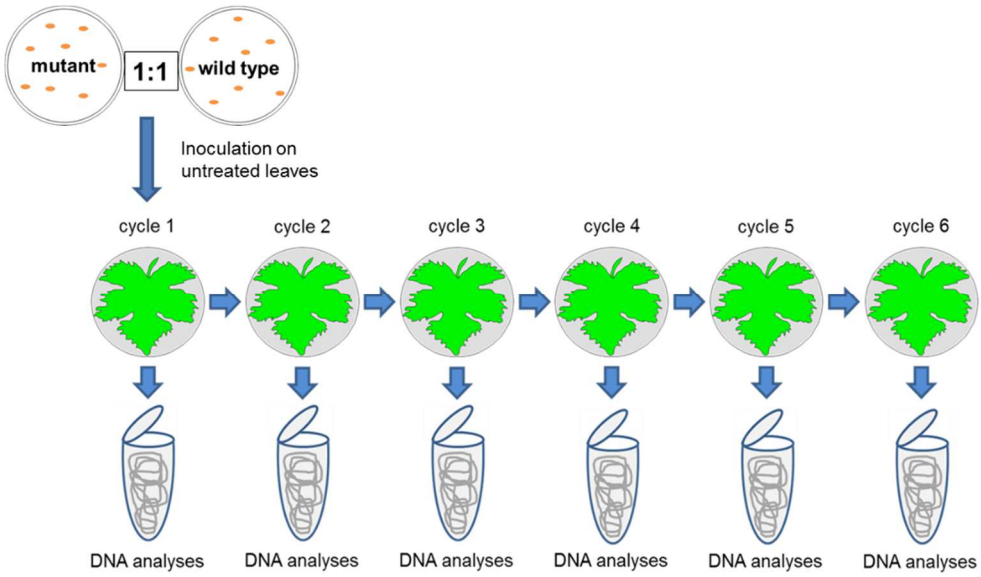


Figure 7: Schematic work flow of the fitness tests with artificial paired mixtures. Inoculation was done on untreated grapevine leaves and after each cycle the frequency of resistance, represented by a molecular marker, was determined by pyrosequencing.

2.12 Homology modelling

The SDH protein homology modelling of subunits SDH-B, SDH-C and SDH-D of *E. necator* was done by Dr. Janosch Achenbach (BASF SE) and Dr. Antje Wolf (BASF SE). The construction was done using standard settings of the modelling tool in MOE (Molecular Operating Environment, v.2010.1; Chemical Computing Group Inc., Montreal, Canada). Since the SDH X-ray structure of the SDH complex from *E. necator* is not available the SDH X-ray structure of the SDH complex (resolution of 2.1 Å) of *Gallus gallus* (PDB 2WQY) was used as a structural template. Sequence identity to *E. necator* for subunits B, C and D was determined as 69.8, 27.7 and 31.1%. Amino acid sequences were aligned with the BLOSUM62 substitution matrix. The implementation of mutations leading to amino acid substitutions B-H242R, B-I244V and C-G169D/S into the three-dimensional structure of the reference protein was done manually.

2.13 Bioinformatic and statistical analyses

2.13.1 Standard bioinformatic and statistical analysis

The design of oligonucleotides, blasts and alignments was done using an internal bioinformatic site from BASF (Bioinformatics@BASF), which includes public databases, such as NCBI, or by using the DNASTAR Lasergene 12 Core Suite software. For pyrosequencing assays, oligonucleotides were designed using the manufacturers´ Pyrosequencing Assay Design Software.

In order to validate the presence or absence of fitness penalties associated with a resistant phenotype, a linear regression model combined with a recursive partitioning algorithm was applied to the available sets of data (Zeileis *et al*. 2008). With the support of Dr. Iain Proctor (BASF SE), a linear-model-tree function from the statistical computing software R Studio (version 3.2.0, partykit package) was used. The graphical outcomes are shown in the supplementary material (Supplementary material, Figure 48-Figure 51).

2.13.2 Bioinformatic analysis of whole genome sequence data

The *de novo* sequencing of the *B. graminis* f.sp. *tritici* strain 2588 was accomplished by DNA Landmarks (Quebec, Canada) using 2x100 bp paired end (PE) reads. *De novo* sequencing refers to sequencing a novel genome, where no reference genome is available or the quality of the reference genome is not sufficient. The resequencing of sensitive and adapted wheat powdery mildew isolates was done by the Bejing Genomics Institute (BGI; Hong Kong, China) on a Hiseq2000 (Illumina) machine with 2x150 bp paired end (PE) reads. The sequencing and assembly was done using the reference genomes (strain 96224, Wicket *et al.* 2013 and strain 2588 during these studies) as templates.

The resulting sequencing data were analysed and evaluated by Dr. Holger Hartmann (BASF SE). Analysis was accomplished by using a BASF in-house Pipeline. The resulting reads were mapped to the previously sequenced reference genomes. Duplicate reads were removed and an initial list of genetic variants was created. The quality of single nucleotide polymorphism (SNP) mutations was improved by quality recalibration, subtracting the initial list of genetic variants. The quality of insertion and deletions (INDELs) was improved by realigning the reads from all strains simultaneously. The final list of SNPs and INDELs was generated using the UnifiedGenotyper method. The reference genome was functionally annotated against the Uniprot database release uniprot20_2012_3. Finally, the annotated reference genome and all identified genetic variants were uploaded to the in-house developed genome portal (Bioinformatics@BASF) for data inspection, analysis and blasts.

3. Results

It is important to characterise resistant isolates and identify the effects of the acquired resistance, for implementation of suitable resistance management strategies. This includes the elucidation of the resistance mechanisms, the level of resistance and the knowledge of fitness costs associated with a resistant phenotype, which helps to predict the speed of occurrence and spread of resistant *E. necator* populations.

3.1 Determination of the baseline sensitivity and sampling methods of *B. graminis* f.sp. *tritici* and *E. necator* for the European sensitivity monitoring

Before the market launch of metrafenone and boscalid, preliminary sensitivity studies were performed by the BASF resistance research laboratory in order to define the baseline sensitivity of *B. graminis* f.sp. *tritici* and *E. necator* populations. Baseline sensitivity describes the sensitivity of a representative number of isolates of a pathogen, which have never been exposed to the fungicide of interest. *In vivo* detached leaf tests with numerous isolates were performed and the mean EC_{50} and EC_{95} values were calculated (Table 12). Based on these baseline studies and the resulting EC_{95} values of sensitive isolates, discriminatory doses of metrafenone, boscalid and fluxapyroxad were determined in order to distinguish between sensitive and potentially upcoming resistant isolates for the sensitivity monitoring program.

Table 12: Results of the baseline sensitivity studies of *B. graminis* f.sp. *tritici* and *E. necator* for metrafenone, boscalid and fluxapyroxad. n=number of analysed isolates.

	B. graminis* f.sp *tritici	***E. necator***		
	Metrafenone		**Boscalid**	**Fluxapyroxad**
Year	2000 (n = 140)	2000 – 2001 (n = 110)	2000 – 2001 (n = 67)	2011 (n = 5)
Range of EC_{50} values	0.0031 - 0.0124 mg/l	0.09 - 0.39 mg/l	0.19 - 1.7 mg/l	0.19 – 0.34 mg/l
Mean EC_{50} value	0.0053 mg/l	0.23 mg/l	0.6 mg/l	0.25 mg/l

Two service providers are contracted by BASF to run the annual routine sensitivity monitoring: The company EpiLogic (Freising-Weihenstephan, Germany) for the European monitoring of *B. graminis* f.sp. *tritici* and *E. necator* (except France) and the company Coniphy (Quincieux, France) for the French sensitivity monitoring of *E. necator*. Samples for the European sensitivity monitoring were collected by two different sampling methods: field sampling and airborne sampling. For field sampling, infected leaves were collected by field technicians

directly from experimental trial locations or standard commercial sites. In contrast, the airborne monitoring is based on sampling of conidia from the air, using a spore trap mounted on a car driving through predefined routes in different European regions (performed by EpiLogic). The methods and the discriminatory doses used for the sensitivity characterisation are depending on the fungicide used and the service provider (Table 13).

Table 13: Discriminatory doses of metrafenone, boscalid and fluxapyroxad used for the European and the French sensitivity monitoring.

	EpiLogic		Coniphy
	B. graminis* f.sp *tritici	***E. necator***	***E. necator***
Metrafenone	0.02 and 0.08 mg/l	10 mg/l	50 mg/l
Boscalid	-	20 mg/l (2005 - 2008) 30 mg/l (from 2009 on)	15 and 100 mg/l
Fluxapyroxad	-	1 mg/l	10 mg/l

EpiLogic method:

For each field sample and each sample from a region of the airborne monitoring, a defined number of single chain isolates was prepared from infected plant material. Sensitivity characterisation with the respective discriminatory dose of the fungicide (Table 13) was done via an *in vivo* leaf disc test. Inoculation was at leaf discs preventively (application 1 day before inoculation) treated with the fungicide of interest. Isolates with growth > 50% at the respective discriminatory doses, were classified as less sensitive and sent as living isolate to the BASF laboratories for further characterisation.

Coniphy method:

For each field sample, *E. necator* conidia were harvested from several infected leaves of one sample. Sensitivity analysis was done as population mixtures, using an *in vivo* leaf disc test with the respective discriminatory dose (Table 13) for each fungicide. The sporulation rate, relative to an untreated control, was evaluated and the fungicide efficacy was calculated as efficacy on sporulation (%). Populations with an efficacy below 60% were classified as less sensitive and sent to the BASF laboratories for further characterisation.

3.2 Aryl-phenyl-ketone sensitivity and mode of action studies

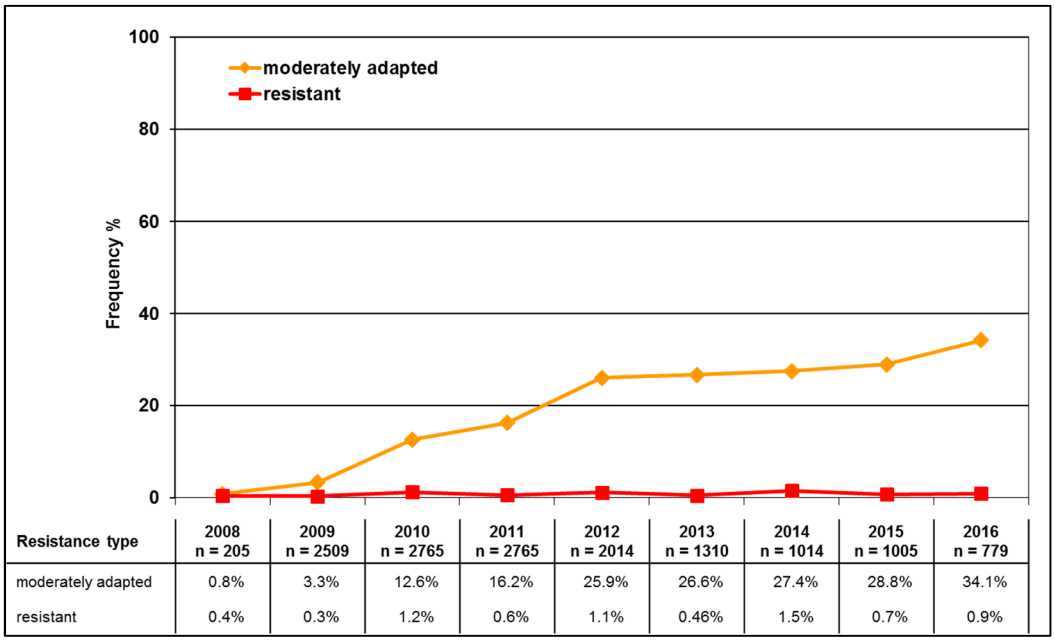
The focus was set on metrafenone in these studies, as it is the first and most frequently used compound of this fungicide class. Sensitivity tests and morphological analysis were done to characterise resistant *B. graminis* f.sp. *tritici* and *E. necator* isolates. The competitive ability of the resistant strains was evaluated and potential fitness costs associated with metrafenone

resistance determined. Attempts were done to elucidate the resistance mechanism of metrafenone by comparative genome analysis of sensitive and resistant *B. graminis* f.sp. *tritici* and *A. nidulans* isolates.

3.2.1 Reassessment and classification of adapted *B. graminis* f.sp. *tritici* isolates

In 2008, four years after market introduction, first isolates of *B. graminis* f.sp. *tritici* with a lower sensitivity towards metrafenone were identified for the first time and revealed two different resistance phenotypes, described as ´moderately adapted´ and ´resistant´ (Felsenstein *et al.* 2010).

Based on the monitoring data from 2008 – 2013 and the data from 2014 – 2016 (processed and evaluated during this study), the development of the frequency of metrafenone resistance for both resistance types was investigated (Figure 8). After an increase between 2008 and 2012, the frequency of moderately adapted isolates stabilised at a level below 30%. In 2016, a slight increase to 34% was observed. The frequency of resistant isolates stayed over all the years at very low levels, below 2%, in the population.



Resistance type	2008 n = 205	2009 n = 2509	2010 n = 2765	2011 n = 2765	2012 n = 2014	2013 n = 1310	2014 n = 1014	2015 n = 1005	2016 n = 779
moderately adapted	0.8%	3.3%	12.6%	16.2%	25.9%	26.6%	27.4%	28.8%	34.1%
resistant	0.4%	0.3%	1.2%	0.6%	1.1%	0.46%	1.5%	0.7%	0.9%

Figure 8: Development of the frequency of metrafenone resistance in *B. graminis* f.sp. *tritici* from 2008 to 2016. The sensitivity of the isolates was determined using a detached leaf test (EpiLogic), n=number of isolates analysed.

In order to confirm the observations made in previous years, isolates classified as less sensitive in 2014, were rechecked on metrafenone treated leaves for further analysis (Figure 9).

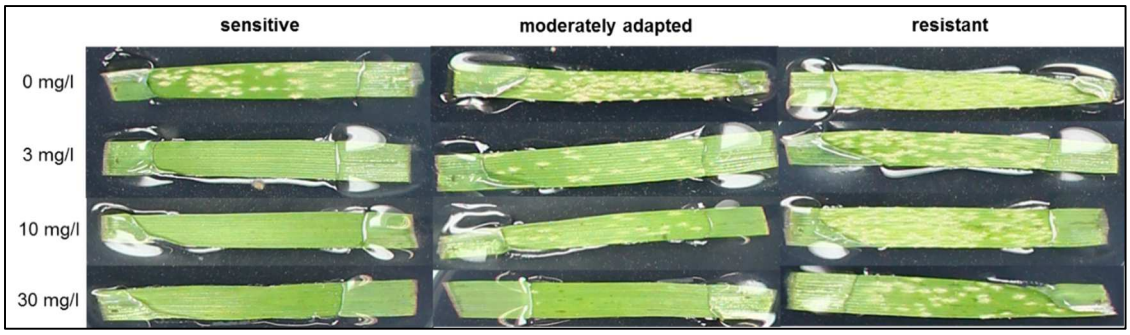


Figure 9: Differences in the infection behaviour of sensitive, moderately adapted and resistant isolates of *B. graminis* f.sp. *tritici* isolates on metrafenone treated wheat leaves (7 dpi).

Infection symptoms of sensitive isolates were only observed on the untreated control. Isolates characterised as moderately adapted developed lesions on leaves treated with 3 mg/l of metrafenone, lesions were not or slightly detectable at 10 mg/l. No growth inhibition could be observed for resistant isolates at all tested metrafenone concentrations. Sensitive, moderately adapted and resistant isolates showed a similar infection behaviour on untreated wheat leaves. No obvious differences were observed with respect to the latent period and sporulation behaviour.

Based on the results of these detached leaf tests, representative sensitive isolates and isolates of each resistance phenotype were selected for a whole genome sequencing approach, which aimed to identify genes correlated with metrafenone resistance. The results of the whole genome sequencing approaches are described in chapter 3.2.5.

3.2.2 Sensitivity of *E. necator* towards metrafenone

Three years after market introduction, in 2010 and 2012, single *E. necator* isolates classified as less sensitive were identified for the first time by BASF. In 2013, further isolates from Italy, Hungary and Austria were characterised as resistant (Stammler *et al*. 2014; Kunova *et al.* 2016; Graf *et al*. 2017). The further development of metrafenone resistance in European grape powdery mildew populations was evaluated in the present study. Isolates classified as resistant were cultivated as living strains and the level of the sensitivity loss was investigated.

3.2.2.1 European monitoring results

The development of metrafenone resistance is shown as a summary of the European sensitivity monitoring from 2010 to 2016 (Figure 10). The monitoring results from 2010 – 2013 were included as available data and the results of the sensitivity monitoring from 2014 - 2016 are processed and evaluated during these studies.

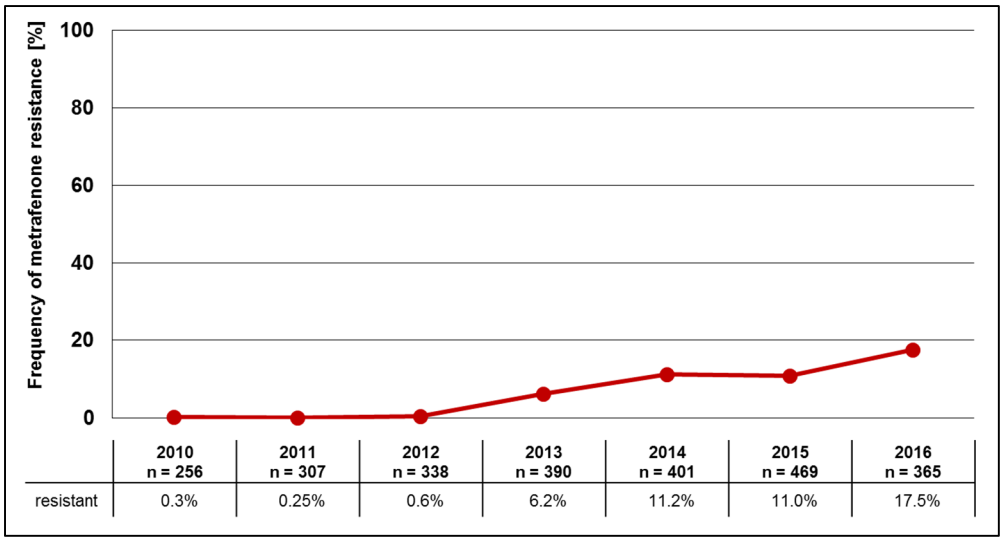


Figure 10: Development of the frequency of metrafenone resistance in *E. necator* from 2010 to 2016. The sensitivity of the isolates was determined using a detached leaf test. The frequency is represented as percentage resistant isolates, n=number of isolates analysed.

The frequency of resistant isolates was below 1% in the populations from 2010 – 2012, increased to 6.2% in 2013 and showed a further increase from 2013 - 2016 to 17.5%.

For the sensitivity monitoring from 2014 - 2016, detailed information about the distribution of metrafenone resistance and the number of analysed samples for each country is provided in Figure 11.

Figure 11: Frequency of metrafenone resistance in *E. necator* from 2014 to 2016. The results are shown as country split and the frequency is represented by the number of sensitive (blue) and resistant (red) isolates for the years 2014 (A), 2015 (B) and 2016 (C). The number of analysed isolates and the sampling method is shown in a table (right upper corner); field samples: samples and isolates; airborne monitoring: regions and populations.

Samples from France, Greece, Spain, Portugal and Slovenia showed an overall sensitive population. In Switzerland and Germany, low frequencies of resistant isolates (7% and 2%) were detected in 2014; samples from 2015 and 2016 were again fully sensitive. Higher frequencies of resistant isolates were observed in Slovakia (52 - 64%) and Czech Republic (38%). Fluctuating resistance frequencies were observed in Hungary with 40% in 2014, 88% in 2015 and 15% in 2016. The resistance frequencies in Austria and Italy showed an increase of resistant isolates from 2014 to 2016 (AT: 10% to 37%; IT: 15% to 24%).

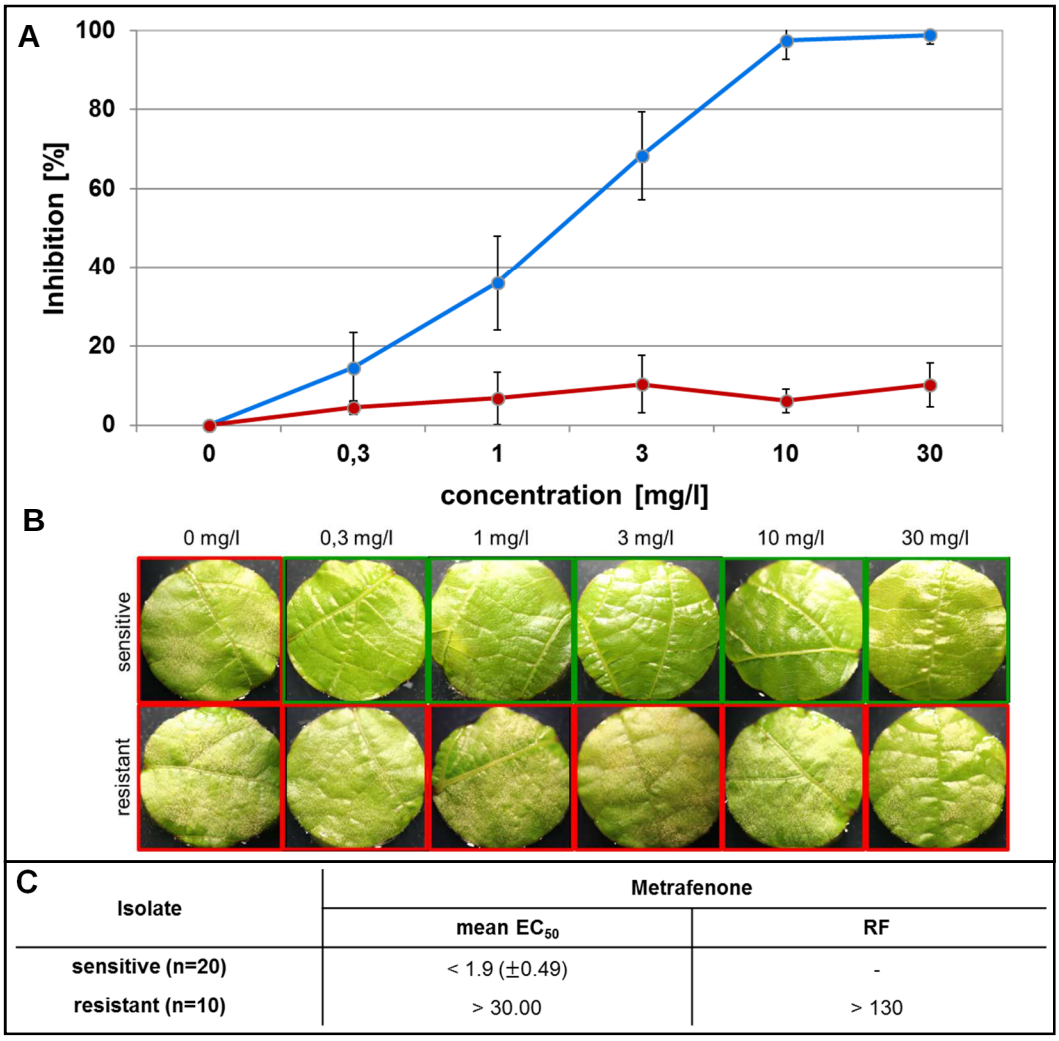
The frequency of metrafenone resistant isolates of *E. necator* is strongly dependent on the origin of the samples, and metrafenone resistance is not homogeneously distributed in European countries. High portions of resistance have been detected in the eastern regions of Europe. Four ´hot-spot regions´ can be identified, with respect to the emergence and the spread of metrafenone resistant isolates: Hungary, Czech Republic, Slovakia and Italy.

3.2.2.2 Characterisation of metrafenone resistant *E. necator* isolates

Some sensitive isolates and all isolates classified by EpiLogic as less sensitive during the European sensitivity monitoring in 2014 and 2015 were sent to BASF and cultivated. Randomly selected isolates were maintained as living isolate for further characterisation. The germination rate, the infection and growth behaviour of these *E. necator* isolates under metrafenone treatment were analysed.

Similar germination rates were observed for sensitive (84% – 92%) and resistant (75% – 88%) isolates in the untreated control (Supplementary material, Figure 41). No dose response depending on the tested metrafenone dilutions was observed, neither for the germination of the sensitive nor for the resistant isolates. These results indicate that metrafenone does not influence the germination of *E. necator*.

To assess the sensitivity loss of grape powdery mildew towards metrafenone, *in vivo* leaf disc tests were performed with several sensitive and resistant isolates (Figure 12).



C

Isolate	Metrafenone	
	mean EC_{50}	RF
sensitive (n=20)	< 1.9 (±0.49)	-
resistant (n=10)	> 30.00	> 130

Figure 12: Characterisation of metrafenone sensitive and resistant *E. necator* isolates. A: Inhibition curves of sensitive and resistant isolates on metrafenone dilutions using leaf disc tests. Mean EC_{50} values of sensitive isolates (n=20) are represented in blue and resistant isolates (n=10) in red (C). Error bars = 2xSE. B: Inoculated leaf discs treated with different metrafenone concentrations of a representative sensitive and resistant *E. necator* isolate after 10 d incubation; diseased leaf discs are represented by a frame in red (sporulation detected) and green (no sporulation). C: Mean EC_{50} values and resulting RF-value, compared to the baseline sensitivity.

Metrafenone sensitive and resistant *E. necator* isolates showed a similar infection behaviour on untreated grapevine leaves. Regarding the germination rate, the latent period and the sporulation behaviour, no differences were observed (data not shown). Infection symptoms of the majority of sensitive isolates were only observed on the untreated control. Some isolates characterised as sensitive showed slight infection symptoms on 1 and 3 mg/l. The EC_{50} values of sensitive isolates ranged from < 1 to 4.7 mg/l. Compared to the baseline sensitivity (0.09 – 0.39 mg/l) the range of higher EC_{50} values was observed. Growth and sporulation was not inhibited for resistant isolates. Infection symptoms were observed on leaf discs treated with

0.3, 1, 3, 10 and 30 mg/l metrafenone. Leaf disc tests of resistant isolates resulted in EC_{50} values of > 30 mg/l and RF values of > 130.

3.2.3 Phenotypical analysis of *E. necator* isolates under metrafenone treatment

Microscopy is a valuable tool to examine effects of fungicide treatment on the infection process of fungal pathogens. It has also been used to understand the biological effects of metrafenone on *B. graminis* f.sp. *tritici* (Opalski *et al.* 2006). Microscopic studies were performed with *E. necator* to analyse the biological effects of metrafenone. The activity of metrafenone on the infection process before (preventive) and after inoculation (curative) were assessed.

A: Developmental stages of *E. necator* under laboratory conditions on untreated grapevine leaves

The developmental stages of *E. necator* during the infection on untreated grapevine leaves and the staining were done in accordance to Leinhos *et al.* (1997) and Delp (1954). The infection process was evaluated 24 h, 48 h post-inoculation (hpi), 3 d post-inoculation (dpi), 4 dpi and 7 dpi (Figure 13).

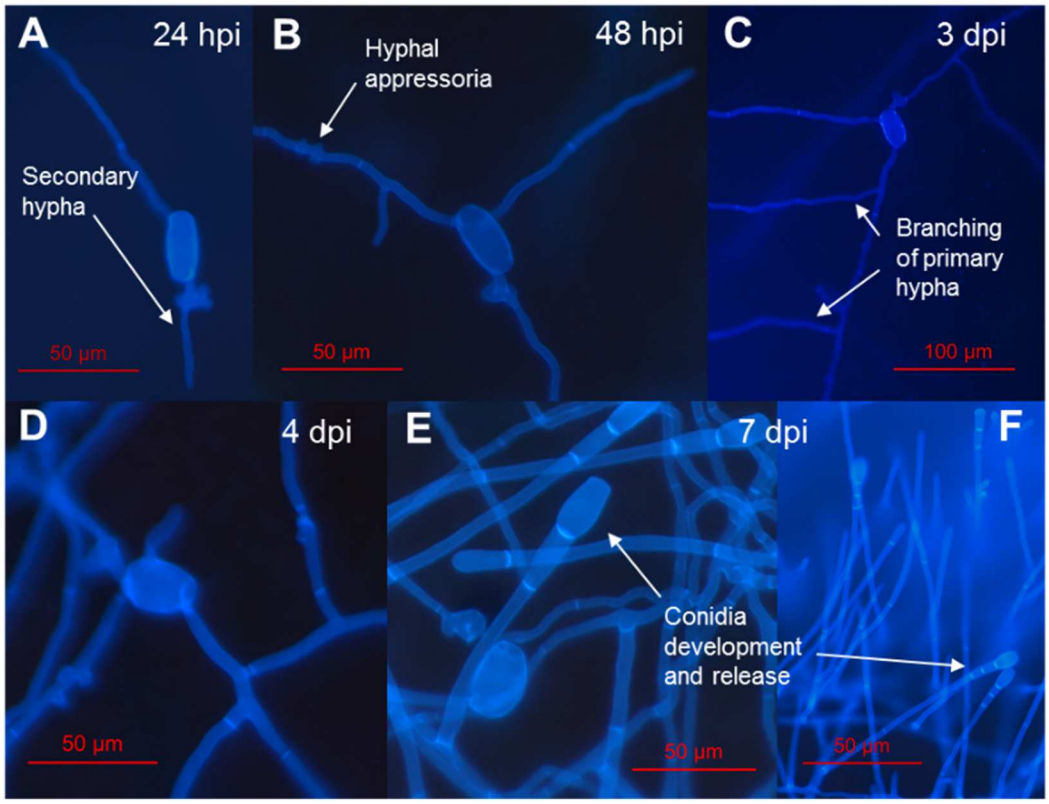


Figure 13*:* Infection process of *E. necator* on untreated grapevine leaves using Uvitex 2B staining. Evaluation was done 24 hpi, 48 hpi, 3, 4 and 7 dpi; characteristic structures are indicated with an arrow.

After 24 hpi, typical multilobed primary appressoria had developed, the primary hypha emerged from the opposite end of the conidium and the secondary hypha was extended from the appressorium (Figure 13A). First hyphal appressoria were built and branching of primary hypha was observed 48 hpi (Figure 13B). Further hyphal appressoria and branching of primary hypha was initiated 3 dpi and 4 dpi (Figure 13C and D). Multiseptated conidiophores with mature conidia were formed and released at 7 dpi (Figure 13E and F). The development in the absence of metrafenone is consistent with observations made by Leinhos *et al.* (1997).

B: Developmental changes of *E. necator* under preventive and curative metrafenone treatment on grapevine leaves

Preventive application of metrafenone was done 1 day before inoculation (dbi). Evaluation of the preventive activity was carried out 24 hpi, 48 hpi and 7 dpi. At 24 hpi, germination was not significantly reduced on metrafenone treated leaves. Further development was frequently stopped (90%) after the formation of primary appressoria. Primary appressoria were often malformed (Figure 14A and C) and sometimes secondary hyphae were directly formed (Figure 14D). At 48 hpi, elongation and branching of secondary hyphae was rarely observed after metrafenone treatment. Even after 7 dpi, no further progress of conidial development was observed (Figure 14B and E). In addition to the observation of morphological changes after preventive treatment, two curative tests were performed. Detached leaves were inoculated with the reference strain and either at 2 dpi (K2), when haustoria and hyphal development should normally have been set or 5 dpi (K5), just before conidia initiation, they were treated with 10 mg/l metrafenone. For K2, the subsequent fungal development was assessed 3, 4, 7 and 8 dpi. At 3 dpi no obvious differences were observed between untreated and treated grapevine leaves. Since Uvitex 2B staining does not clearly distinguish between dead and living hyphae, the evaluation of hyphal cell death after metrafenone application was not assessed. At 4 dpi some hyphal tips started to grow in a curved manner, suggesting they had lost proper orientation. For some hyphae, a coalescent growth was observed (Figure 14P). At 7 and 8 dpi, obvious effects of metrafenone treatment were visible. Malformed hyphae were frequently observed, showing more curved and coalescent growth (Figure 14G and H). The diameter of hyphae was greater than that of untreated hyphae (Figure 14K) and mycelium penetration sites were more abundant and frequently malformed (Figure 14F, I and J). Hyphal tips were sometimes swollen and Uvitex 2B staining accumulated there, whereas for some hyphae the staining was very weak or failed (Figure 14K and L).

Under K5 conditions, morphological changes on the conidiation of *E. necator* were determined and subsequent fungal development was assessed 6 dpi and 7 dpi. At 6 dpi multiseptated conidiophores were present but conidia formation was reduced and slightly delayed compared to the untreated control. Frequently, conidiophores had an irregular septation pattern and

subsequent conidia development was rarely observed at 7 dpi (Figure 14M). When conidiation was initiated, conidia had a tubular or curved shape (Figure 14N and O). As observed in K2, hyphae were thicker and had a strong tendency for a coalescent growth (Figure 14P).

Taken the results of the preventive and curative conditions used together, abnormalities in appressoria formation, hyphal growth and malformation of conidiophores and conidia were observed for *E. necator* under metrafenone treatment.

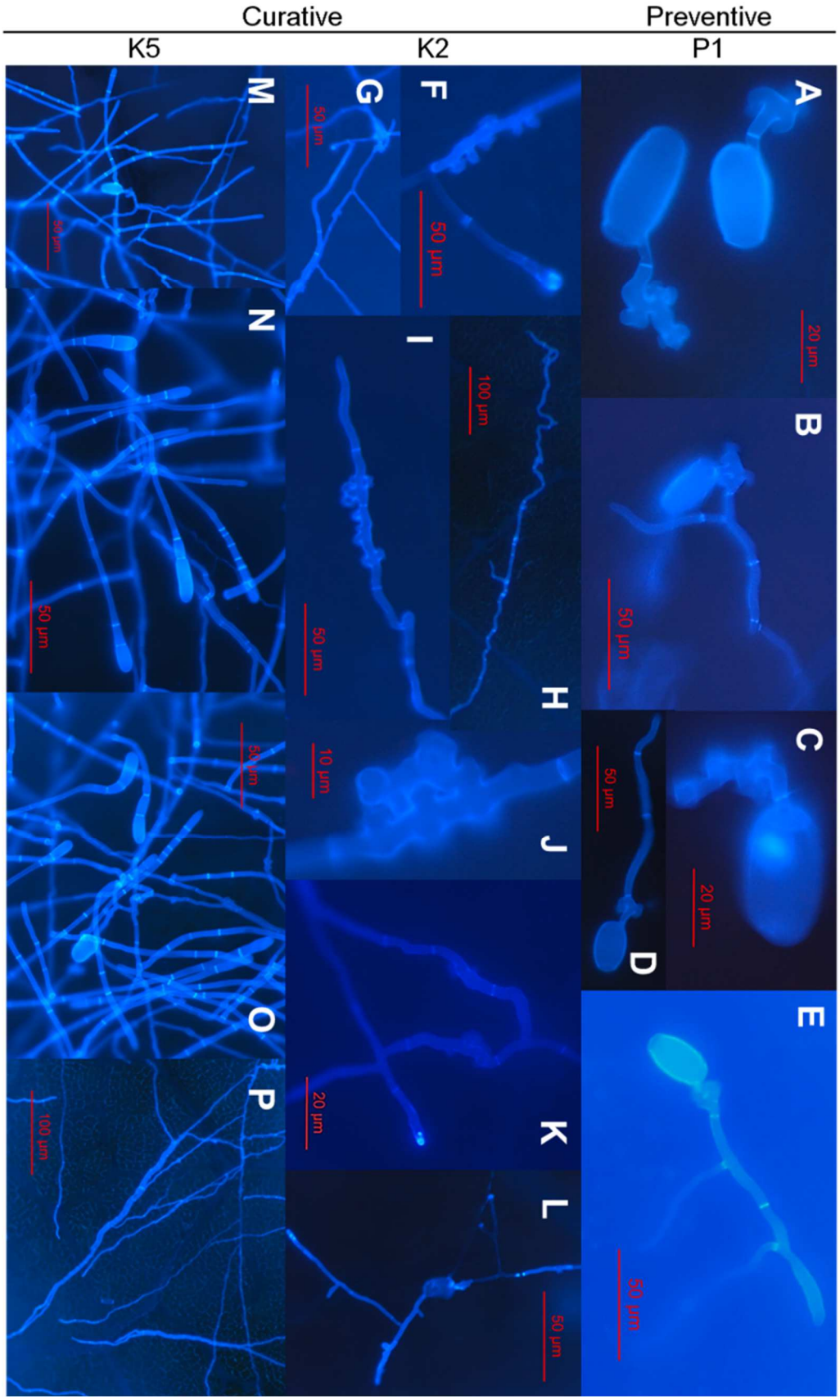


Figure 14: Morphological effects of preventive and curative application of 10 mg/l metrafenone on the development of *E. necator* using Uvitex 2B staining. Application was done 1d before inoculation (P1; A - E), 2dpi (K2; F - L) or 5 dpi (K5; M - P).

C: Developmental changes of metrafenone resistant *E. necator* isolates on metrafenone treated grapevine leaves

Morphological effects of metrafenone treatment were additionally analysed for a metrafenone resistant *E. necator* isolate (1104) (Figure 15). The analysis was done simultaneously with the sensitive strain using the same preventive and curative conditions.

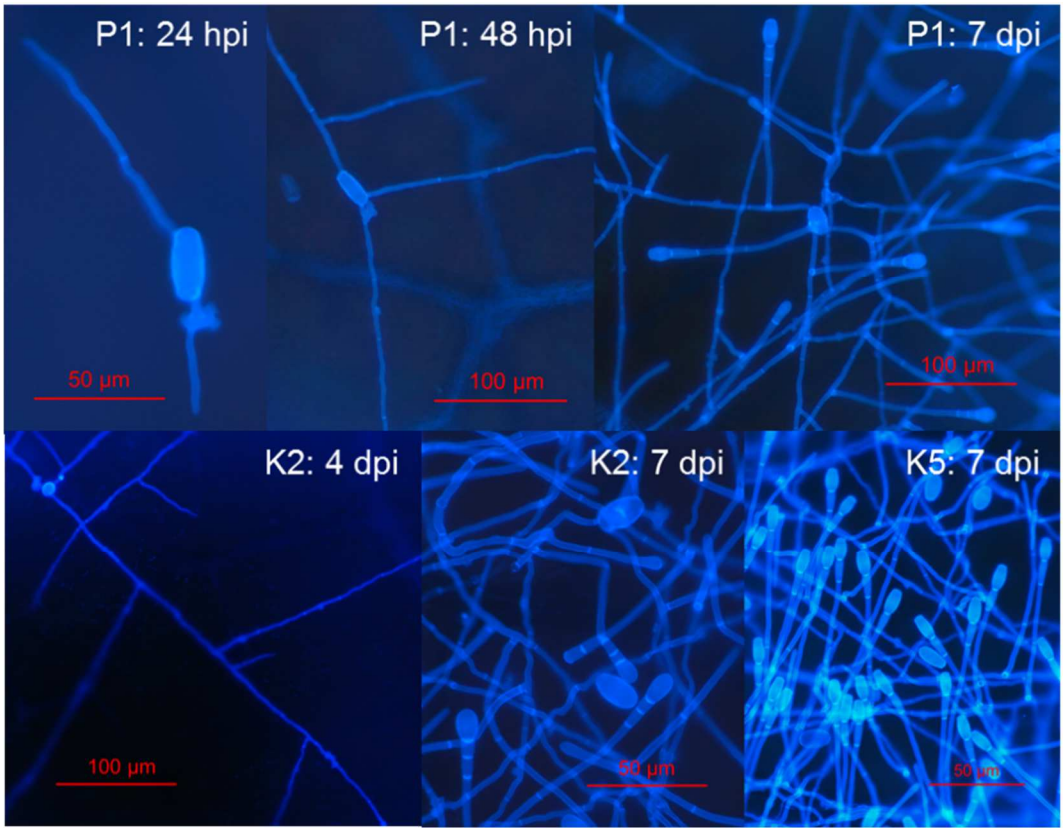


Figure 15: Development of a metrafenone resistant *E. necator* isolate (1104) on treated grapevine leaves using Uvitex 2B staining. P1: application 1 dbi; K2: application 2 dpi; K5: application 5 dpi.

The application conditions used (preventive and curative) of metrafenone had no influence on the infection process and morphology of the resistant reference isolate 1104. The infection process and the morphology of the infection structures were identical to the sensitive reference strain 1111 on untreated leaves. This contributes to the observations made during the characterisation of *E. necator* isolates, and concludes that there are no morphological differences between metrafenone sensitive and resistant isolates.

D: Comparison of developmental changes between *E. necator* and *B. graminis* f.sp. *tritici* under metrafenone treatment

Developmental changes triggered by metrafenone treatment on grape powdery mildew, as shown in Figure 14 were compared with the effects of metrafenone observed by Opalski *et al.* (2006) on wheat powdery mildew (Table 14).

Table 14: Comparison of developmental changes after metrafenone treatment between *B. graminis* f.sp. *tritici* (4 mg/l metrafenone) and *E. necator* (10 mg/l metrafenone).

		Erysiphe necator	***Blumeria graminis*** f.sp. ***tritici*** **(Opalski *et al.* 2006)**
Preventive	24 h, 48 h and 7 dpi	Growth stopped after primary appressoria formation	Strongly reduced haustoria formation
		Sometimes malformed primary appressoria	Multilobed, sometimes malformed appressoria
		Rare formation of primary hyphae, sometimes directly 2nd hyphae	Rare elongation of 2nd hyphae
Curative	4, 7 and 8 dpi	Staining failed	Mycelium collapsed
		Hyphal tips swollen	Hyphal tips swollen and burst or bifurcated
		Hyphal appressoria and mycelium penetration sites abundant, closely spaced, frequent and malformed	Secondary appressoria abundant, closely spaced and frequent bifurcated
		Hyphae elongated, curved and with greater diameter	Diameter of hyphae greater
	6 and 7 dpi	Conidiophores elongated and with a malformed septation pattern	Conidiophores elongated with malformed septation
		Conidia formation rare; tubular or curved	fail to form conidia

Under preventive conditions, similar effects of metrafenone treatment were observed for both powdery mildew species. Differences were only observed due to developmental properties specific for each species. Appressoria of *E. necator* are naturally multilobed, whereas multilobed appressoria of *B. graminis* f.sp. *tritici* indicate a malformation due to metrafenone treatment. Hyphal collapse under curative application could not been evaluated for *E. necator*, but can be proposed as Uvitex 2B staining failed for some hyphae. A slightly curved hyphal growth occurs naturally in the development of *E. necator* under untreated conditions, but is strongly enhanced under metrafenone treatment. For both organisms, conidiation is delayed or inhibited and aberrant conidiophores were formed. Conidiophores showed an irregular septation pattern, elongated tubes and the resulting conidia were tubular or curved. In summary, the effects of metrafenone application on the infection process of *B. graminis* f.sp. *tritici* and *E. necator* are very similar, suggesting a similar mode of action of this compound on both organisms.

3.2.4 Fitness costs associated with metrafenone resistance in *E. necator*

The knowledge about fitness costs associated with fungicide resistance helps to predict the speed of the development and the spread of fungicide resistance, and to imply appropriate anti-resistance strategies. Observations in the laboratory with the first resistant *E. necator* isolates indicated that these isolates were difficult to maintain in culture over time. In fact, single metrafenone resistant *E. necator* isolates where shown to be fully sensitive after several cycles of cultivation on untreated grapevine leaves (Stammler *et al*. 2014). The loss of resistance and indications for fitness penalties associated with metrafenone resistance in *B. graminis* f.sp. *tritici* (Figure 4) suggested that metrafenone resistance in *E. necator* may also be associated with fitness penalties. On this account, the fitness of metrafenone resistant *E. necator* isolates in the absence of selection pressure was investigated. Mixtures of sensitive and resistant *E. necator* isolates were examined in growth competition tests. The competitiveness assays were conducted under optimal growth conditions on young detached grapevine leaves, which are known to be more susceptible to *E. necator*. In this study, two different types of fitness tests were performed (chapter 2.11): In the first test, pairs of single sensitive and resistant isolates (paired) were tested. In the second test, a combination of several sensitive and resistant isolates (populations) was used.

3.2.4.1 Growth competition of paired mixtures

Considering that the target site of metrafenone and therefore, the precise genetic resistance mechanism for metrafenone is not known, a direct quantification of the frequency of metrafenone resistant isolates was not possible. On the other hand, several metrafenone resistant isolates also carry a single target site mutation in the cytochrome *b* gene, the G143A. This mutation can cause high resistance towards QoI-fungicides, which is well known and widespread in various fungal plant pathogens without causing any fitness defect.

The G143A mutation was therefore used as a molecular marker for the detection of the frequency of metrafenone resistant isolates. The isolates used in the fitness test were chosen with respect to their QoI and metrafenone resistance backgrounds. Paired mixtures of metrafenone sensitive and resistant *E. necator* isolates were used to observe the development of metrafenone resistance. Isolates sensitive towards metrafenone and QoIs (without G143A) were mixed with isolates resistant to metrafenone and QoIs (with G143A) in a ration 1:1 (Scenario 1). In a second scenario (Scenario 2), isolates sensitive towards metrafenone and resistant to QoIs (with G143A) were mixed with isolates resistant to metrafenone and sensitive to QoIs (without G134A) (Table 15).

Table 15: *E. necator* isolates used for the G143A based competitiveness test. Resistance background and mixtures used in the growth competition test; MFN-res: metrafenone resistant.

Test		Isolate ID	Isolate-mixture	Resistance phenotype	QoI	MFN	SDHI
Frequency of metrafenone resistance via G143A	Scenario 1	1030	I a, b	wild-type	sensitive	sensitive	sensitive
		1111	I c, d	wild-type			
		1050	I a, I c	MFN-res	G143A	resistant	
		1106	I b, I d	MFN-res			
	Scenario 2	1054	II a, b	wild-type	G143A	sensitive	sensitive
		1057	II c, d	wild-type			
		1112	II e, f	wild-type			
		1038	II a, c, e	MFN-res	sensitive	resistant	
		1105	II b, d, f	MFN-res			

The mixtures were cultivated over 12 growth cycles and single isolates were used as a control. In all performed fitness tests, a growth cycle is defined as the time from the inoculation to the evaluation of the diseased leaf area. The frequency of metrafenone resistance was thereby calculated using the percentage of G143A after each growth cycle with the help of pyrosequencing. For both scenarios, the development of metrafenone resistance over time was observed (Figure 16).

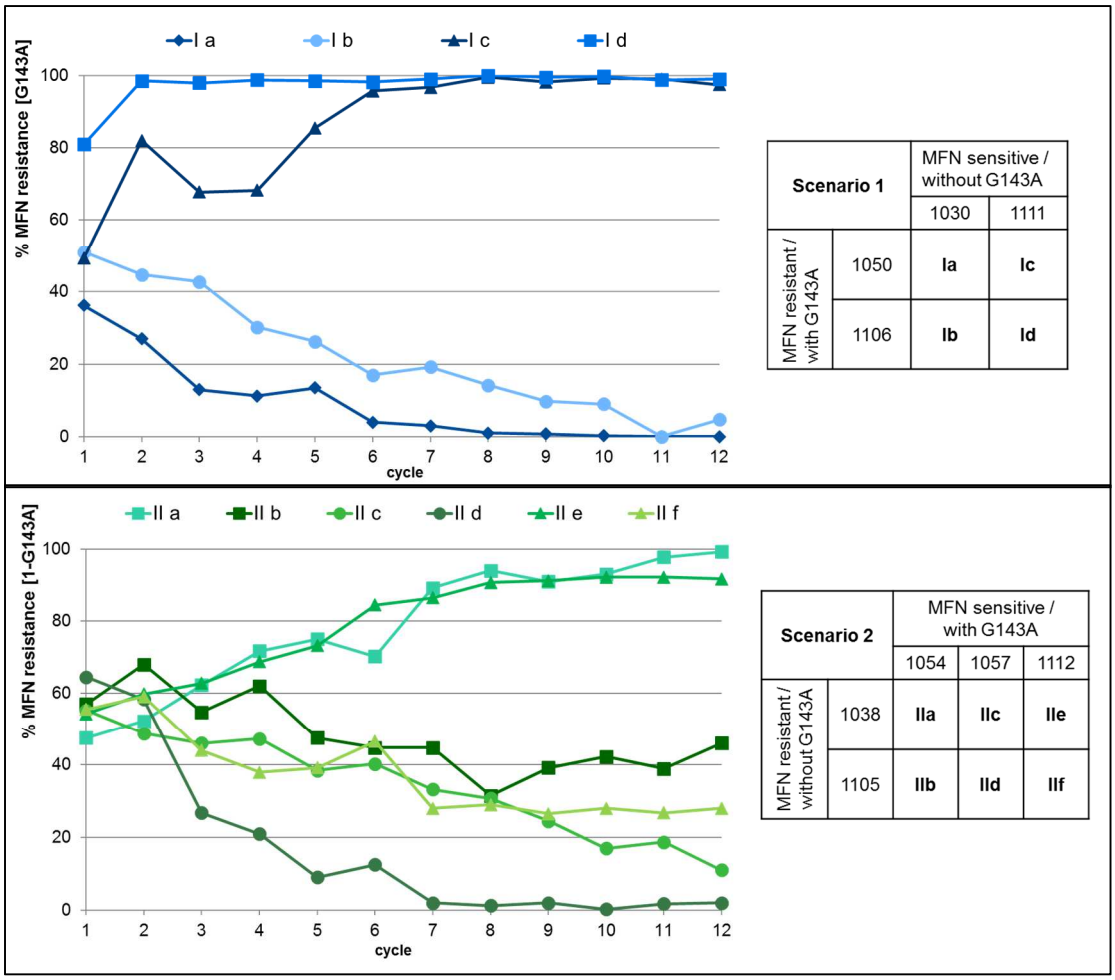


Figure 16: Changes over time in the frequency of metrafenone resistance in artificial mixtures of metrafenone resistant and sensitive isolates of *E. necator* cycled on untreated grapevine leaves. The frequency of metrafenone resistance was indirectly determined as percentage of G143A by pyrosequencing. Each cycle represents 10 days of incubation. Scenario 1 (Mixtures of metrafenone sensitive (without G143A) and metrafenone resistant (with G143A) isolates); Scenario 2 (Mixtures of metrafenone sensitive (with G143A) and metrafenone resistant (without G143A) isolates); in scenario 2, the frequency of metrafenone resistance is the inverse of the quantified G143A frequency [1-G143A].

For scenario 1, mixtures with strain 1030 (MFN Ia and Ib) showed a significant decrease of metrafenone resistance down to 0% over time. The mixtures Ia and Ib were classified by the partitioning algorithm in the same group, also called ´node´, and a negative regression coefficient of -4,25 was estimated for these mixtures (Supplementary material, Figure 48). In contrast, mixtures with the second sensitive isolate 1111 (MFN Ic and Id) showed a strong increase of G143A and reached 100% after the 8th cycle. Also, here the partitioning algorithm classified the mixtures Ic and Id in the same node and a positive regression coefficient of +2,31 was estimated (Supplementary material, Figure 48). The results for scenario 1 give no evidence for effects of metrafenone resistance on fitness and are dominated by intrinsic differences in the competitiveness of single isolates.

Four of the analysed mixtures in scenario 2 (MFN II b, c, d and f) showed a significant decrease of metrafenone resistance, where the resistant partner seems to be less competitive than the sensitive isolate. The two remaining mixtures (MFN II a and e) show an increase of 92% and 100% of metrafenone resistance over time. In these mixtures, the resistant partner seems to be more competitive than the sensitive strain. Two of three mixtures with the metrafenone resistant isolate 1038 (MFN II a and e) show a positive increase of resistance frequencies with a regression coefficient of +4,27. In contrast, all the mixtures with the resistant isolate 1105 showed in all mixtures a significant decrease of resistance over time (Supplementary material, Figure 48). This leads to the suggestion that the change in the development of metrafenone resistance is not only dependent on the sensitive isolate used but also on the resistant isolate used.

In contrast to the competitiveness tests of *B. graminis* f.sp. *tritici*, the presence of fitness penalties associated with metrafenone resistance in *E. necator* can neither be proved nor negated by growth competition tests using paired mixtures and the molecular marker G143A. For the fitness tests with *B. graminis* f.sp. *tritici,* only one sensitive wheat powdery mildew isolate had been used as mixing partner with resistant isolates, which may be intrinsically less competitive and lead to the obtained results.

3.2.4.2 Competitive ability of population mixtures

Another approach, with more than one pair of isolates, was conducted in order to further investigate possible costs connected with metrafenone resistance. The development of metrafenone resistance in artificial population mixtures was directly observed as diseased leaf area on untreated and metrafenone treated leaves. Three different population mixtures were tested: a sensitive-mixture, a resistant-mixture and a sensitive-resistant-mixture and all isolates were mixed in the same portions (Figure 6). The percentage of diseased leaf area on untreated grapevine leaves was assessed after each cycle to monitor the intensity of sporulation. Changes in the frequency of metrafenone resistance were checked on metrafenone treated leaves after the first, the fifth and the ninth cycle. The same mixtures were used for inoculation of both untreated and treated grapevine leaves. In the case of a fitness cost connected with metrafenone resistance, it was expected that the sensitive-resistant-mixture would show a decrease of resistance over time. Three independent tests were performed: A, B and C. The isolates used for the population mixtures are shown in Table 16.

Table 16: *E. necator* isolates used for the population based competitiveness test. The resistance background and the test in which the isolates were used is shown; MFN-res: metrafenone resistant.

Test	Isolate ID	Resistance phenotype	Test
Frequency of metrafenone resistance via diseased leaf area	1054	wild-type	A, B
	1055	wild-type	A, B
	1056	wild-type	A, B
	1057	wild-type	A, B
	1058	wild-type	A, B
	1109	wild-type	C
	1110	wild-type	C
	1111	wild-type	C
	1112	wild-type	C
	1113	wild-type	C
	1050	MFN-res	A, B
	1051	MFN-res	A, B
	1052	MFN-res	A, B
	1053	MFN-res	A, B
	1059	MFN-res	A, B
	1105	MFN-res	C
	1106	MFN-res	C
	1107	MFN-res	C
	1108	MFN-res	C
	1117	MFN-res	C

The development of metrafenone resistance was calculated relative to the untreated control (Figure 17).

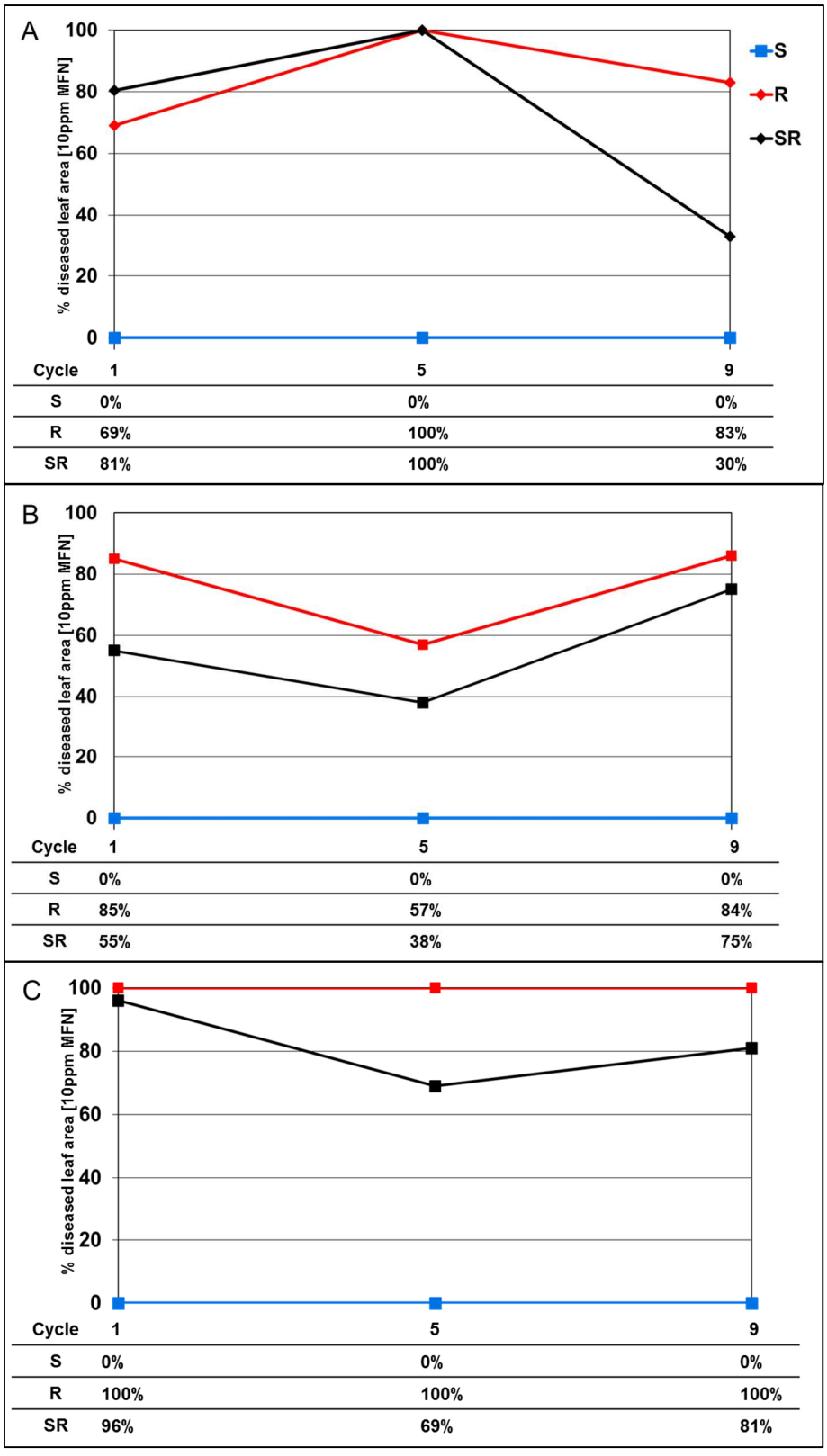
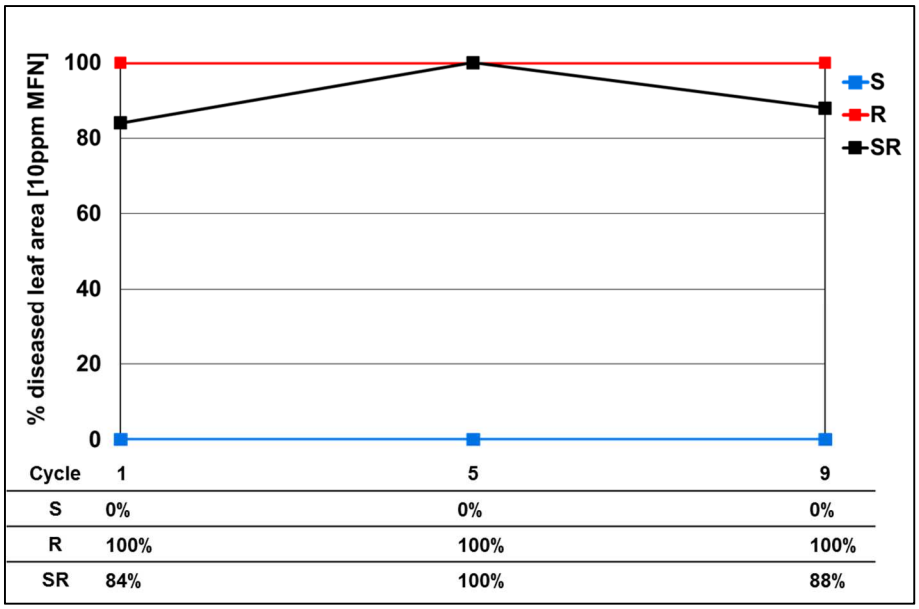


Figure 17: Changes in the metrafenone resistance frequency in artificial population mixtures of sensitive and resistant *E. necator* isolates determined as diseased leaf area on metrafenone treated leaves. Each cycle represents 10 d incubation on untreated grapevine leaves. After cycle 1, 5 and 9 the development of metrafenone resistance was evaluated on metrafenone treated leaves. S: Mixture of sensitive isolates, R: Mixture of resistant isolates and SR: Mixture with sensitive and resistant isolates. Three independently repeated tests are shown as A, B and C.

In all three tests, the sensitive-mixture did not show any disease symptoms on metrafenone treated leaves. The frequency of metrafenone resistance in the SR-mixture in test A showed an increase to cycle 5 and a strong decrease to cycle 9, which is consistent with the development of resistance in the R-mixture. The decrease in mixture SR is stronger than the decrease in mixture R, which may indicate that the resistant fraction in this mixture less competitive than the sensitive fraction. A similar development of metrafenone resistance was observed for mixtures SR and R in test B. The frequency decreases after cycle 5 and increases to cycle 9, which indicates that both fractions have similar competitive abilities. In test C, despite of a stable and high frequency of resistance in the R-mixture, the portion of resistance slightly decreases in mixture SR. Altogether, there is only a slight tendency, indicating a reduced competitiveness (A and C) of metrafenone resistant isolates. As in the experiments with single isolate mixtures, no clear tendency linked to fitness costs associated with metrafenone resistance could be observed with this test series.

3.2.4.3 Competitive ability of population mixtures under sulphur treatment

Previous competitiveness assays were conducted under optimal growth conditions. It could be argued, that these ideal conditions for an infection of grape powdery mildew may compensate or mask potential fitness costs of metrafenone resistance. To test whether fitness costs is resistant isolates become visible in a more stressful situation, the multisite fungicide sulphur (Kumulus®) was used in the following test, to result in a more stressful situation. It was shown in a preliminary test, that with a concentration of 50 mg/l sulphur, most of the isolates were inhibited ≤ 50%. This sublethal concentration was therefore considered as suitable for the purpose of the test. The same experimental conditions, except the use of sulphur treated leaves instead of untreated, as described in 2.11.1, were applied. Thereby the mixtures were inoculated on sulphur treated leaves and grown for several cycles. Isolates used in this test were the same as in test C and are listed in Table 16. Also in this test, growth was evaluated after each cycle on sulphur treated leaves and after the first, the fifth and ninth cycle on metrafenone treated leaves. The frequency of metrafenone resistance, relative to the untreated control, on metrafenone treated leaves is shown in Figure 18. The resistance frequency of the R-mixture remains at a high level over all cycles. For the SR-mixture, an increase of metrafenone resistance from cycle 1 to cycle 5 and a slight decrease to cycle 9 was observed. Resistant isolates in the SR-mixture showed neither a tendency to decrease nor an increase. The results of this test indicate a similar competitive ability of sensitive and resistant isolates in these mixtures and under the stress conditions used.



Cycle	1	5	9
S	0%	0%	0%
R	100%	100%	100%
SR	84%	100%	88%

Figure 18: Changes in metrafenone resistance frequency in artificial population mixtures of sensitive and resistant *E. necator* isolates determined as diseased leaf area on metrafenone treated leaves. Each cycle represents 10 d incubation on sulphur treated grapevine leaves. After cycle 1, 5 and 9 the development of metrafenone resistance was additionally evaluated on metrafenone treated leaves.

Altogether, the results of the performed fitness tests (3.2.4.1, 3.2.4.2 and 3.2.4.3) do not indicate that metrafenone resistant *E. necator* isolates are less competitive than sensitive isolates. Clear conclusions about fitness costs connected with metrafenone resistance cannot be drawn based on these results.

3.2.5 Investigation of the resistance mechanism of metrafenone by whole genome sequencing

The aryl-phenyl-ketones belong to a small group of powdery mildew specific fungicides. Despite the fact that resistant wheat and grape powdery mildew populations have already been identified, the exact mode of action of metrafenone is still unknown. Nowadays complete genome sequences of several pathogenic bacteria and fungi are available. Due to the high resolution of the generated data combined with decreasing analysis costs, whole genome sequencing (WGS) is becoming a valuable tool for the detection of drug-resistance in microorganisms (Köser *et al.* 2014). In this part of the study, the aim was to elucidate the resistance mechanism of metrafenone by comparative WGS.

3.2.5.1 Whole genome sequencing of *B. graminis* f.sp. *tritici*

As a model organism for obligate biotrophic fungi, numerous studies, including determination of genome sequences, have been done for *B. graminis* f.sp. *tritici*. These analyses provide information about differences and problems between the sequencing of non-biotrophic and biotrophic ascomycetes. The estimated genome size of powdery mildews varies between 120 - 180 mega bases (Mb), which is up to four times larger than the genome size of related non-biotrophic fungi (30-40 Mb). The large genome size is due to a massive proliferation of transposable elements (Spanu *et al.* 2010; Wicker *et al.* 2013). The reproduction of powdery mildew populations seems to be predominantly clonal, which results in distinct haplo-groups and nonrandomly associated alleles among loci (Wicker *et al.* 2013; Pirondi *et al.* 2015). This enhances the probability to identify resistance associated genes by comparative genome analysis of sensitive and resistant powdery mildew isolates.

In this study, whole genome sequencing was performed for the detection of single-nucleotide polymorphisms (SNP) between sensitive, moderately adapted and resistant isolates of *B. graminis* f.sp. *tritici*. The aim was to identify genes that might contribute to metrafenone resistance and which may be linked to the mode of action of this compound. Sequencing was done by DNA Landmarks (Quebec, Canada) or BGI Hong Kong (Hong Kong, China) using illumina sequencing on a HiSeq machine with 2x150 bp paired end (PE) reads. The sequencing data was analysed and evaluated by the internal bioinformatic services of BASF SE.

Except for isolate B (sensitive reference strain), all isolates originated from the German airborne monitoring performed in 2014 (Table 17).

Table 17: Origin and resistance type of *B. graminis* f.sp. *tritici* isolates. De novo sequencing of strain 2588 and whole genome sequencing of isolates A - F.

Isolate	Reference genome	A	B	C	D	E	F
ID	2588	2591	EP3	2596	2593	2608	2605
Origin	Lübeck-Rostock	Lübeck-Rostock	Limburgerhof	Lübeck-Rostock	Lübeck-Rostock	Nyborg-Kopenhagen	Flensburg-Kappeln-Eckernförde
Resistance type	sensitive	sensitive	sensitive	moderately adapted	moderately adapted	resistant	resistant

The genomes of these strains were very similar to each other and the sequencing depth was between 50 – 100 fold. To identify differences between the strains, reads had to be mapped to an annotated reference genome. The best publicly available reference genome (strain 96224, Wicket *et al.* 2013) has a genome size of 158 Mb, separated in 1868 contigs larger than 2000 bp, and 6525 annotated genes. De novo sequencing and annotation of another sensitive strain (2588) was done, in order to prevent omitting interesting mutations. The genome of this strain was used as second reference genome. With the HiSeq, 2x150 bp PE reads as a basis for the assembly, the final genome had a size of 191 Mb, split over 140.778 contigs larger than 300 bp (N50: 2649bp) and 63.038 annotated genes.

Compared to the two reference genomes, the total number of polymorphisms in all sequenced isolates is shown in table 18. The six isolates differed at 151.695 to 190.922 polymorphisms from the reference genome 2588. Compared with the reference strain 96224, the number of polymorphisms varied between 160.471 and 175.404 in the resequenced isolates. Isolate B, which was isolated in the 1990s, shows a higher number of polymorphisms compared to both reference genomes.

Table 18: Number of polymorphisms for the whole genome sequencing of metrafenone sensitive and adapted (moderately adapted and resistant) isolates of *B. graminis* f.sp. *tritici*. The isolates were compared with the reference genomes 96224 and 2588.

2588	A	B	C	D	E	F	Total
Total	166.798	190.922	167.142	151.695	165.360	166.701	411.993
SNPs	162.352	185.822	162.726	147.581	160.926	162.240	401.301
INDEL	4.446	5.100	4.416	4.114	4.434	4.461	10.692
SNPs							
Intergenic	125.687	142.943	125.445	114.590	124.364	124.855	307.611
genetic	34.789	35.934	35.629	32.991	35.307	35.795	45.567
In CDS	30.266	31.441	30.970	28.786	30.783	31.215	40.001
Non silent	20.249	21.126	20.849	19.304	20.460	20.956	26.465

96224	A	B	C	D	E	F	Total
Total	160.471	175.404	160.939	162.640	163.961	163.579	357.626
SNPs	154.028	168.626	154.551	156.059	157.359	157.003	346.034
INDEL	6.443	6.778	6.388	6.581	6.572	6.576	11.592
SNPs							
Intergenic	148.523	162.541	149.140	150.562	151.834	151.304	333.644
genetic	5.505	6.085	5.411	5.497	5.555	5.699	14.092
In CDS	4.790	5.341	4.710	4.819	4.863	5.014	12.390
Non silent	2.194	2.476	2.140	2.195	2.203	2.304	5.602

A total of 5.602 SNPs (excluding synonymous mutations) in coding sequences (CDS) were identified compared to 96224. Between the reference strain 2588 and the sequenced isolates, the number of non-silent genetic mutations was much higher (26.465), which can be mainly explained with the higher number of annotated genes in the respective genome. Many of these SNPs were identified in one, two or three of the used isolates, including the sensitive isolates. To filter for SNPs with a likely effect on resistance, only those SNPs present in all resistant strains (C, D, E, F), but not in the sensitive isolates were selected. This resulted in only 31 non-silent SNPs for the reference strain 96224 and 245 non-silent SNPs for the reference genome 2588 in CDS. Only six genes with a non-silent SNP in CDS regions were identified for both reference genomes (Supplementary material, Table 28). The predicted functions of the coding proteins were analysed to find connections or hints regarding the biological effects of metrafenone. Two of them, annotated as an o-acyltransferase and a Rsc (remodelling the structure of chromatin) complex subunit, where chosen for further investigations (Table 19). The investigation results of the Rsc complex subunit are described below in chapter 3.2.5.3.

Table 19: Mutations of genes in the four resequenced adapted (moderately adapted and resistant) *B. graminis* f.sp. *tritici* isolates compared with both reference genomes (96224 and 2588).

Annotation (e-value)	Predicted function	Strains	Amino acid exchange	Position
Rsc complex subunit (0)	Chromatin remodelling	C, D, E, F	R → K	152
O-acyltransferase (1.1e-228)	Phospholipid remodelling	C, D, E, F	V → I	415

Analysis of the gene coding for a o-acyltransferase

A promising target site mutation could be identified in the gene of a o-acyltransferase. The gene structure and the corresponding mutations are shown in figure 19.

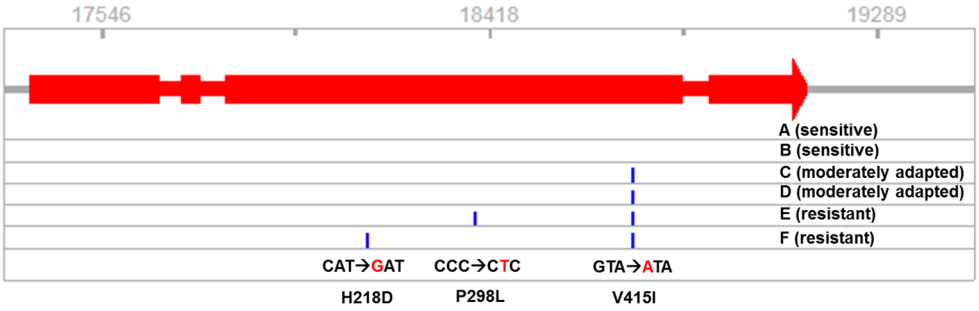


Figure 19: Gene structure the o-acyltransferase gene of the sequenced *B. graminis* f.sp. *tritici* isolates compared to the reference genome 96224. The identified nucleotide exchanges and the resulting amino acid substitutions are shown.

The sequence of the o-acyltransferase gene of the sequenced sensitive isolates was identical, compared to both reference strains. An amino acid substitution from valine to isoleucine at position 415 was identified in strains C and D (moderately adapted) and in the resistant isolates E and F. In addition to this mutation, isolates with the resistance phenotype both showed another mutation. In isolate E, an amino acid exchange from proline to leucine at position 298 and in isolate F, the substitution from histidine to aspartic acid at position 218.

The o-acyltransferase gene was amplified and sequenced for further three sensitive, three moderately adapted and four resistant wheat powdery mildew isolates. The majority of the isolates were identical in their sequence and showed no exchanges in their amino acid sequence. However, the sensitive isolate 2611 and the resistant isolate 2626 showed the V415I mutation. Besides this amino acid substitution, the sensitive strain 2611 showed the mutation P298L, whereas the H218D mutation could be detected in the resistant isolate 2626 (Supplementary material, Table 29).

The o-acyltransferase protein sequence of *B. graminis* f.sp. *tritici* was compared with that of other fungal pathogens (Figure 20).

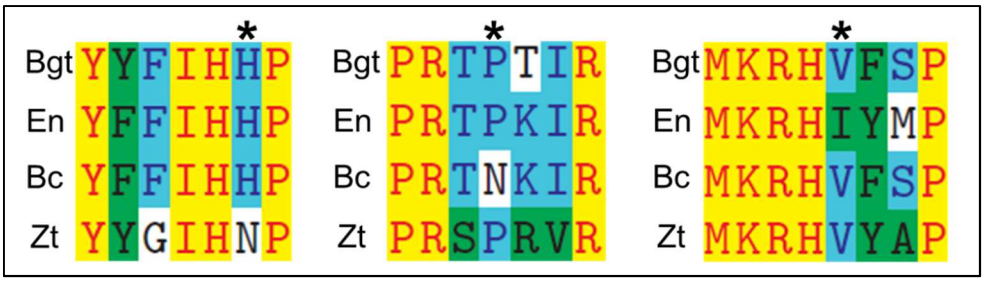


Figure 20: Degree of conservation across different species of substituted amino acids in the o-acyltransferase protein sequence of *B. graminis* f.sp. *tritici*. The three picture details show surrounding regions of the substituted amino acids. Conserved residues are coloured in yellow (all match), blue (≥ 50%), green (similar) and white (non conserved). Substituted amino acids are marked with an asterisk. Sequences originate from *B. graminis* f.sp. *tritici* (Bgt), *E. necator* (En), *B. cinerea* (Bc),) and *Z. tritici* (Zt).

The degree of conservation of amino acids histidine (H218), proline (P298) and valine (V415) of *B. graminis* f.sp. *tritici* compared to protein sequences of *E. necator*, *B. cinerea* and *Z. tritici* resulted in ≥ 50% identity. The positions of these amino acid exchanges are not highly conserved across the species analysed. Simultaneously, the homologous gene of the o-acyltransferase of *E. necator* was amplified for three sensitive and three resistant isolates. The sequences were compared and no differences between metrafenone sensitive and resistant were observed (supplementary material, Figure 42). Furthermore, all sequenced isolates already showed an isoleucine at homologous position of the V415I of *B. graminis* f.sp. *tritici* (Figure 20; supplementary material, Figure 43).

These results indicate that the o-acyltransferase protein is unlikely to be linked to the mode of resistance to metrafenone and does not seem to contribute to metrafenone resistance in wheat and grape powdery mildew.

3.2.5.2 Effect of metrafenone on non-biotrophic ascomycetes

Besides powdery mildews, metrafenone is used for the control of *Oculimacula* spp., the causal agent of the eye spot disease on wheat. The effect of metrafenone on the development of *Oculimacula* spp. was assessed in a microtiter and an agar plate test. Furthermore, the activity of metrafenone on the sporulation of the fungal model organism *A. nidulans* seems to be affected (Schmitt *et al*. 2006) and was also investigated.

Activity of metrafenone on *Oculimacula* spp.

Two reference strains were chosen for these investigations (Isolate 1 and 2). The resulting inhibition curves result from two independent microtiter tests and are shown in figure 21.

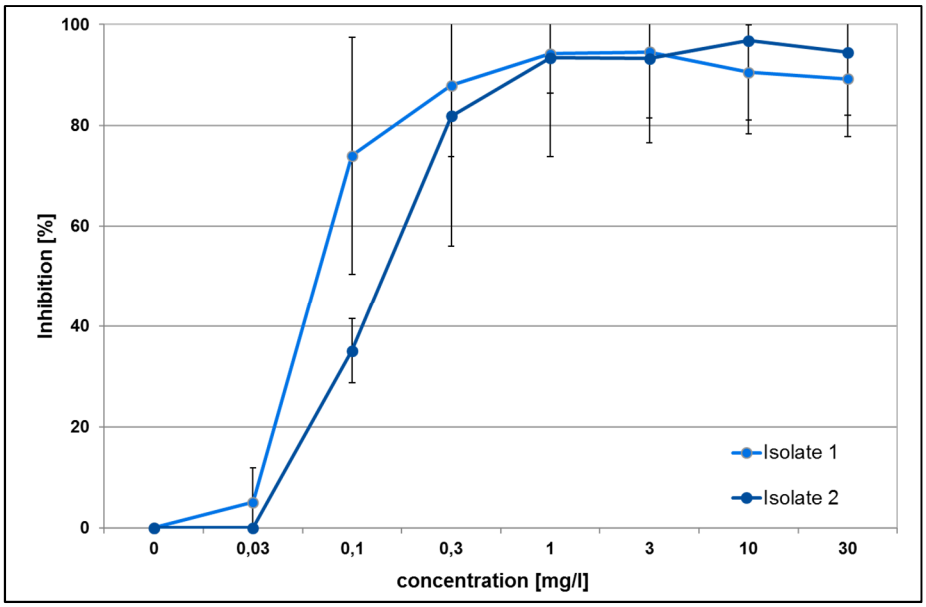


Figure 21: Inhibition curves of *Oculimacula* spp. reference strains on metrafenone dilutions using a microtiter test. After five days incubation, growth inhibition was calculated from two independent repeated tests; Error bars = 2xSE.

The EC_{50} values determined are 0.1 mg/l for isolate 1 and 0.31 mg/l for isolate 2. The growth of both reference strains is strongly inhibited with a concentration of 0.3 mg/l metrafenone, whereas even on 30 mg/l full inhibition was not achieved for both isolates.

Changes in the sporulation behaviour under metrafenone treatment were assessed in an agar plate test with different metrafenone concentrations (Figure 22).

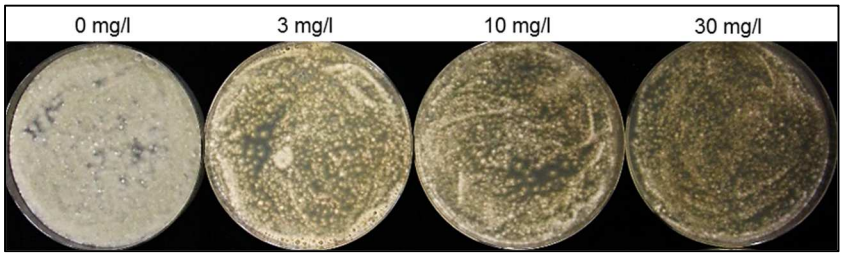


Figure 22: Developmental changes of *Oculimacula* spp. under metrafenone treatment on metrafenone embedded agar plates. Plates were inoculated with isolate 1 and incubated for 14 days at 14°C.

Growth and sporulation of *Oculimacula* isolates were strongly inhibited at 3 mg/l metrafenone compared to the untreated control and a slight dose response was visible. At 30 mg/l restricted mycelial growth was observed and sporulation was hardly detectable.

Activity of metrafenone on *A. nidulans*

The effect of metrafenone on the development of *A. nidulans* (wildtype strain 1035) was analysed in an agar plate test using different concentrations of metrafenone (Figure 23).

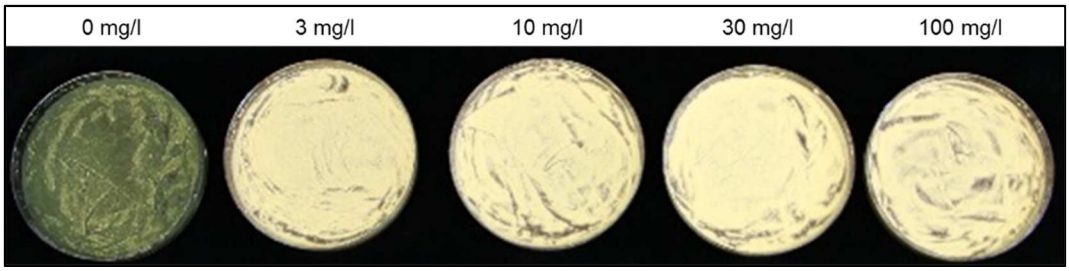


Figure 23: Activity of metrafenone on the development of *A. nidulans* using different concentrations of metrafenone in an agar plate test. Plates were incubated for 4 days at 37°C.

In contrast to the control plate without metrafenone, typical green coloured conidia of *A. nidulans* were not observed on all metrafenone dilutions used. Metrafenone treatment had no influence on mycelial growth and no dose response was observed at the concentrations used.

Both, *Oculimacula* spp. and *A. nidulans* showed changes under metrafenone treatment. A strong effect on the sporulation was observed for both fungal organisms, which confirmed the observations made by Schmitt *et al*. 2006.

3.2.5.3 Whole genome sequencing of *A. nidulans*

An influence on sporulation under metrafenone treatment is also described for *B. graminis* f.sp. *tritici* (Opalski *et al*. 2006) and was observed during the phenotypical analyses of *E. necator* (3.2.3). With a genome size of 30 Mb and as one of the best characterized model organisms for the research of filamentous fungi, numerous information, including WGS data, is available for *A. nidulans*.

Metrafenone adapted strains were generated by spontaneous mutagenesis in the presence of metrafenone. Selection was performed on metrafenone embedded agar plates (30 and 100 mg/l) with a high spore density. Adapted isolates were obtained from sporulating spots and cultivated on metrafenone agar plates. After a growth cycle in the absence of metrafenone, generated isolates were again transferred to metrafenone containing agar plates, in order to see if the mutagenesis resulted in stable adapted isolates. A total of 20 isolates with different levels of adaption were obtained. In this context, the word ´adapted´ is used to characterize the ability to sporulate on agar plates at different metrafenone concentrations. The comparative genome analysis of the sensitive wildtype isolate (1035) of *A. nidulans* and the generated progeny was used as new access for the identification of the resistance mechanism of metrafenone.

Five adapted isolates (adapted 1 - 5), with the highest levels of adaption, were chosen for a whole genome sequencing approach. The decrease in sporulation of these strains on different metrafenone concentrations was assessed (Figure 24).

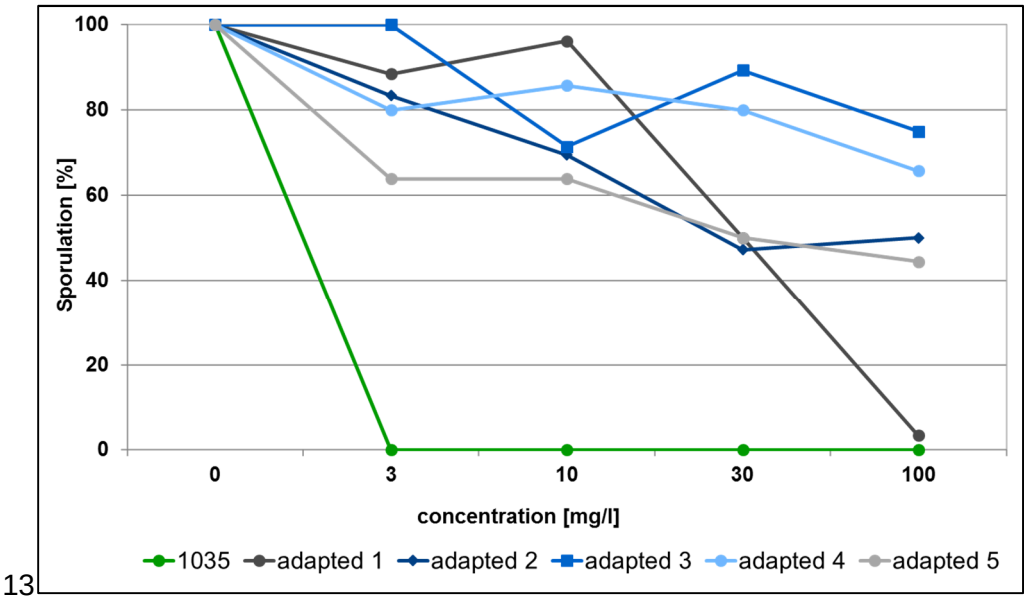


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Figure 24: Characterisation of the sporulation of metrafenone adapted *A. nidulans* isolates on different metrafenone concentrations. The decrease of the sporulation of the sensitive parental strain 1035 (green) and five adapted isolates (blue and grey) is shown.

Sporulation of the parental strain (1035) was only observed on agar plates without metrafenone. The sporulation was completely inhibited on 3, 10, 30 and 100 mg/l metrafenone. In contrast, the adapted isolates, except adapted isolate 1, showed more than 40% sporulation, even at 100 mg/l compared to the untreated control. The sporulation of the adapted isolate 1 was > 50% at the concentrations of 3, 10 and 30 mg/l, and strongly inhibited at 100 mg/l metrafenone.

Genomic DNA of the parental stain and the respective adapted isolates was analysed by whole genome sequencing. Sequencing was accomplished by BGI Hong Kong using the illumina sequencing method on a HiSeq machine with 2x150 bp paired end (PE) reads. The resulting sequencing data were analysed and evaluated by internal bioinformatic services (BASF SE).

The genomes of the parental strain and the progeny were nearly identical and resulted in a genome size of about 30 Mb. The genomes mapped to the reference genome (strain FGSC A4; Cerqueira *et al.* 2014) with 50 - 100-fold coverage. Compared with the reference genome and the parental strain, a total of 29 SNPs (including synonymous mutations) were identified in the adapted progeny, 18 in coding sequences (CDS) and 11 in noncoding regions. Mutations in CDS regions lead to amino acid substitutions in nine genes. Only two genes were identified, in which all adapted isolates showed a SNP at the same position. These genes code for an isoflavone reductase family protein and an aldehyde dehydrogenase protein (Figure 25).

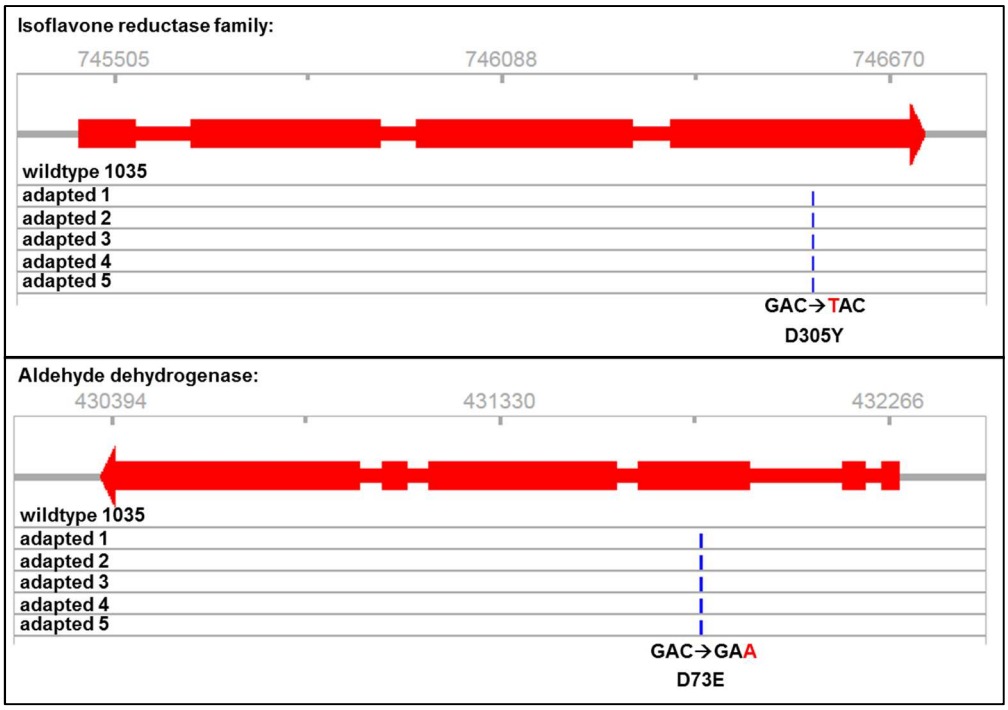


Figure 25: Gene structure of an isoflavone reductase family protein and an aldehyde dehydrogenase protein of the sequenced *A. nidulans* strains compared to the reference genome FGSC A4. The identified nucleotide exchanges and the resulting amino acid substitutions are shown in the picture.

An amino acid substitution from aspartic acid to tyrosine at position 305 was identified in the sequence of the isoflavone reductase. In the sequence of the aldehyde dehydrogenase, an amino acid exchange from aspartic acid to glutamine acid was detected at position 73.

Sequences of these two proteins were used to search for homologs within the reference genomes of wheat powdery mildew (96224 and 2588) and in a *E. necator* genome (Jones *et al.* 2014). No proteins were identified for the isoflavone reductase for both powdery mildews. For the aldehyde dehydrogenase, numerous homologous proteins were identified in both powdery mildews genomes. The highest hits were rechecked in the genome data of the sequenced *B. graminis* f.sp. *tritici* isolates for SNP identification. Sequences were identical for all isolates; no SNP was identified.

In intergenic regions, five SNPs were identified in all adapted *A. nidulans* isolates except the wildtype 1035, compared to the reference genome. One of them is potentially localised in the promotor region of a Rsc complex subunit (Figure 26).

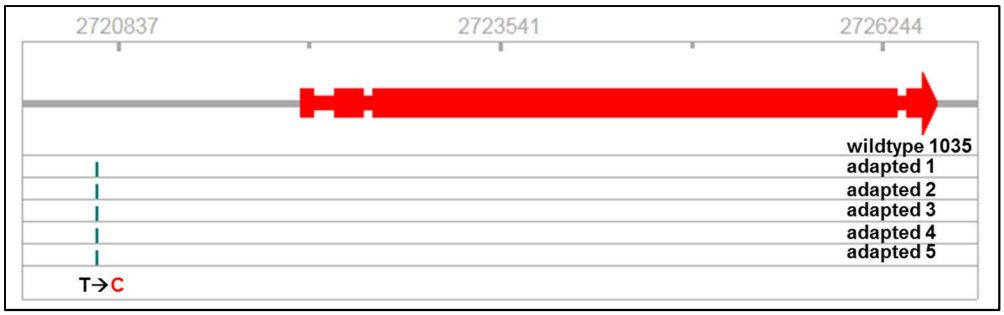


Figure 26: Gene structure and surrounding region of a Rsc protein of the sequenced *A. nidulans* strains compared to the reference genome FGSC A4. The identified nucleotide exchange and its position are shown in the picture.

As shown in chapter 1.1.5.1, the comparative whole genome sequencing approach of *B. graminis* f.sp. *tritici* isolates identified a target site mutation in a Rsc complex subunit gene, which was revealed to be an orthologous protein to the Rsc complex of *A. nidulans*. Additionally, intergenic mutations in the potential promotor region were detected (Figure 27).

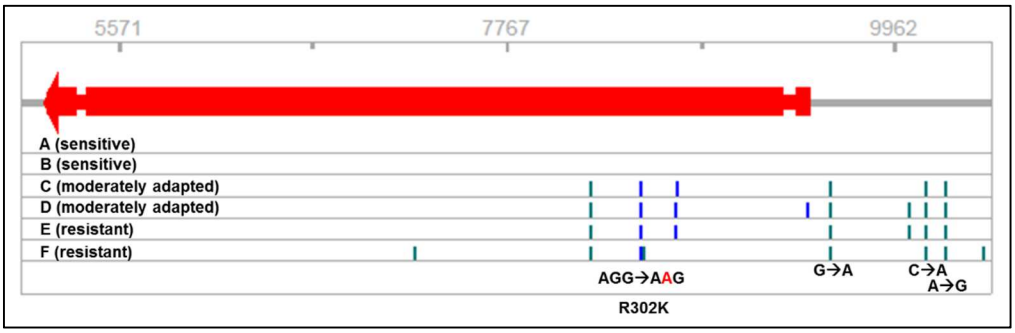


Figure 27: Gene structure the Rsc complex subunit of the sequenced *B. graminis* f.sp. *tritici* isolates compared to the reference genome 2588. The identified nucleotide exchanges and the resulting amino acid substitution and its position are shown in the picture. Synonymous SNPs are shown in blue and non-synonymous SNPs in green.

The mutation in the Rsc ortholog of *B. graminis* f.sp. *tritici* leads to an amino acid exchange from arginine to lysine at position 302. An orthologous protein could also be identified for *E. necator*. The amino acid sequence of the Rsc complex subunit proteins were compared with two other plant pathogenic fungi (Figure 28).

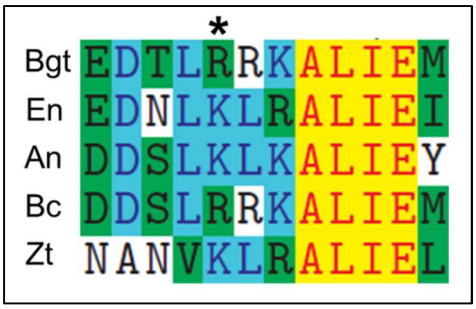


Figure 28: Degree of conservation across different species of the substituted amino acid in the Rsc complex subunit protein sequence of *B. graminis* f.sp. *tritici*. The picture detail shows the surrounding region of the substituted amino acid. Conserved residues are coloured in yellow (all match), blue (≥ 50%), green (similar) and white (non conserved). The substituted amino acid is marked with an asterisk. Sequences originate from *B. graminis* f.sp. *tritici* (Bgt), *E. necator* (En), *A. nidulans* (An), *B. cinerea* (Bc),) and *Z. tritici* (Zt).

The amino acid sequences of *E. necator*, *A. nidulans* and *Z. tritici* already showed a lysine at the homologous position, meaning the R302K mutation is not highly conserved across the analysed species (supplementary material, Figure 44). With the sequence information of these two powdery mildews orthologs, primer were designed to compare the sequences of further sensitive and resistant isolates. For *B. graminis* f.sp. *tritici,* the Rsc complex subunit gene was amplified and sequenced for a sensitive and two further resistant wheat powdery mildew isolates. The target site mutation R302K was identified in all sequences, including the sequence of the sensitive strain. The respective nucleotide changes in the potential promotor region were detected in the sensitive, as well as one resistant isolate. For *E. necator,* the orthologous gene of the Rsc complex subunit was amplified and sequenced for a sensitive and a resistant isolate. Except for one silent SNP in CDS sequences, no differences in the nucleotide sequence of the Rsc complex subunit ortholog were detected between the sensitive and resistant *E. necator* isolates. These results do not support the hypothesis, that the Rsc complex subunit contributes to metrafenone resistance in wheat, as well as grape powdery mildew.

3.3 Sensitivity towards SDHIs in *E. necator*

In Europe, the use of SDHIs in grapevine until now is restricted to boscalid and fluopyram. With fluxapyroxad, which will be launched in 2017, three SDHIs will be available. Field isolates with resistances against SDHIs are already known for various plant pathogens affecting cereal, fruit and vegetable crops (Stammler *et al.* 2007, Avenot and Michailides 2010, Miles *et al.* 2014, Rehfus *et al.* 2016).

3.3.1 European monitoring results

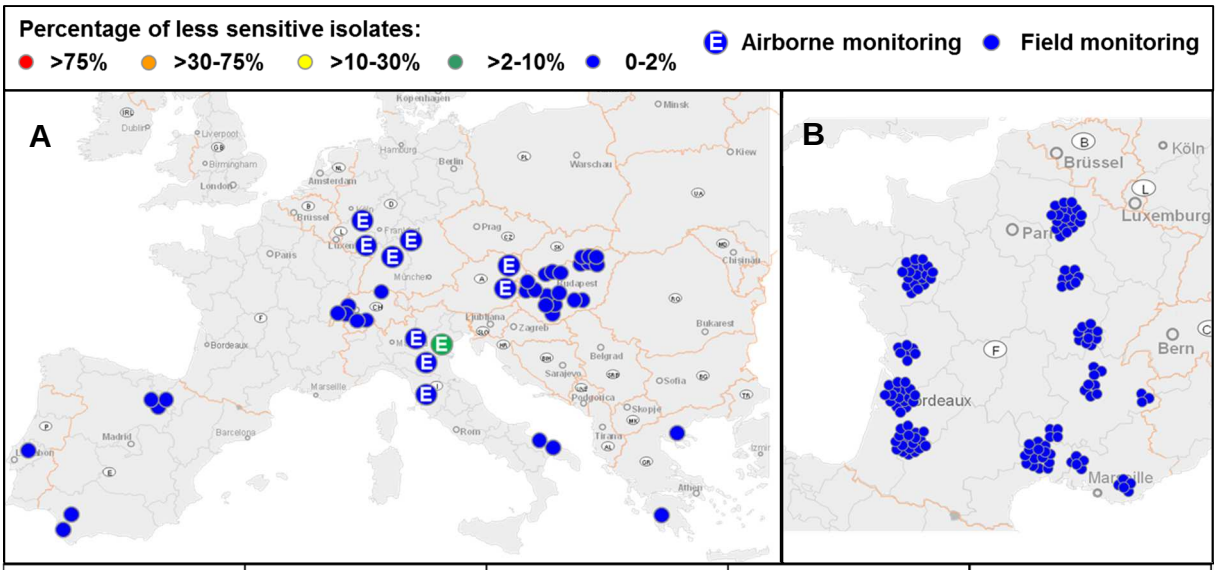
Similar to the analysis made with metrafenone, the results of the SDHI sensitivity monitoring from 2014 to 2016 were processed and evaluated during this study. To illustrate the development of the sensitivity situation of *E. necator* towards SDHIs, data from the sensitivity monitoring from 2007 to 2013 were included. Over the years, two different SDHIs, boscalid and fluxapyroxad, were used as reference active ingredient for sensitivity characterisation (Table 20).

Table 20: Development of the frequency of *E. necator* isolates with a reduced SDHI sensitivity in Europe and France.

	Origin	2007	2008 n=376	2009 n=346	2010 n=277	2011 n=347	2012 n=367	2013 n=305	2014 n=401	2015 n=506	2016 n=368
Boscalid	Europe	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	NA	NA	21,1%*	14,6%
	France	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	1,4%	5,0%
	Mean	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	NA	NA	9,2%	13,6%
Fluxapyroxad	Europe	NA	NA	NA	NA	NA	NA	0,0%	0,4%	2,4%	NA
	France	NA	NA	NA	NA	NA	NA	NA	NA	NA	0,0%
	Mean	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

*: samples on demand (out of 95 field trial samples)

In contrast to the European SDHI monitoring in 2014 and 2015, which was performed with fluxapyroxad as reference compound, the French sensitivity monitoring was done with the SDHI boscalid. The discriminatory doses for boscalid and fluxapyroxad, used for the sensitivity monitoring, are shown in table 13.



2014	Field samples	Air borne monitoring	French monitoring	Total
Samples / regions	37	10	-	47
Isolates / populations	161	100	140	401

2015	Field samples	Air borne monitoring	French monitoring	Total
Samples / regions	49	10	-	59
Isolates / populations	224	100	182	506

Figure 29: European sensitivity situation of *E. necator* towards SDHIs in 2015 and 2016. A: European sensitivity monitoring with fluxapyroxad in 2014. B: French sensitivity monitoring with boscalid in 2014. C: European sensitivity monitoring with fluxapyroxad in 2015. D: French sensitivity monitoring with boscalid in 2015. The sampling method used and the portion of resistant isolates in the samples is represented by a colour code shown in the legend.

In 2014, a single *E. necator* isolate with a reduced fluxapyroxad sensitivity was identified for the first time (Figure 29A). For this isolate, which originated from the airborne monitoring in Northern Italy in the Veneto region, growth > 50% was observed on 1 mg/l fluxapyroxad. All remaining isolates obtained from field samples or airborne monitoring showed no growth and were classified as sensitive. The French monitoring revealed an overall sensitive situation in 2014 (Figure 29B). In 2015, all analysed isolates from Italy were sensitive. Single isolates with reduced sensitivity towards fluxapyroxad were identified from field samples in Slovenia and Greece (Figure 29C). From Slovenia, two of five isolates, obtained from one sample, showed

> 50% growth on 1 mg/l fluxapyroxad. Four of five isolates from a Greek sample were also less sensitive. Samples from other European countries included in the monitoring were classified as sensitive. For the French monitoring in 2015 (Figure 29D), 145 samples from commercial sites and 37 samples from trial sites were analysed. An overall sensitive situation was observed for commercial sites. Two samples from trial sites in Southern France were identified with a reduced sensitivity towards boscalid. Growth could be observed at 15 mg/l but not at 100 mg/l for these two isolates.

Additionally, field trials with boscalid were performed in 2015. In contrast to the routine SDHI monitoring, which was performed with fluxapyroxad as reference compound, isolates obtained from these samples were characterized with a discriminatory dose of 30 mg/l boscalid. 95 isolates obtained from 17 field samples from different countries were analysed (Figure 30).

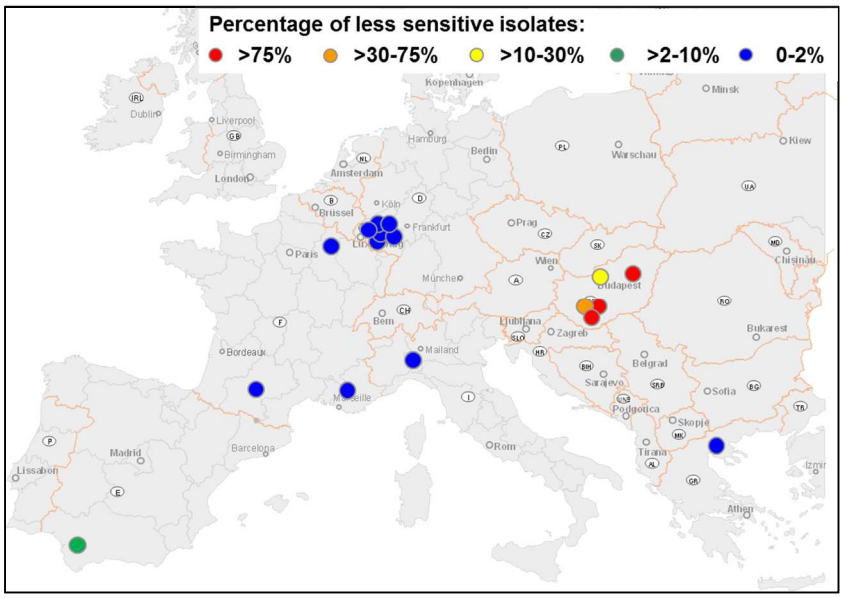


Figure 30: Extra sensitivity monitoring of *E. necator* towards the SDHI boscalid in 2015. The sensitivity of field samples on demand from trial sites are shown and the portion of resistant isolates within the samples is represented by a colour code shown in the legend.

Samples from France, Germany, Italy and Greece were characterised as sensitive, whereas all samples from Hungary showed less sensitive portions of isolates. Three of these samples showed more than 75% isolates with a reduced sensitivity, one 58% and the last 20%. The sample from Spain contained low frequencies of less sensitive isolates at 0.5%.

Isolates with a reduced sensitivity towards boscalid were detected in Hungary, which were not detected during the fluxapyroxad monitoring. This leads to the suggestion, that some isolates may have a reduced sensitivity to boscalid but not towards fluxapyroxad. Consequently, to get a more precise overview about the sensitivity situation towards SDHIs in Europe, boscalid was used for the next sensitivity monitoring.

The entire European sensitivity monitoring in 2016 (Figure 31) was accomplished with a discriminatory dose of the SDHI boscalid (Table 13). A total of 368 isolates, 208 obtained from field samples, 120 from the airborne monitoring and 40 French samples were monitored.

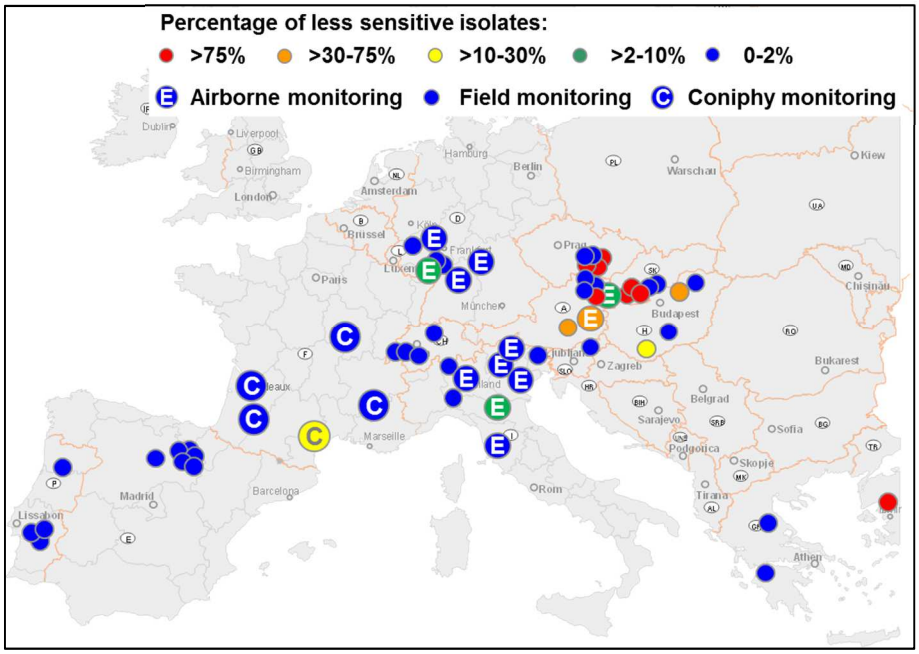


Figure 31: European sensitivity situation of *E. necator* towards the SDHI boscalid in 2016. Samples originate from two different sampling methods (airborne and field sampling). The sampling method and portion of resistant isolates within the samples is represented by a colour code shown in the legend.

The majority of isolates from South-West Europe could be classified as sensitive. Isolates with a lower boscalid sensitivity were detected in Southern France, in two of seven samples. Samples from the Eastern part of Europe showed higher frequencies of reduced sensitivity. Of five analysed field samples from Austria, two showed a reduced sensitivity with a frequency of 60% and 100%. In Czech Republic and Slovakia, three of five analysed samples with a frequency of 75% less sensitive isolates were observed. Some isolates from field samples, which originated from Hungary were classified as less sensitive: Of four analysed samples, two were classified as sensitive, one had 20% of less sensitive isolates and the other with 60% was detected. The two samples from Greece were sensitive and one sample from Turkey had high frequencies of less sensitive isolates with 80%. For the airborne monitoring, one out of ten isolates from the palatinate region in Germany and one out of ten isolates from the region of South Tyrol in Italy showed growth and were characterised as less sensitive. In Austria, both regions from the airborne monitoring showed isolates with reduced sensitivity. One isolate of ten from Weinviertel region and two isolates of ten from Burgenland were thereby classified as less sensitive.

The occurrence of less sensitive isolates within the European population is not evenly distributed over the different countries. Whereas southwestern Europe is still mainly sensitive,

higher frequencies of isolates with a sensitivity loss could be observed in eastern Europe. Similar to the distribution of metrafenone resistance in *E. necator,* Hungary, Czech Republic, Slovakia and Austria show higher frequencies of isolates with a reduced SDHI sensitivity.

All isolates, which were characterised as less sensitive towards SDHIs (fluxapyroxad or boscalid) were cultivated as living strains on detached grapevine leaves for further analysis.

3.3.2 Identification of mutations associated with a reduced SDHI sensitivity

The analysis of other SDHI resistant fungal organisms revealed point mutations in the *sdh* genes, which have been identified as the responsible resistance mechanisms. All *E. necator* isolates obtained in the European resistance monitoring and which had been classified as less sensitive were further analysed to elucidate the resistance mechanism leading to the sensitivity loss. For comparative sequence analysis, subunit genes *sdh*B, *sdh*C and *sdh*D were amplified with *E. necator* specific oligonucleotides (supplementary material, Figure 45 - Figure 47). All isolates used for comparative sequence analysis are shown in table 21 with their *sdh*-genotype.

Table 21: Overview of *E. necator* isolates used for comparative sequence analysis of *sdh* subunits B, C and D. Detailed overview of their genetic properties and SDH substitutions; IT: Italy; AU: Austria; HU: Hungary; GR: Greece; SL: Slovenia; DE: Germany.

Isolate	Boscalid	Origin	SDHB		SDHC		SDHD
			substitution	codon	substitution	codon	
Hst	sensitive		no	-	no	-	no
1105	sensitive	IT	no	-	no	-	no
1110	sensitive	AU	no	-	no	-	no
1112	sensitive	IT	no	-	no	-	no
1104	resistant	HU	H242R	cat > cgt	no	-	no
1138	resistant	HU	H242R	cat > cgt	no	-	no
1140	resistant	HU	H242R	cat > cgt	no	-	no
1152	resistant	HU	H242R	cat > cgt	no	-	no
1134	resistant	GR	no	-	G169D	ggc > gac	no
1136	resistant	GR	no	-	G169D	ggc > gac	no
1143	resistant	SL	no	-	G169D	ggc > gac	no
1144	resistant	SL	no	-	G169D	ggc > gac	no
1131	resistant	SK	no	-	G169S	ggc > agc	no
1232	resistant	DE	I244V	ata > gta	no	-	no

Two mutations in the *sdh*-genes were identified in 2014, which lead to amino acid exchanges from histidine to arginine at position 242 in subunit B or an exchange from glycine to aspartic acid in subunit C at position 169. In 2015 and 2016, two further amino acid exchanges were identified in isolates with a reduced SDHI sensitivity. One isolate (1131) showed a substitution

from glycine to serine at position 169 in subunit C and another isolate (1232) showed an exchange from isoleucine to valine at position 244 in subunit B. Sequence analysis of subunit C revealed an arginine to glycine substitution at position 25 (agg > ggg), which was identified in several sensitive and less sensitive isolates. Therefore, this mutation does not seem to be connected with a reduced SDHI sensitivity and was not further analysed. Sequences of sensitive and resistant isolates were identical for *sdh*D and no mutation was identified. None of the isolates showed a substitution in more than one subunit, meaning double mutations were not observed.

The amino acid substitutions, which lead to resistance against SDHIs are located at highly conserved positions of the SDH enzyme and some are directly involved in enzyme activity. Alignments of the SDH subunits of *E. necator* with plant pathogenic fungi and other species showed that the positions of these amino acid changes are highly conserved (Figure 32).

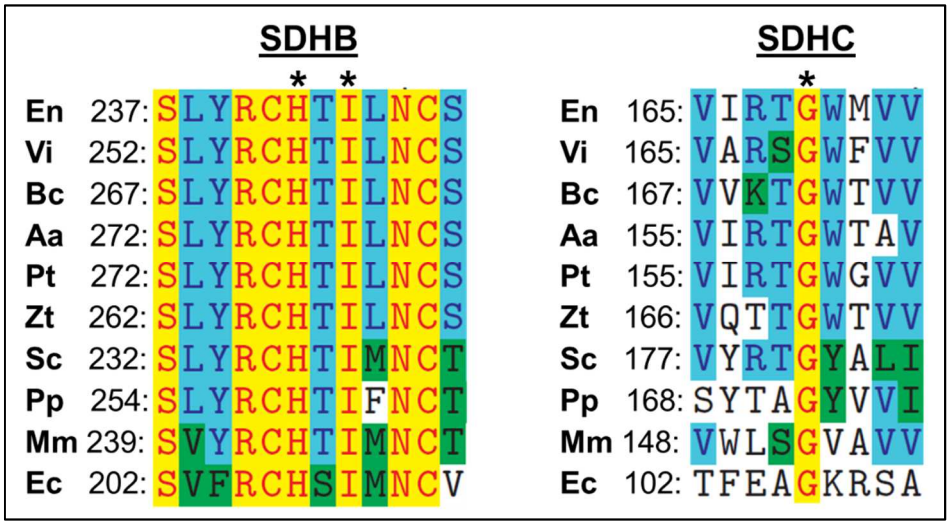
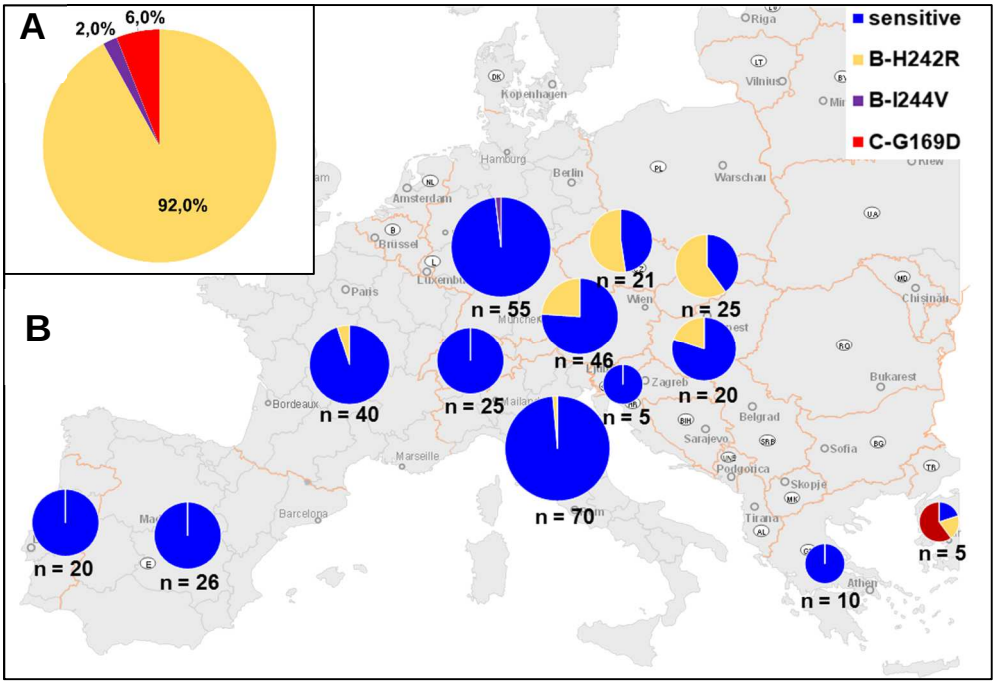


Figure 32: Degree of conservation across different species and localization of substituted amino acids in SDH subunits B and C of *E. necator*. Conserved residues are coloured in yellow (all match), blue (≥ 50%), green (similar) and white (non conserved). Substituted amino acids are marked with an asterisk. Sequences originate from *E. necator* (En), *Venturia inaequalis* (Vi), *B. cinerea* (Bc), *Alternaria alternata* (Aa), *Pyrenophora teres* (Pt), *Z. tritici* (Zt), *Saccharomyces cerevisiae* (Sc), *Mus musculus* (Mm) and *Escherichia coli* (Ec).

Many other plant pathogens have already evolved resistance towards SDHIs, caused by target site mutations in the *sdh* genes. Sequence alignments were also done in comparison with other SDHI resistant plant pathogenic fungi. The H242R substitution in *E. necator* was found to be at the homologous position to H277/Y (*P. teres*), H272R/L/T (*B. cinerea*) and H267R (*Z. tritici*). Another substitution, I269V, identified in lab mutants of *Z. tritici,* was found to be homologous to I244V in *E. necator*. No homologous mutation in other fungi has been described for the substitutions from glycine to aspartic acid or serine (G169D/S).

3.3.3 Frequency and distribution of *sdh*-mutations

Based on the sequences of less sensitive *E. necator* isolates, a molecular monitoring method was developed. The detection of point mutations B-H242R and C-G169D/S in the *sdh*-genes was accomplished by pyrosequencing assays. All isolates classified as less sensitive towards boscalid in 2016 were cultivated as living isolates on detached leaves, analysed and changes in the *sdh*-genes were determined. The corresponding mechanisms, which lead to a reduced sensitivity, were identified by pyrosequencing assays or sequence analysis. For the visualisation of the distribution and frequencies of less sensitive isolates with the corresponding *sdh*-mutation, isolates characterised as sensitive were also included (Figure 33). With 86.4% of the monitored isolates, the fraction of sensitive isolates represents the majority of analysed isolates. 50 isolates with a lower sensitivity were identified, representing 13.6% of the monitored population (Figure 33C). The H244R is the most frequent detected mutation at 92% of the resistant population. Three isolates, representing 6% of isolates with a reduced sensitivity, carried the G169D mutation (Figure 33A). In 2016, no isolate was detected with the G169S. For one isolate from Germany with a reduced SDHI sensitivity, pyrosequencing could not detect a mutation at positions 242 in subunit B and 169 in subunit C. Sequencing of the *sdh*-genes revealed a point mutation, which lead to an amino acid exchange from isoleucine to valine at position 244 in subunit B (2%). This mutation was observed for the first time. All *E. necator* isolates analysed and classified as less sensitive to SDHIs, show a single target site mutation in the *sdh*-genes. Double mutations in the *sdh*-genes were not detected.



C

Origin	sensitive	H242R	I244V	G169D	G169S
DE	98,2%	0,0%	1,8%	0,0%	0,0%
CZ	47,6%	52,4%	0,0%	0,0%	0,0%
AU	76,1%	23,9%	0,0%	0,0%	0,0%
SK	40,0%	60,0%	0,0%	0,0%	0,0%
SL	100,0%	0,0%	0,0%	0,0%	0,0%
HU	80,0%	20,0%	0,0%	0,0%	0,0%
IT	98,6%	1,4%	0,0%	0,0%	0,0%
CH	100,0%	0,0%	0,0%	0,0%	0,0%
FR	95,0%	5,0%	0,0%	0,0%	0,0%
SP	100,0%	0,0%	0,0%	0,0%	0,0%
PT	100,0%	0,0%	0,0%	0,0%	0,0%
GR	100,0%	0,0%	0,0%	0,0%	0,0%
TR	20,0%	20,0%	0,0%	60,0%	0,0%
EU	86,4%	12,5%	0,3%	0,8%	0,0%

Figure 33: Distribution and frequency of *sdh*-genotypes in *E. necator* (n=368) in various European countries in 2016, determined by pyrosequencing. A: Percentage of *sdh*-mutations within the portion of isolates characterised as less sensitive; B: Percentage of *sdh*-genotypes within all analysed isolates shown as country split; C: Percentage of *sdh*-mutations for each country and Europe.

Homology model of SDH with mutations associated with a loss of SDHI sensitivity

The identified amino acid substitutions, B-H242R, B-I244V and C-G169D/S, were analysed with respect to their location within the SDH protein. The positions were identified with the help of a homology model of the *E. necator* wildtype enzyme (Figure 34). The modelling, as well as the implementation of the amino acid substitutions were conducted by internal services (BASF SE). Sequence identity to *E. necator* for subunits B, C and D was determined and resulted in 69.8, 27.7 and 31.1%.

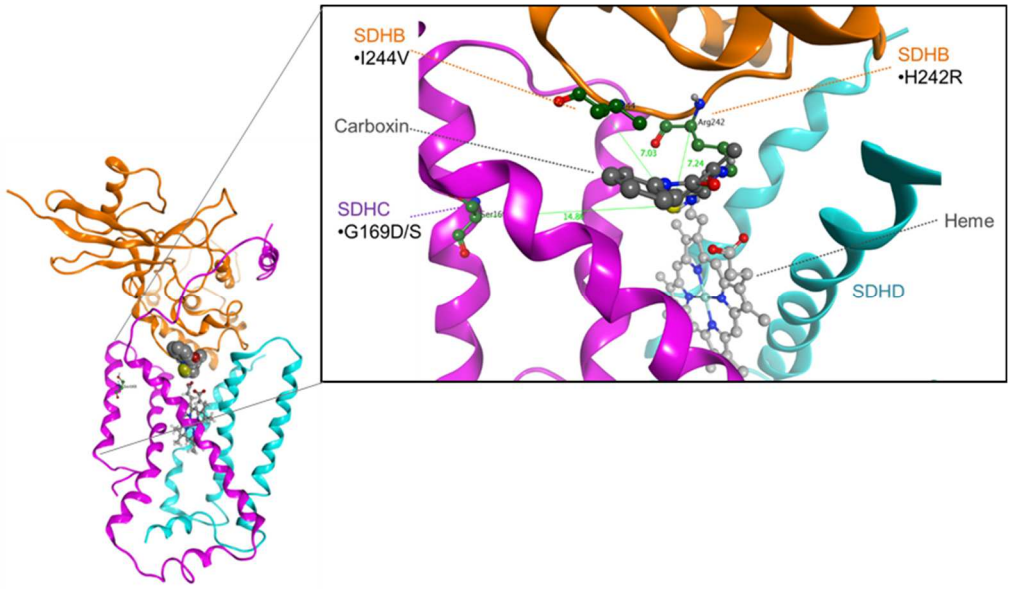


Figure 34: Homology model of the succinate dehydrogenase subunits B, C and D from *E. necator* based on X-ray from *Gallus gallus* (PDB 2WQY) with the locations of the amino acid substitutions B-H242R, B-I244V, C-G169D and C-G169S. SDH-B (orange), SDH-C (lilac) and SDH-D (blue) are shown in cartoon style; the position of carboxin within the binding pocket indicates the ubiquinone binding site. Substituted amino acids, the carboxin and haem b group are shown as ball and stick model.

As shown previously, the substitutions B-H242R and B-I244V were found to be in direct vicinity to the interaction site of SDHIs (7.24 and 7.03 Å), the ubiquinone-binding site. The location of the novel C-G169D/S substitution is in larger distance from the binding pocket with 14.86 Å. None of the substitutions identified is near the haem b group.

3.3.4 Phenotypic characterisation of *E. necator* isolates with a reduced SDHI sensitivity

It is known that different target site mutations in the SDH genes can cause different levels of resistance in plant pathogenic fungi. Furthermore, the level of resistance seems also to be species specific and therefore dependent on the pathogen (Scalliet *et al.* 2012; Veloukas *et al.* 2013). Therefore, the impact of the mutation B-H242R, B-I244V, C-G169D and C-G169S on the sensitivity of *E. necator* towards the SDHIs boscalid, fluxapyroxad and fluopyram was investigated by germination and leaf disc tests. Sensitive and representative isolates of each *sdh*-genotype were tested.

Germination tests

The effect of SDHIs on germination was determined in germination tests one day after inoculation. The germination rate on different concentrations of boscalid, fluxapyroxad and fluopyram, relative to the untreated control, was calculated as inhibition of the germination and is shown for representative isolates (Figure 35). All analysed isolates, sensitive and less sensitive towards SDHIs, had similar germination rates, in the absence of fungicide. Germination of sensitive isolates was completely inhibited at 0.3 mg/l fluxapyroxad, 1 mg/l boscalid and 1 mg/l fluopyram (Figure 35A and B). Boscalid had low effects on the germination of isolates with the B-H242R mutation, only a slight dose response was observed. The germination rate of these isolates was reduced to 50% at 1 to 3 mg/l for both fluxapyroxad and fluopyram and fully inhibited at 30 mg/l. Treatment with boscalid and fluopyram resulted in a similar germination rate for isolates carrying the I244V mutation. The germination rate was reduced by 50% at 3 mg/l and complete inhibition was observed with 30 mg/ml. The efficacy of fluxapyroxad was higher with a 70% reduction of the germination at 1 mg/l. In contrast, isolates carrying the C-G169D substitution were less affected by all tested SDHIs. Tested isolates showed no or only a slight decrease of the germination rate with 30 mg/ml boscalid but a ≥ 50% decrease at 30 mg/l fluxapyroxad and fluopyram, germination was not, however, fully inhibited. Treatment with 1 mg/ml fluxapyroxad decreased the germination rate of the isolate with the G169S substitution by 50% and complete inhibition was achieved with 3 mg/ml. Boscalid and fluopyram were more strongly affected with a reduction of the germination rate to 50% at 3 mg/ml and full inhibition at 10 mg/ml.

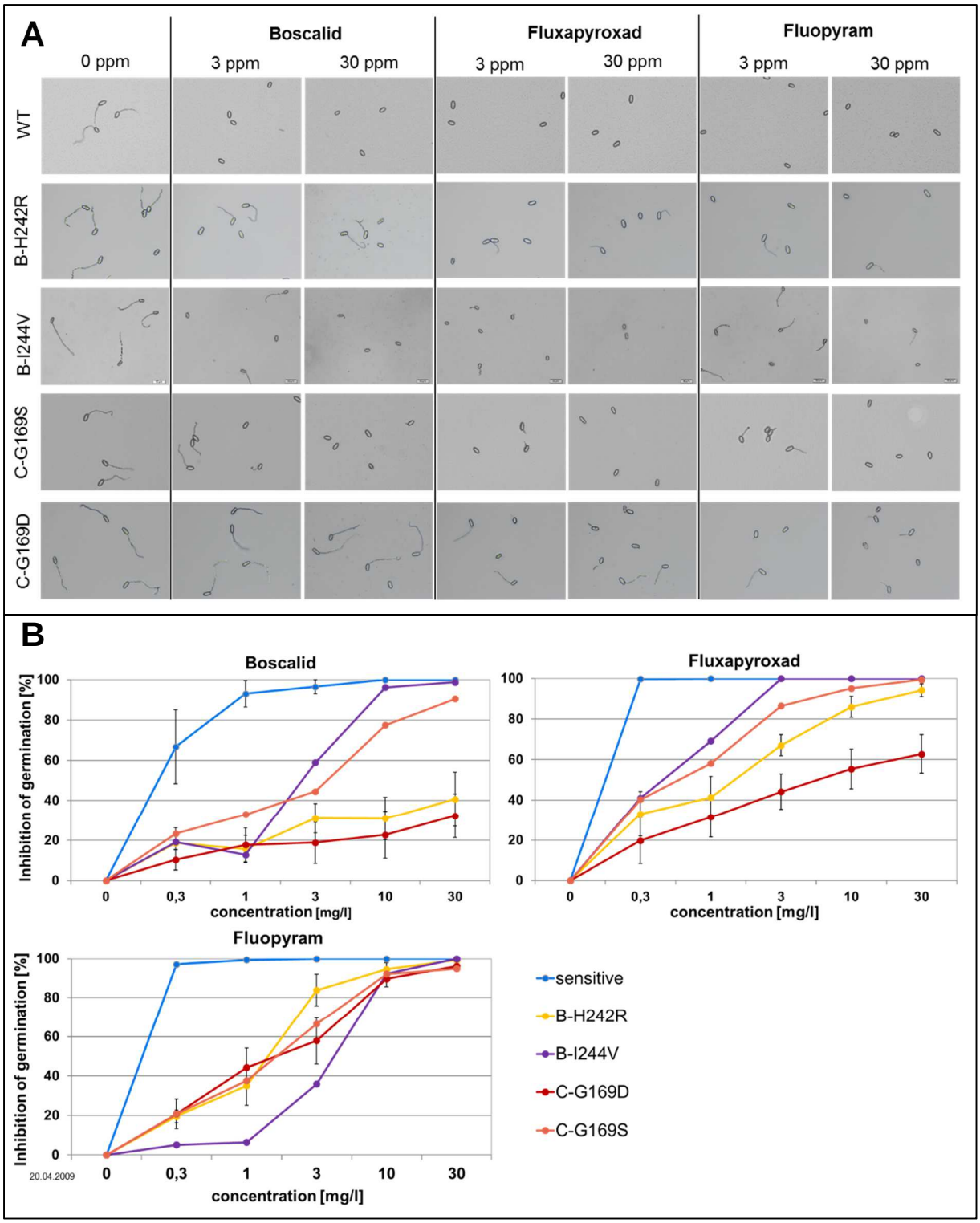


Figure 35: Germination of *E. necator* isolates with different mutations in *sdh*-genes on different concentrations of SDHI fungicides. A: Changes in the germination of isolates with *sdh*-mutations in *E. necator* on different SDHI concentrations. 0, 3 and 30 mg/l boscalid, fluxapyroxad and fluopyram were used. Pictures were taken 24h after inoculation at 10x magnification; B: Inhibition of the germination of *E. necator* isolates on different concentrations of boscalid, fluxapyroxad and fluopyram. Each isolate was analysed 2 times and the germination rate was assessed 24h after inoculation. Sensitive n=4, B-H242R n=5, B-I244V n=1, C-G169D n=5 and C-G169S n=1; Error bars = 2xSE.

Leaf disc tests

Further characterisation of isolates with the different *sdh*-genotypes was done with *in vivo* leaf disc tests. The efficacy of SDHIs could be assessed on the host surface by evaluation of the diseased leaf area. As a result, EC_{50} values and respective resistance factors (RF) were calculated (Table 22). For the calculation of the EC_{50} range and the resistance factors, EC_{50} values used were as mean of two independent replicates for each isolate.

Table 22: Overview of EC_{50} and RF values of *E. necator* isolates with different *sdh*-mutations.

				Boscalid		Fluxapyroxad		Fluopyram	
Isolate	**SDHB**	**SDHC**	**SDHD**	**EC_{50}**	**RF**	**EC_{50}**	**RF**	**EC_{50}**	**RF**
Wildtype (n=4)	no	no	no	0.53 (±0.14)	-	< 0.33 (±0.04)	-	0.82 (±0.28)	-
Adapted (n=7)	H242R	no	no	> 30.00 (±0.00)	> 55	0.44 (±0.08)	> 1	2.49 (±0.9)	3
Adapted (n=1)	I244V	no	no	4.29	8	1.57	> 5	> 30.00	> 36
Adapted (n=8)	no	G169D	no	> 30.00 (±0.00)	> 55	13.92 (±4.26)	> 41	7.52 (±1.80)	9
Adapted (n=1)	no	G169S	no	> 30.00	> 55	1.51	> 4	> 30.00	> 36

The impact of isolates with the different *sdh*-genotypes on the efficacy of boscalid, fluxapyroxad and fluopyram is compared and visualised in Figure 36.

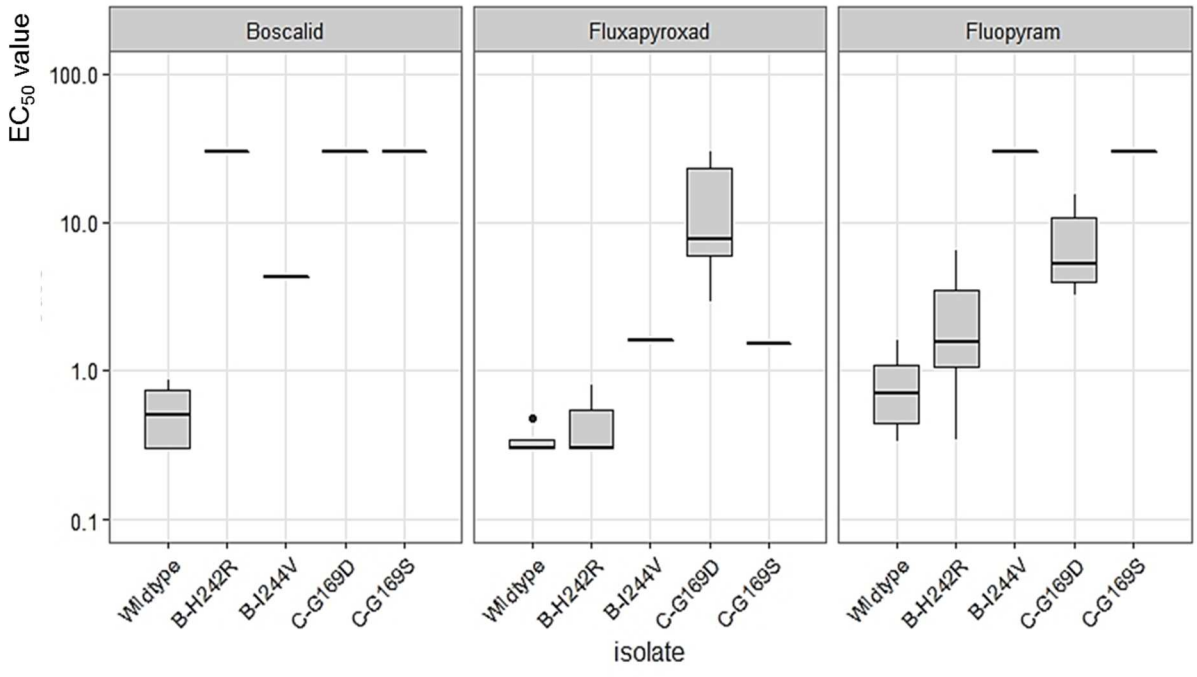


Figure 36: Sensitivity of *E. necator* isolates with different mutations in *sdh*-genes to SDHI fungicides. SDHIs were used as formulations and dissolved in water. Each isolate was analysed 2 times. Mean EC50 values are shown on a logarithmic scale as boxplots, sensitive n=4, B-H242R n=7, B-I244V n=1, C-G169D n=7 and C-G169S n=1.

The infection behaviour and sporulation of sensitive as well as less sensitive isolates was similar on untreated leaf discs, no phenotypical differences were observed. The analysis of

sensitive isolates in the leaf disc tests resulted in EC_{50} values ranging from < 0.3 to 0.81 mg/l boscalid, < 0.3 mg/l fluxapyroxad and 0.3 to 1.6 mg/l fluopyram. Isolates with the B-H242R substitution showed an increase of EC_{50} values compared with sensitive isolates with > 30 mg/l for boscalid. Fluxapyroxad and fluopyram were less affected with EC_{50} values < 0.3 to 0.56 mg/l and 0.34 to 4.43 mg/l. All tested SDHIs were less effective in tests with isolates carrying the C-G169D substitution. These showed EC_{50} values of > 30 mg/l boscalid, from 2.9 to > 30 mg/l fluxapyroxad and 3.3 to 15.5 mg/l fluopyram. The substitutions C-G169S and B-I244V were each observed in one isolate, these were tested in two independent tests. For the C-G169S isolate, fluxapyroxad treatment led to slightly increased EC_{50} values compared to the sensitive isolates, with 1.51 mg/l and resulted in EC_{50} values of >30 mg/ml boscalid and > 30 mg/ml fluopyram. The isolate carrying the B-I244V substitution was inhibited by fluxapyroxad treatment with an EC_{50} value of 1.57 mg/l. For boscalid slightly higher EC_{50} values were observed for this isolate with 4.29 mg/l and for fluopyram, the EC_{50} value was most strongly increased with > 30 mg/l.

For the chemical control of grape powdery mildew, three SDHIs (boscalid, fluopyram and fluxapyroxad) are or soon will be registered in Europe. In cereals, several other SDHI compounds like isopyrazam, penthiopyrad and benzovindiflupyr, are available. A reference strain for both most frequent mutations (B-H242R and C-G169D) was chosen for further characterisation using these SDHI compounds (Figure 37).

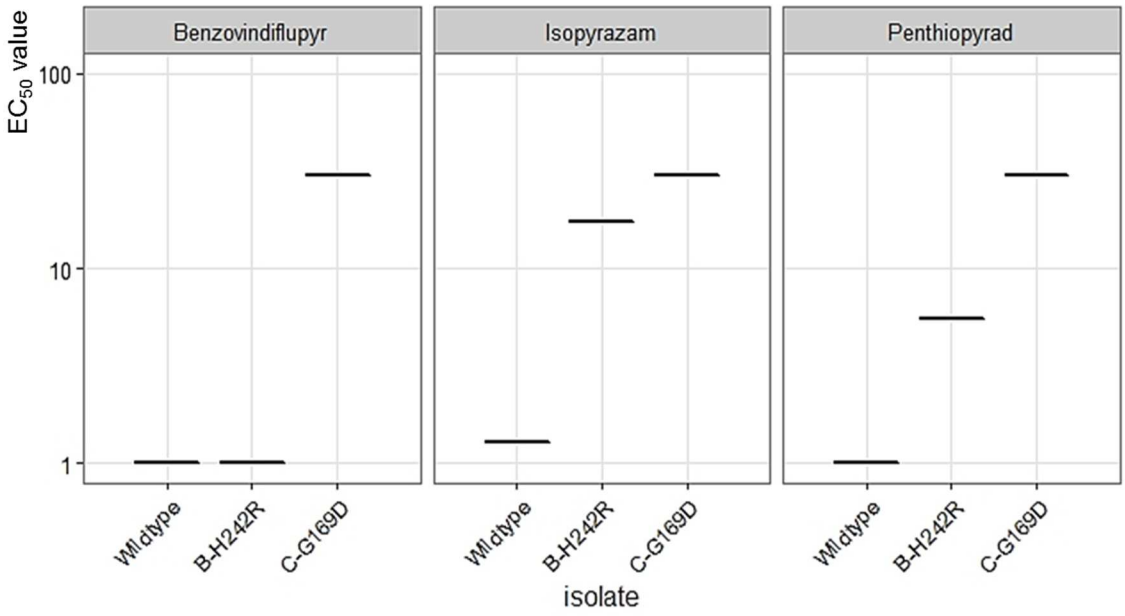


Figure 37: Sensitivity of *E. necator* isolates with mutations B-H242R and C-G169D to SDHI fungicides, not registered for the use in grapevine. Benzovindiflupyr, isopyrazam and penthiopyrad were used as active ingredient dissolved in DMSO and diluted in water. Each isolate was analysed 2 times and the mean EC_{50} values are shown on a logarithmic scale.

EC_{50} values of sensitive isolates were < 1 mg/l for benzovindiflupyr and penthiopyrad, and 1.3 mg/l for isopyrazam. For the B-H242R strain EC_{50} values were < 1 mg/l benzovindiflupyr, 17.3 mg/l isopyrazam and 5.5 mg/l penthiopyrad. EC_{50} values of the C-G169D strain were increased for all SDHIs analysed with EC_{50} values > 30 mg/l compared to the sensitive strain.

Altogether, these results indicate cross resistance between the analysed SDHIs, even if the efficacy of the tested SDHIs is differently affected by the analysed *sdh*-genotypes.

3.3.5 Competitive ability of *E. necator* isolates with a reduced SDHI sensitivity

The competitive ability of isolates carrying the most frequent mutations, the B-H242R and the C-G169D, was analysed in several mixtures of sensitive and resistant isolates. The mixtures were grown without selection pressure for six growth cycles. The germination and leaf disc test conducted indicated no phenotypical differences between sensitive isolates and isolates with a *sdh*-genotype under untreated conditions. For the selection of isolates the sensitivity towards QoIs, MFN and SDHIs was checked before and the germination rate was determined. Only sensitive and resistant isolates with similar germination rates and identical resistance background (except for SDHI) were used in the mixtures (Table 23).

Table 23: *E. necator* isolates and their *sdh*-genotype used for mixtures of SDHI sensitive and less sensitive isolates for the *sdh*-genotype based competitiveness test. Overview of their resistance properties and mixtures used in the growth competition test.

Test	Isolate ID	In mixture	Resistance phenotype	SDHI	MFN	QoI
Frequency of *sdh*-genotype	1108	I a, b, c, d	wild-type	sensitive	resistant	G143A
	1120	II a, b, c, d	wild-type			
	1104	I a, II a	*sdh*-genotype	B-H242R		
	1125	I b, II b	*sdh*-genotype			
	1129	I c, II c	*sdh*-genotype			
	1152	I d, II d	*sdh*-genotype			
	1012	III a, b, c, d	wild-type	sensitive	sensitive	G143A
	1109	IV a, b, c, d	wild-type			
	1113	III a, IV a	*sdh*-genotype	C-G169D		
	1133	III b, IV b	*sdh*-genotype			
	1143	III c, IV c	*sdh*-genotype			
	1144	III d, IV d	*sdh*-genotype			

Spore suspensions of single isolates were used as controls. The frequencies of the *sdh*-mutations determined after each cycle of the competition assays are shown in Figure 38.

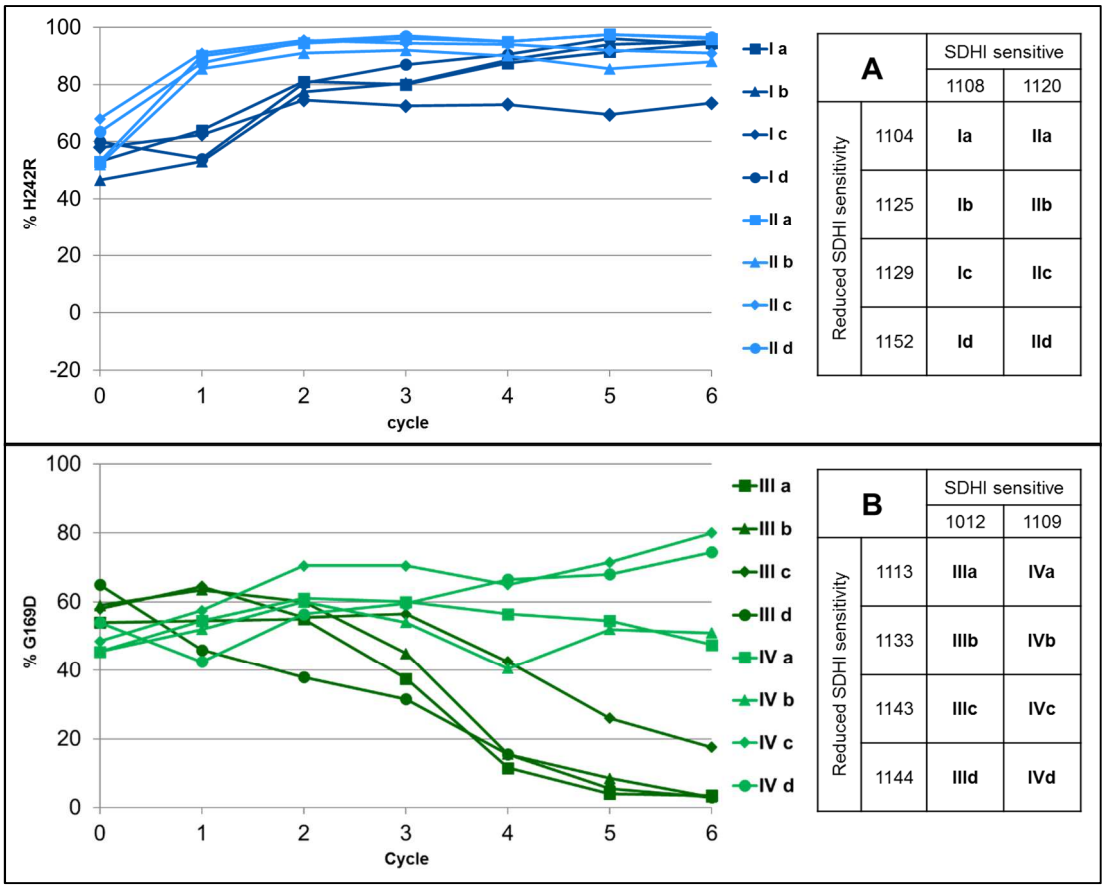


Figure 38: Changes in percent *sdh*-genotype (H242R or G169D) determined by pyrosequencing of artificial mixtures of sensitive and less sensitive *E. necator* isolates. Mixtures were cycled on untreated grapevine leaves and each cycle represents 10 days of incubation. A: Mixtures of sensitive isolates and isolates carrying the H242R; B: Mixtures of sensitive isolates and isolates carrying the G169D.

An increase in the frequency of H242R in mixtures with isolates of this *sdh*-genotype was observed for all analysed mixtures. After the first two cycles, the H242R frequency in mixtures IIa, b, c and d increased to 92 – 97% and persisted at the same level for all cycles. Mixtures Ia, b and d showed a steady increase of the resistant portion to 94 – 95% up to cycle 5. An increase until cycle two (73%) could also be detected for mixture Ic, but no further increase was observed. Isolates carrying the H242R substitution are competitive with the sensitive isolates used in this test system. For all analysed mixtures, a significant positive increase (Ia, b, c, d: reg.coef. = +6.07; IIa, b, c, d: reg.coef. = +3.93) of H242R was detected (supplementary material, Figure 49).

In contrast, different observations were made in the test where isolates with the G169D mutation were analysed. Mixtures IIIa, b, c and d showed a steady and significant (reg.coef. = - 9.99) decrease of the resistant allele to 3 – 17%. Isolates with the G169D mixed with this sensitive strain are less competitive. Only slight increases for mixtures IV a, b, c and d were observed (reg.coef. = +2.13) (supplementary material, Figure 50). Taken together, the frequency of G169D tends to stay at the same level for mixtures IV with the same sensitive

strain, meaning that under these conditions, there is no difference in the competitiveness of this sensitive strain and the isolates with the G169D mutation.

3.4 Fitness studies of multiple resistant *E. necator* isolates

The frequency of resistance towards metrafenone and the number of isolates with a reduced sensitivity towards SDHIs is strongly dependent on the origin. High portions of resistant isolates were identified in the eastern part of Europe. During these studies, the detection of *E. necator* isolates with resistances towards more than one fungicide class became obvious in these regions. Besides resistances towards QoIs and metrafenone, these isolates also showed a target site mutation, which led to a reduced SDHI sensitivity. To estimate the risk of spread of isolates with multiple resistances, a competitiveness test with multiple resistant *E. necator* isolates was performed. Isolates sensitive towards the three fungicide classes above mentioned were mixed with metrafenone resistant isolates, which carried G143A (QoI resistant) and B-H242R (SDHI resistant), shown in table 24.

Table 24: *E. necator* isolates and their resistance background used for mixtures of sensitive and multiple resistant isolates for the *sdh*-genotype and G143A based competitiveness test. Overview of their resistance properties; FR: France; IT: Italy; HU: Hungary; SK: Slovakia.

Test	Isolate ID	Origin	In mixture	Resistance phenotype	QoI	SDHI	MFN
Frequency of multiple resistance	1030	FR	V a, b, c, d	wild-type	sensitive	sensitive	sensitive
	1111	IT	VI a, b, c, d	wild-type			
	1104	HU	V a, VI a	multiple resistant	G143A	H242R	resistant
	1122	SK	V b, VI b	multiple resistant			
	1125	SK	V c, VI c	multiple resistant			
	1152	HU	V d, VI d	multiple resistant			

Isolate mixtures were grown without selection pressure for six cycles and after each cycle the frequency of G143A and B-H242R was determined for each mixture by pyrosequencing (Figure 39). Additionally, frequencies for single isolates were determined after each cycle as a control.

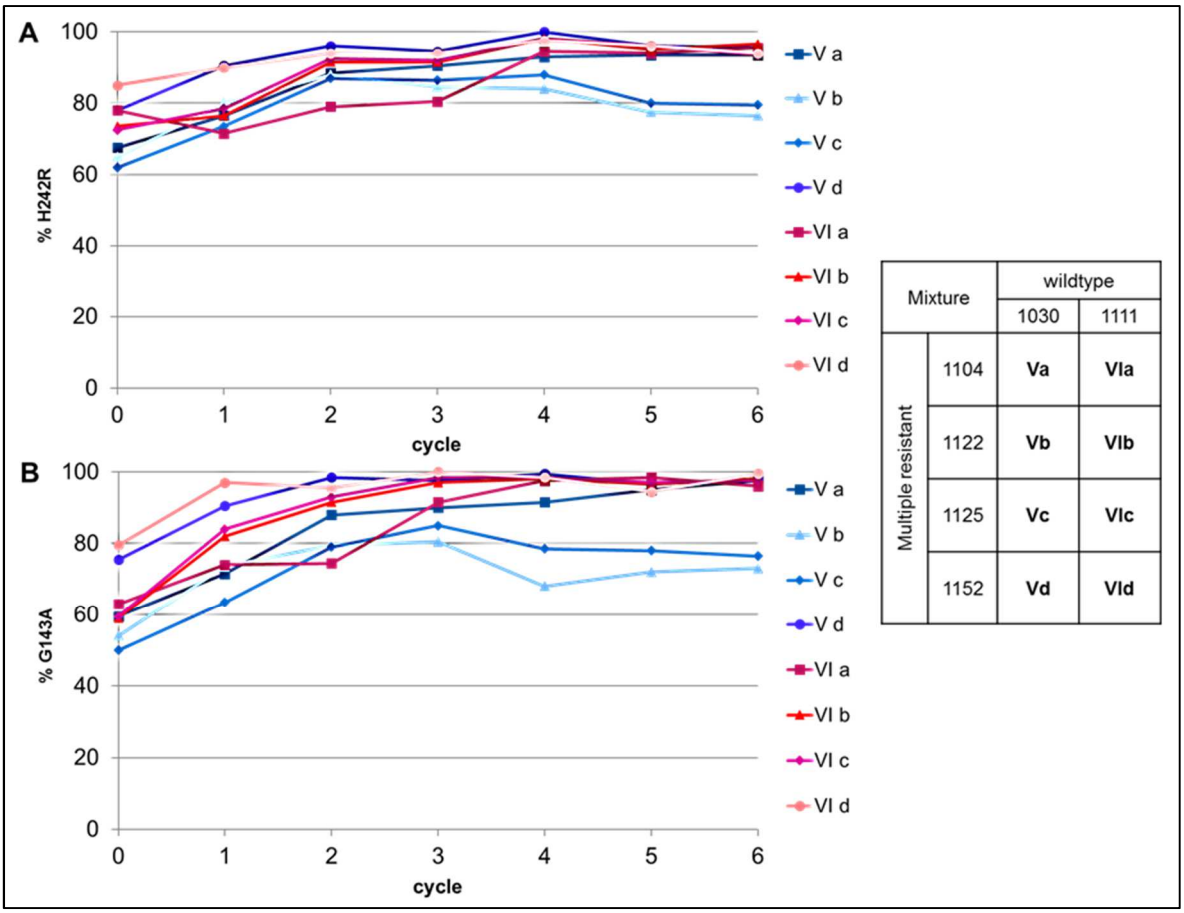


Figure 39: Changes in percent G143A and B-H242R determined by pyrosequencing of artificial mixtures of multiple resistant and sensitive isolates of *E. necator*. Mixtures were of cycled on untreated grapevine and each cycle represents 10 days of incubation. A: Frequency of H242R; B: Frequency of G143A.

All analysed mixtures show a strong increase in the frequency of both mutations (G143A and B-H242R) after the first two cycles. Except for the isolate mixtures V b and V c, all other mixtures showed a further increase to 94 – 97% H242R and to 96 – 100% G143A. For all analysed mixtures, a significant positive increase (V b, c and VI a: reg.coef. = +2.44; V a, d, VI b, c and d: reg.coef. = +3.16) of H242R and G143A was detected (supplementary material, Figure 51). Under the test conditions, multiple resistant isolates were not less competitive than the sensitive mixing partner used.

4. Discussion

Plant pathogens, particularly species with mixed sexual and asexual reproduction, possess the ability to rapidly overcome chemical control (Jones *et al*. 2014). *E. necator* has a high asexual reproductive frequency, which increases the intrinsical risk for the development of fungicide resistance. In addition to the multi-site fungicide sulphur, the number of modes of action available to control powdery mildew is limited and for some fungicide classes, such as QoIs and DMIs, resistances are already known for grape powdery mildew.

Considering the fact that repeated fungicide applications are needed to control powdery mildew, a diversity of different fungicide classes is essential to ensure a proper resistance management, e.g. developing a spray application program by alternating or mixing different modes of action. To protect the available fungicide classes from further activity loss and implement appropriate product use recommendations, it is essential to understand the properties of resistant isolates.

4.1 Sensitivity situation of *E. necator* towards aryl-phenyl-ketones and SDHIs

The chemical class of aryl-phenyl-ketones is represented by two members, the benzophenone derived compounds metrafenone and pyriofenone, which are assigned to the same cross-resistance group (FRAC 2017, code list). In contrast, SDHIs are composed of numerous and diverse active ingredients. However, only three SDHIs are currently relevant in the control of grapevine diseases: boscalid, fluxapyroxad and fluopyram. Due to the high structural complexity of SDHI compounds, a complex resistance and cross-resistance pattern has been observed. This is the reason why isolates with a reduced SDHI sensitivity were termed as `less sensitive` in the current thesis. The term `resistant` would not be an appropriate description for some mutations and SDHI compounds.

4.1.1 Emergence and frequency of metrafenone resistance in *E. necator*

The registration of metrafenone in Europe was obtained by BASF in 2004, first for the control of powdery mildews and eye spot disease in cereals. From then on, it was registered in other crops and frequently used in cereals, cucurbits and grapevine. In 2008, the fifth season of metrafenone application, first resistant wheat powdery mildew isolate were observed. Two different resistance phenotypes, classified as ´moderately adapted´ and ´resistant´, were identified during the BASF monitoring program (Felsenstein *et al*. 2010). Investigations of Felsenstein *et al*. (2010) and Stammler *et al*. (2014), with isolates of both resistance

phenotypes indicated that `moderately adapted` isolates could be efficiently controlled with the registered field dose rate of 0.5 l/ha (150 g a.i./ha) of Flexity® (300 g metrafenone/l) and that conidia, produced on treated leaves were not viable and therefore unable to infect new leaves. Although a slight dose response was observed for resistant isolates, they could not be completely controlled with high doses of metrafenone. Resulting spores from the resistant phenotype were viable and able to infect new leaves (Felsenstein *et al*. 2010; Stammler *et al*. 2014).

After the market launch of metrafenone for its use in grapevine, the sensitivity of *E. necator* was monitored on a yearly basis in *in vivo* tests by using a discriminatory dose of 10 mg/l metrafenone. With Vivando® (500 g metrafenone/l), which was introduced in 2007, and Kusabi® (300 g pyriofenone/l), introduced in 2014, two aryl-phenyl-ketones are used in European vineyards. In 2010, single *E. necator* isolates with a reduced sensitivity towards metrafenone were identified for the first time (Stammler *et al*. 2014). Further resistant isolates were detected in Hungary, Austria (BASF sensitivity monitoring studies) and Italy in 2013 (Kunova *et al.* 2016; Graf *et al*. 2017). Studies with metrafenone resistant isolates showed that they were able to grow and sporulate even on leaves treated with a concentration ten times higher than the registered field rate (Kunova *et al*. 2016). These results could be confirmed during the current studies. All tested isolates classified as resistant showed EC_{50} values > 30 mg/l, and thus resulted in a RF value higher than 130.

In contrast to the observations made in wheat powdery mildew, moderately adapted isolates could not be identified during the grape powdery mildew monitoring. This difference is due to the different testing methods, which had been chosen for wheat and grape powdery mildew monitoring. Whereas, in the wheat powdery mildew monitoring, different concentrations were used to determine the sensitivity of each isolate, for grape powdery mildew only one discriminatory dose was tested. Using one discriminatory dose of metrafenone is useful to identify isolates clearly outside the baseline sensitivity, but not sufficient to detect isolates with a slightly reduced sensitivity. This applies also to the discriminatory dose of 50 mg/l for the French monitoring, which is even higher than the highest concentration used in the leaf disc tests. Hence, the discrimination between moderately adapted and resistant phenotypes, which was done for wheat powdery mildew (Felsenstein *et al*. 2010), is not possible for *E. necator*. During these investigations, sensitivity studies with isolates from 2014 and 2015, which had been characterised as sensitive resulted in an EC_{50} range of < 1 – 4.7 mg/l (mean EC_{50} of < 1,9 mg/l). Before the launch of metrafenone in grapevine, baseline sensitivity studies with 110 *E. necator* isolates revealed an EC_{50} range of 0.09 – 0.39 mg/l metrafenone (mean EC_{50} of 0.23 mg/l). The broader EC_{50} range may indicate a shifting to a slightly reduced sensitivity for some isolates within the sensitive fraction. The registered full rate of metrafenone (Vivando®) in grapes in Europe is on average, 0.2 l/ha (100 g a.i./ha) and was in principle sufficient to

control isolates with a slightly reduced sensitivity. In conclusion, even if the portion of slightly adapted isolates seems to be low, the occurrence of a moderately adapted phenotype for *E. necator* cannot be excluded. Conclusions about the frequency of potentially existing moderately adapted *E. necator* isolates and their spread cannot be drawn, but could be evaluated by using a second lower discriminatory dose, like it was done for wheat powdery mildew.

The frequency of moderately adapted *B. graminis* f.sp. *tritici* isolates in European wheat growing regions is characterised by an increase to 25% from 2009 to 2012 (Stammler *et al*. 2014; Graf *et al*. 2017). Since 2012, the frequency has stabilised and only a slight increase to a level of 34% in 2016 was observed. The frequency of resistant isolates remained over all these years at very low levels and represented only up to 1.5% of the monitored population. Competition experiments, conducted with mixtures of sensitive and moderately adapted or resistant isolates, showed a decrease of metrafenone resistance in all tested isolate mixtures. Both the development of the frequency of less sensitive isolates and the results of the competitiveness test indicate the presence of fitness costs associated with moderately adapted and resistant wheat powdery mildew isolates. In contrast to the development of metrafenone resistant wheat powdery mildew isolates, the frequency of resistant *E. necator* isolates is increasing. In 2016, the distribution of resistant isolates was very heterogenous and the resistant fraction represented 17.5% of the monitored population.

In summary, there are major differences in the development of metrafenone resistance between wheat and grape powdery mildew. These differences may indicate a distinct evolution of metrafenone resistance in the two powdery mildew species, which could be based on different resistance mechanisms.

4.1.2 Emergence and frequency of *E. necator* isolates with a reduced SDHI sensitivity

SDHIs are currently one of the most important and fastest growing fungicide classes. Because of their broad activity spectrum and high efficacy at low rates, they are frequently used for the chemical control of fungal diseases (Stammler *et al*. 2015). Resistances towards SDHIs are already known for more than 15 plant pathogenic fungi (FRAC 2017). Besides diseases on cereals, such as *P. teres* on barley (Rehfus *et al*. 2016) and *Z. tritici* on wheat (FRAC 2017), resistances have been identified in pathogens responsible for diseases on fruit and vegetable crops, such as *B. cinerea* on strawberries and grapevine (Stammler *et al.* 2007; Veloukas *et al.* 2011), *P. xanthii* on cucurbits (Miyamoto *et al.* 2010b), *A. alternata* (Avenot und Michailides, 2007) on pistachio and *V. inaequalis* on apple (Huf 2016; Toffolatti *et al*. 2016). Boscalid was the first SDHI with a broad activity spectrum and the first SDHI registered for the use in

grapevine. With the registration of fluopyram in 2014 and fluxapyroxad in 2017, only three SDHIs are available for the use in grapevine. SDHI solo products, such as the boscalid containing fungicide Cantus® (introduced in 2006) and Luna Privilege® (introduced in 2012) containing fluopyram, are also registered for the control of *B. cinerea* in grapevine. Sercadis® (introduced in 2017) is the first SDHI solo product only registered for the control of *E. necator*. The control of grape powdery mildew with SDHI containing products is mainly based on mixtures, such as Collis® (kresoxim-methyl and boscalid; introduced in 2006) and Luna® Experience (tebuconazole and fluopyram; introduced in 2013). In 2014, a single *E. necator* isolate with a reduced SDHI sensitivity was detected for the first time in BASF monitoring studies (FRAC 2017, minutes of the SDHI Meeting 2015; Graf *et al*. 2017). The European SDHI sensitivity monitoring from 2013 to 2015 identified only single isolates with a reduced fluxapyroxad sensitivity in Italy and Greece. A special trial program in 2015, in which samples were analysed with a discriminatory dose of the SDHI boscalid, identified isolates with a reduced boscalid sensitivity in Hungary, which were not detected with the respective dose of fluxapyroxad. Therefore, the suggestion was made that some isolates may have a reduced sensitivity towards boscalid but not towards fluxapyroxad and were not detected in 2013 to 2015. It seems, that there is no full cross resistance between the two SDHIs boscalid and fluxapyroxad, which was also observed for less sensitive *A. alternata* isolates (Avenot *et al*. 2014). The year of emergence of the first boscalid less sensitive *E. necator* isolates cannot be exactly determined, but can be suggested to be between 2012 and 2014. Consequently, in order to detect all isolates with a reduced SDHI sensitivity, the sensitivity monitoring in 2016 was accomplished with boscalid as discriminatory ingredient and revealed moderate frequencies of isolates with a reduced boscalid sensitivity. In 2016, the distribution of less sensitive isolates was heterogenous and represented 12.5% of the analysed European population.

4.2 Emergence and distribution of resistance in European grape powdery mildew populations

Five years after the detection of the first isolate with reduced metrafenone sensitivity, the resistant portion represents about one fifth of the monitored population, however the dispersal of metrafenone resistance is unevenly distributed over the different European counties. Whereas the north-western part is still sensitive, higher resistance frequencies have been observed in east European countries, such as Hungary, Czech Republic and Slovakia. Interestingly, a similar distribution pattern could be observed for isolates with a reduced SDHI sensitivity. In addition, QoI resistance frequencies are also stable at high levels in these countries (BASF sensitivity monitoring studies). Regarding the occurrence and distribution of

fungicide resistance in *E. necator* populations, Eastern Europe appears to be a hot spot region. It seems that the frequency and further development of metrafenone and SDHI resistance is strongly dependent on the European region considered. With these observations, the question rises, as to if there is a reason, which could justify these differences between the sensitivity status of the north-western and the eastern part of Europe. Besides the intrinsic risk of the fungal pathogen and the fungicide itself, there are different aspects that could influence the emergence of fungicide resistance:

1. The practical use of the fungicide, meaning the frequency of applications per season, the application dose, using fungicide mixtures or alternating different fungicide classes and the timing of the application.

2. The climatic conditions, that might be favourable for a severe disease pressure and a high population size.

3. The history and genetic variability of the fungal population, that may have already developed fungicide resistances.

In the following, these aspects will be discussed based on the case examples Germany and Hungary.

1. Throughout Europe, practical recommendations for the use of fungicides can differ between countries. For a fungicide product, the manufacturer specifies the directions for use with a recommended dose, a maximum number of applications per season and a spray interval. Furthermore, indications may be given by the manufacturers or local advisory to alternate or use mixtures of products with different modes of action, in order to reduce the risk of resistance development. This is in accordance with current resistance management models, in which alternating of different modes of action or the usage of fungicide mixtures has given evidence to decrease the selection for fungicide resistance (van der Bosch *et al*. 2014). The application frequency with an average of eight applications per season, is similar for Germany and Hungary, and the recommended dose rates are only slightly different. In Hungary and other European countries, moderate dose rates, e.g. 0.2 – 0.25 l/ha Vivando® and 0.3 – 0.4 l/ha Collis® are recommended. In Germany, the recommended dose rate is dependent on the phenological developmental stage of grapevine and varies between 0.08 – 0.32 l/ha Vivando® and 0.16 – 0.64 l/ha Collis®. A lower dose rate is applied from the beginning of the season until flowering and higher doses should be applied in the period of bud break, bloom and fructification. A fungal population consists of sensitive and resistant strains, which arise through mutation and slowly invade the population (van den Berg *et al.* 2013). With a high fungicide dose, the sensitive fraction is greatly removed from the population, whereas the selection pressure is increased and the resistant fraction

survives. Hence, by using a lower fungicide dose, a higher density of sensitive isolates ensures a competitive environment and the probability is less likely that the resistant fraction is able to increase (van den Bosch *et al*. 2014). The spray timing is another aspect which can also be slightly different among the countries, but is very similar for Hungary and Germany. The exact time point for applications is adjusted by the phenological stage or weather driven forecast models (Gubler *et al*. 1999; Kast 1997), whereas the timing of the first application is the most important (Caffi *et al*. 2011). Chemical control of grape powdery mildew was shown to be more effective when applied in preventive conditions rather than curative conditions (Gubler *et al*. 1999). For a suitable resistance management, a preventive or protective fungicide use is proposed and post-infection, curative treatments should be avoided (FRAC 2017). The chance for the development of fungicide resistance is dependent on the number of produced spores and the probability that a mutation can occur (van den Berg *et al*. 2013). It is hypothesised, that when a fungicide application is delayed, the infection process may have proceeded to sporulating lesions which result in a larger initial population, and which is exposed to the fungicide (Brent and Hollomon 2007b). Overall, the practice between Germany and Hungary seems to be comparable. With a similar application start, spray interval and dose rate, the practice does not seem to be the main factor, which differentiates the emergence of fungicide resistance.

2. The efficacy of a fungicide treatment strongly depends on the climatic conditions of the respective grape growing region and is linked to the occurring seasonal disease pressure. Optimal weather conditions for a severe *E. necator* infection are moderate temperatures (23 to 30°C) combined with high humidity. According to this, Germany is considered to be a region with a moderate powdery mildew disease pressure. Whereas in Hungary, high disease pressures with strong *E. necator* infections are on average expected every two years (Personal communication: Peter Hoffmann, BASF SE Hungary). The intensity of the disease pressure within a season is also dependent on the frequency and the time when the first symptoms occur. If the primary infection occurs early in the season, vast numbers of conidia are produced throughout the season, which result in an elongated disease cycle (Cortesi *et al.* 1997; Magaray *et al.* 1997). High infection rates in untreated field plots, with up to 100% infection have been observed in Hungary, when the first powdery mildew symptoms were detected early in the growing season (unpublished data, BASF SE). Under favourable conditions, as in Hungary, the time between spore germination and conidiation can be shortened to 5 days, which leads to multiple infection cycles (Gubler *et al*. 1999). The differences in disease severity and therefore the selection pressure is very high and may explain the differences observed in terms of resistance emergence and frequencies. High disease

pressure means more difficulty to control the disease and higher number of individuals and therefore also a higher genetic variability. Selection of resistant individuals is therefore more likely in a region with high disease pressure compared to a country like Germany with a moderate disease pressure.

3. Over the years, *E. necator* has been exposed to various fungicides, which were frequently used for control. In 2012 and 2014, when *E. necator* isolates, with a reduced sensitivity towards metrafenone and SDHIs were detected for the first time, resistances towards other fungicide classes such as DMIs and QoIs had already emerged in Europe (Dufour *et al*. 2011). Higher frequencies of QoI resistant *E. necator* isolates were also present in the Hungarian population (FRAC 2017). As one of the few powdery mildew specific fungicides, Vivando® was frequently used in German and Hungarian vineyards. After market launch, it was applied up to 4 times per season. The time span over which the population is exposed to a fungicide is increased, when the fungicide is frequently used in several applications. This may have led to an increased selection for fungicide resistance (van den Bosch *et al*. 2014). The use of fungicide mixtures is in line with a suitable resistance management. The mixing partner, which should be a representative of another fungicide class, reduces the risk of fungicide resistance development. However, if the population is already resistant to one of the mixing partners, the risk is increased for the other. Collis® is composed of a QoI (kresoxim-methyl) and a SDHI (boscalid). It could be speculated, that, with the emergence of QoI resistance, the activity of Collis® was mainly relying on boscalid and thus inducing a selection pressure for isolates with a reduced SDHI sensitivity. Furthermore, in 2013, in order to control *B. cinerea* infections at the end of the season, an additional application of Cantus® (boscalid) was recommended in Hungary. This might have resulted in further SDHI selection pressure. In summary, the historic presence of QoI resistance at relatively high frequencies made QoIs less useful as a resistance management partner and did not decrease the selection pressure on other modes of action included in the spray program, as for example boscalid and metrafenone. This leads to the conclusion that the emergence of resistance for one mode of action cannot delay the resistance emergence for the other remaining mode of actions and enhances the need for a diverse set of different modes of action and the strict practice of other anti-resistance management strategies.

4.3 Identification of molecular mechanisms connected to a sensitivity loss towards metrafenone and SDHIs in *E. necator* isolates

The sensitivity monitoring of obligate biotrophs, such as *E. necator*, is often done with *in vivo* test methods. Sensitivity tests are conducted with a discriminatory concentration or several concentrations to identify changes in the sensitivity compared to the reference population. Such methods are time consuming and do not provide the identification of the mechanism, which leads to the sensitivity loss. The elucidation of the resistance mechanism is mandatory for the development of molecular genetic monitoring methods and delivers more precise information about resistance situations in European vineyards. In the future, the *in vivo* monitoring method can be complemented or even replaced by molecular techniques for the rapid analysis of leaf and bunch samples.

4.3.1 Elucidation of the resistance mechanism associated with metrafenone resistance: phenotypical and whole genome sequencing studies

The biochemical mode of action of metrafenone is still unknown, although numerous attempts have been done to identify the molecular target (Köhle *et al.* 2004; Schmitt *et al.* 2006; Opalski *et al.* 2006). Phenotypical observations about the influence of metrafenone on the development of *B. graminis* f.sp. *tritici* and *E. necator*, provides a first indication on the biological processes inhibited by the fungicide. This information allows also to draw hypothesis about the potential group of proteins that are targeted by metrafenone.

The germination of *B. graminis* f.sp. *tritici* was slightly reduced under metrafenone treatment (Opalski *et al*. 2006), which was not observed for *E. necator* (Kunova *et al*. 2016). In fact, metrafenone has no influence on the germination of *E. necator*. This could be confirmed during the present studies. Microscopy studies revealed that metrafenone induces morphogenic abnormalities for *B. graminis* f.sp. *tritici* and *B. graminis* f.sp. *hordei*, like malformed and multilobed appressoria, aberrant conidiophores and bursting of hyphal tips (Schmitt *et al.* 2006; Opalski *et al.* 2006). Similar observations were made with *E. necator* and *E. pisi* (Schmitt *et al.* 2006). Phenotypical investigations of *E. necator* under preventive and curative conditions confirmed these results and revealed abnormalities in the appressoria formation, hyphal growth and malformation of conidiophores and conidia.

Based on the observed morphological aberrations induced by metrafenone, the molecular target seems to be involved in hyphal morphogenesis, polarised hyphal growth as well as the establishment and maintenance of cell polarity (during this study; Schmitt *et al.* 2006; Opalski

et al. 2006). The search for mutants from other fungal species with similar morphological characteristics allowed the identification of numerous proteins that are involved in the establishment and maintenance of cell polarity and hyphal morphogenesis in filamentous fungi, such as Ras and Rho GTPases (Opalski *et al*. 2006). Actin delocalization and swollen hyphal tips were also observed for the Rho mutant *Agrho*3 of *Ashbya gossypii* and the constitutive activation of a Ras protein in *Colletotrichium trifolii* caused pleiotrophic effects such as bursting of hyphal tips and conidiation defects (Wendland and Philippsen 2001; Truesdell *et al*. 1999). Aberrant and hyperbranched hyphae and misshaped conidiophores were also observed in a deletion of a Rac homolog of *Penicillium marneffei* (Δ*cflB*) (Boyce *et al*. 2005). Another effect of metrafenone observed for *E. necator* was a strongly enhanced curved hyphal growth. Dominant negative *cflA* mutants, coding for a Rho GTPase, of *Penicillium marneffei* and *ro* mutants of *Neurospora crassa*, encoding a cytoplasmic dynein or an actin related protein, similarly exhibited curved hyphal growth (Plamann *et al*. 1994; Boyce *et al*. 2005). A comparable hyphal growth behaviour was also observed in deletion mutants of two septin genes (*cdc10* and *cdc11*) of *Candida albicans* (Warenda and Konopka 2002; Warenda *et al*. 2003).

In addition to powdery mildews, metrafenone is registered for the control of *Oculimacula* spp. in winter wheat and treatment resulted in a strongly restricted mycelial growth and sporulation. Furthermore, the sporulation of the non-pathogenic fungal model organism *A. nidulans* is inhibited under metrafenone treatment (Schmitt *et al*. 2006), which could be confirmed during these studies. Even if there are different effects of metrafenone treatment on the different fungal species, some are overlapping. Thus, all analysed organisms, powdery mildews as well as *A. nidulans* and *Oculimacula* spp. exhibited a strong effect on the sporulation under metrafenone treatment. The assumption made by Schmitt *et al*. (2006), that components of the signal transduction chain connected to the target of metrafenone or associated morphological regulating components might be slightly different among species, can be underlined. Phenotypical analyses or growth tests with *Oculimacula* spp., and *A. nidulans* would give further evidence about common aspects and to clarify the effect of metrafenone treatment on the germination, the morphology of hyphal growth and conidiation of these non-biotrophic ascomycetes. Finally, all proteins identified in mutants with similar phenotypic changes, as observed under metrafenone treatment, are somehow involved in polarized growth of filamentous fungi. The lack of cross-resistance with other mode of actions, the powdery mildew specific activity and the interspecific differences make the elucidation of the molecular target of metrafenone on the one hand challenging and on the other hand essential for the development of new fungicidal compounds.

In order to elucidate the molecular mechanism, which confer metrafenone resistance in *B. graminis* f.sp. *tritici* and *E. necator,* a whole genome sequencing approach (WGS) was

applied in this study. With the knowledge of the resistance mechanism the possibility is given to identify the molecular target of metrafenone.

Nowadays, sequencing technologies are constantly improving and the number of available whole genome sequences is steadily increasing. The rapid technological progress has been mainly done to type microbial genomes to improve patient care in medical research (Köser *et al*. 2014) but also to understand how genomes reflect the virulence of filamentous plant pathogens (Raffaele and Kamoun 2012). In 1996, the genome of yeast *Saccharomyces cerevisiae* was the first complete eukaryotic genome (Goffeau *et al*. 1996) and since then several other fungal species such as *Neurospora crassa* (Galagan *et al*. 2003) and *A. nidulans* (Galagan *et al*. 2005) have been sequenced. Besides non-biotrophic plant pathogenic fungi such as *B. cinerea* (Staats and van Kan 2012; van Kan *et al*. 2016), *Mycosphaerella graminicola* (Goodwin *et al*. 2011) and *Fusarium graminearum* (Cuomo *et al*. 2007) some obligate biotrophic ascomycetes like *B. graminis* f.sp. *hordei* (Spanu *et al*. 2010), *B. graminis* f.sp. *tritici* (Wicker *et al*. 2013), *Golovinomyces orontii* (Spanu *et al*. 2010), recently *E. necator* (Jones *et al*. 2014) and the transcriptome of *Podosphaera xanthii* (Vela-Corcía *et al*. 2016) have been sequenced. Together with the progress of WGS technologies, WGS is also becoming an essential tool for the detection of drug-resistance in microorganisms, due to the high resolution of the generated data and progressive reduction of costs and labour. The SNP diversity and spread of azole resistance in the human pathogen *Aspergillus fumigatus* could be already revealed using WGS (Abdolrasouli *et al*. 2015). Furthermore, a WGS approach of phenamacril sensitive and generated resistant *Fusarium graminearum* strains identified the potential target of this fungicide as myosin-5 (Zheng *et al*. 2015).

Compared with saprophytic and other plant pathogenic fungi, powdery mildew fungi show a massive genome expansion (Raffaele and Kamoun 2012). The estimated genome size of powdery mildews is with 120 - 180 Mb up to four times larger than the genome of non-biotrophs (30-40 Mb) and show an immense proliferation of transposable elements with 60 - 90% of the genome size (Spanu *et al*. 2010; Wicker *et al*. 2013; Jones *et al*. 2014). Not only powdery mildews but also other oomycete and basidiomycete biotrophs, like downy mildews or rust fungi, have a reduced gene content compared with related non biotrophs and lack gene families involved in various metabolic processes (Spanu *et al*. 2010; Raffaele and Kamoun 2012; Wicker *et al*. 2013; Vela-Corcía *et al*. 2016). The highly repetitive DNA content, as well as the loss of genes, seems to be in association with the obligate biotrophic life cycle of these pathogens. Chasmothecia, the sexual overwintering structure of powdery mildews, are frequently observed for several species (Gadoury and Pearson 1988, Pirondi *et al*. 2015; Jankovics *et al*. 2015) and it is thought that powdery mildews possess a relatively high sexual recombination rate. In contrast, comparative genome analysis of *B. graminis* f.sp. *hordei* (Hacquard *et al*. 2013) and *B. graminis* f.sp. *tritici* (Wicker *et al*. 2013) isolates indicated a

clonal or near-clonal reproduction, which results in distinct haplo-groups and nonrandomly associated alleles among loci. Recently, the genetic diversity analysis of several isolates revealed that *Podosphaera xanthii* reproduces predominantly asexually (Pirondi *et al*. 2015). Although powdery mildew genomes possess a high percentage of repetitive DNA, the availability of metrafenone resistant wheat powdery mildew isolates and the suggestions for a predominantly clonal reproduction were a promising starting point for WGS studies. Comparative WGS and SNP analysis between two sensitive, two moderately adapted and two resistant isolates of *B. graminis* f.sp. *tritici* was performed to identify genes that might contribute to metrafenone resistance and may be linked to the molecular target.

Besides the publicly available *B. graminis* f.sp. *tritici* genome 96224 (Wicker *et al*. 2013), another sensitive strain (2588) was *de novo* sequenced and used as second reference genome. SNP analysis was performed for the six resequenced isolates and each of the reference genomes. The results of both SNP analysis were taken together and revealed only six genes with non-silent SNPs in coding regions for all less sensitive isolates (moderately adapted and resistant). Based on the predicted function and potential morphological effects, an o-acyltransferase and a Rsc complex subunit were further analysed. The identified o-acyltransferase of *B. graminis* f.sp. *tritici* belongs to the group of acyl-CoA diacylglycerol acyltransferases (DGAT), which are essential enzymes in the lipid biosynthesis (Turchetto-Zolet *et al*. 2011). It was shown to be homologous to the *are1* and *are2* genes from *S. cerevisiae*, which belong to the subgroup DGAT1. Deletion mutants of *gup1*, encoding a yeast o-acyltransferase, exhibited fragile cell walls and a defect in bipolar bud site selection. There might be an interaction or auxiliary role of *are1* and *are2* in the *gup1* mediated remodelling process of glycosylphosphatidylinositol-anchored proteins and may contribute to this phenotype (Bosson *et al*. 2006). An amino acid substitution (V415I) in the o-acyltransferase gene, was identified in all less sensitive isolates (moderately adapted and resistant) sequenced during the first WGS analysis. Unfortunately, the presence of this mutation could not be confirmed by sequencing of other sensitive, moderately adapted and resistant wheat powdery mildew isolates. In addition, sequencing of the *E. necator* o-acyltransferase ortholog for several sensitive and resistant isolates revealed sequence identity. Furthermore, the conservation across different fungal species is low. These observations are leading to the assumption that the o-acyltransferase protein is unlikely to be linked to the mode of action of metrafenone and does not seem to contribute to metrafenone resistance in wheat and grape powdery mildew.

As described above, previous studies have indicated that metrafenone treatment has a strong influence on the sporulation of *A. nidulans*. Since numerous information, including complete sequences of reference strains, is available for *A. nidulans*, the generation of metrafenone adapted isolates may enable the identification of genes linked to metrafenone resistance.

Comparative SNP analysis of the sensitive parental strain and five metrafenone adapted isolates was performed. In two of nine identified genes in coding regions, an amino acid substitution could be identified, which was present in all adapted isolates, but not in the parental strain. These two genes coded for an isoflavone reductase family protein and a hypothetical aldehyde dehydrogenase protein. Isoflavone reductase enzymes are typically involved in the synthesis of defence molecules, which are produced as a response of invading microorganisms in plants (Kuć and Rush 1985). The role in filamentous fungi like *A. nidulans* is still unknown or the similarity to this enzyme class is merely coincidental (Melin *et al*. 2002). No homologous proteins in *B. gramins* f.sp. *tritici* and *E. necator* were identified for the isoflavone reductase family protein. The aldehyde dehydrogenase family is composed of $NAD(P)^+$-dependent enzymes that catalyse the oxidation of a wide spectrum of aldehydes. Besides their roles in various metabolic processes (metabolism of amino acids, carbohydrates and lipids), they play an important role in the detoxification of xenobiotics and drugs (Marchitti *et al*. 2008). A certain subgroup, the benzaldehyde dehydrogenases, are described as specialized aldehyde dehydrogenases for the oxidation of aromatic substrates, such as vanillin and benzaldehyde (Perozich *et al*. 1999). All homologous proteins identified for the hypothetical aldehyde dehydrogenase in wheat powdery mildew were identical in their amino acid sequence for the resequenced isolates. It seems to be unlikely that these enzymes play a role in metrafenone resistance in *B. graminis* f.sp. *tritici*, whereas an overexpression or downregulation, which may contribute to a reduced sensitivity, cannot be excluded.

Another SNP was identified in a potential promotor region of a Rsc complex subunit. Rsc complexes belong to the ATP-dependent chromatin remodelling enzymes, which are essential for cell viability because of their functions in transcriptional regulation (Schubert *et al*. 2013; Wang *et al*. 2014). Yeast knockouts (*swi*2 and *swi*1) exhibited slow growth and were defective in sporulation (Dror and Winston 2004). Recently, an extract with antifungal activity, isolated from a lichen forming fungus, was shown to disturb chromatin remodelling activities (Kwon *et al*. 2016). Interestingly, an orthologous Rsc complex subunit in wheat powdery mildew was shown to have a mutation in the coding sequence for all less sensitive isolates. The conservation across different fungal species was low and sequence comparisons of further wheat and grape powdery mildew isolates were identical. These results contradict the hypothesis that the Rsc complex subunit contributes to metrafenone resistance in wheat as well as grape powdery mildew.

Table 25: Summary of proteins, identified via WGS and SNP analysis of sensitive moderately adapted and resistant *B. graminis* f.sp. *tritici* and *A. nidulans* isolates.

	Hits identified via WGS	Validation study
B. graminis* f.sp. *tritici	O-acyltransferase	- Sequence analysis of further wheat powdery mildew isolates are contradicting - Sequence identity of *E. necator* homologue of sensitive and resistant isolates - Low conservation of mutated position
A. nidulans	Isoflavone reductase family protein	- No homologous proteins in wheat and grape powdery mildew
	Aldehyde dehydrogenase protein	- No differences in homologous proteins of wheat powdery mildew
B. graminis* f.sp. *tritici* and *A. nidulans	Rsc complex subunit	- Sequence analysis of further wheat powdery mildew isolates are contradicting - Sequence identity of *E. necator* homologue of sensitive and resistant isolates - Low conservation of mutated position

The identification of the exact molecular mechanism associated to metrafenone resistance was not successful based on the analysis conducted during these studies. An overview of the identified hits and the performed validation studies is given in Table 25. This is may be due to several causes, that are discussed in the following:

First of all, it might be possible that the resistance mechanism differs between moderately adapted and resistant phenotypes, for instance associated mutations could be located at different positions in one gene or even in several genes. Instead of searching for SNPs at one position for both resistance phenotypes, common mutations should be identified for each phenotype and the resulting genes should be compared. Although preliminary attempts were done to identify mutations specific for each phenotype, this approach could not be implemented because of the limited number of the respective phenotypes. Further analysis via WGS of a higher number of resistant isolates may reveal promising genes that could not be identified during these studies. Furthermore, it might be possible that metrafenone resistance is based on different resistance mechanisms or disturbs several steps of a metabolic pathway wherefore the number of sequenced isolates is not sufficient for the identification of associated genes. Additionally, relevant SNPs could be incorrectly assigned to intergentic regions, due to a poor or defective gene annotation. This could be overcome with whole transcriptome analysis with total RNA-sequencing. A similar approach was done with sensitive and resistant *Phytophthora capsici* isolates towards the carboxylic acid amide fungicide pyrimorph. The proteomic data received revealed several proteins associated with pyrimorph resistance and suggested the inhibition of the cell wall biosynthesis as targeted pathway (Pang *et al*. 2015). The available genome data in combination with transcriptomic information would give further possibilities for the elucidation of the resistance mechanism, such as variation in gene expression levels in sensitive and less sensitive isolates. Even if the WGS approaches did not yet yield in the identification of the resistance mechanism of metrafenone in this study, the

sequencing results received give insights in the genomes of wheat powdery mildew isolates and represent background knowledge for the further elucidation process.

With more than 300 members, natural benzophenones, which are mainly produced by plants and fungi, exhibit a complex structural diversity. Although they are commonly known for their antimicrobial and especially their anticancer activity, rare information about their mode of action is available (Wu *et al*. 2014). The few studies, which investigate the mode of action derive from cancer research and describe numerous targets depending on the analysed substance. Beside a down regulation of signal transduction elements (e.g. MAPK), they seem to inhibit histone acetyltransferases and microtubule assembly (Protiva *et al*. 2008; Pardo-Andreu *et al*. 2011; Díaz-Carballo *et al*. 2012; Wu *et al*. 2014). Based on the common phenol-carbonyl-phenol skeleton, several artificially synthesized benzophenones have been developed. It is noteworthy to say, that even if the structure of benzophenones is very similar, their activities can strongly differ (Hsieh *et al*. 2003). Recently, Winn *et al*. (2017) released a publication about bioreductively activatable prodrug conjugates (BAPCs). Phenstatin based BAPCs represent an essential opportunity for targeted therapeutic interventions in cancer research. These substances are activated based on enzymatic-cleavage at the target localization (Winn *et al*. 2017). The chemical structure of phenstatin, a benzophenone derived potent anticancer agent and tubulin polymerisation inhibitor is very similar to that of metrafenone (Figure 40).

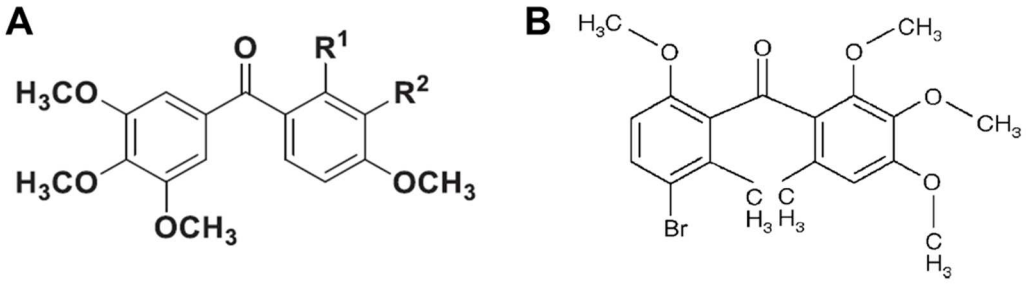


Figure 40: Chemical structure of phenstatin and metrafenone. A: Chemical structure of phenstatin (R1=H; R2=OH) and their prodrug hydroxyphenstatin (R1=R2=OH), source: Winn *et al.* 2017; B: Chemical structure of metrafenone, source: Opalski *et al.* 2006.

Consequently, it could be speculated that metrafenone serves as a prodrug and could be activated by enzyme-mediated oxidation reactions. This hypothesis could be further investigated by analysing the biological effect of phenstatin treatment on wheat and grape powdery mildew. In addition, the sequences available from the resistant strains sequenced during this work, could be analysed with respect to enzymes such as oxidoreductases, which might confer this activation process.

A powerful approach commonly used in proteomics is activity-based protein profiling (ABPP), which enables the labelling and study of specific enzymes (Nodwell and Sieber 2011; Geurink

et al. 2011). This technique is also of great importance to understand the behaviour of bioactive compounds and to identify putative biological targets (Hashimoto and Hatanaka 2008; Pan *et al*. 2016). The captured target could then be identified based on SDS PAGE and tandem mass spectroscopy (MS/MS) (Nodwell and Sieber 2011). Using metrafenone as ABPP probe would give a further possibility to identify binding targets and elucidate the resistance mechanism.

4.3.2 Identification of the resistance mechanism associated with a reduced SDHI sensitivity: Impact and cross-resistance

Different to the case of metrafenone, the target gene of SDHIs is well known and molecular mechanisms, which confer resistance to this class of fungicides have been investigated for several plant pathogens. Molecular analysis of the target genes of the SDHIs, the *sdh*-genes, showed that different mutations in these genes are responsible for a lower SDHI sensitivity. Several mutations at different positions in the SDH-B, SDH-C and SDH-D subunits have been detected, depending on the analysed pathogen. During this work, the emergence of *E. necator* isolates with a reduced SDHI sensitivity was recognised for the first time. Furthermore, the identification of target site mutations, the impact and the cross-resistance pattern of target-site mutations were analysed. Among all isolates analysed with a reduced SDHI sensitivity over three years of *E. necator* monitoring, four different target-site mutations, B-H242R, B-I244V, C-G169D and C-G169S, were identified for the first time in the *sdh*B and *sdh*C genes.

The replacement of histidine by arginine in subunit B at position 242, is one of the most frequent mutated positions associated with SDHI resistance. Considering that the amino acid sequence of the different SDH subunits depends on the fungal species, the numbering of mutations responsible for SDHI resistance differ from pathogen to pathogen (Stammler *et al.* 2015; Mair *et al*. 2016). Homologous substitutions were detected in other fungal plant pathogens like *B. cinerea* (H272), *A. alternata* (H272) and *P. teres* (H277), where substitutions of this histidine to phenylalanine, tyrosine or leucine, depending on the pathogen, have been detected (Stammler *et al*. 2007, Avenot and Michailides 2007, Rehfus *et al*. 2016). Another amino acid exchange in subunit B, the I244V, was identified in one *E. necator* isolate. Field isolates of other fungal pathogens with an amino acid substitution from isoleucine to valine at homologous position are not known, but could be identified in laboratory mutants of *Z. tritici* (I269V) after the selection on carboxin, fluopyram and isopyrazam (Stammler *et al*. 2011; Scalliet *et al*. 2012). This observation would indicate, that this mutation seems to be connected with a reduced SDHI sensitivity in *E. necator*. Interestingly, two different mutations could be identified at position 169 in subunit C*:* the G169D and the G169S. Whereas the substitution from glycine to aspartic acid was identified in several *E. necator* isolates, the substitution to serine was identified in only one isolate. So far, no homologous substitutions to the G169D and G169S

have been identified in other plant pathogenic fungi, neither in field isolates nor in laboratory mutants. The amplification and following transformation of the mutated fragment e.g. in *B. cinerea*, would be a possibility to confirm the effect of the G169S on SDHI sensitivity. Even though, several mutations can confer SDHI resistance to plant pathogens, none of the sequenced *E. necator* isolates possessed two mutations at the same time in the *sdh*-genes. In general, double mutations in *sdh*-genes were not so far detected in field isolates and only rarely observed in single laboratory mutants of *Z. tritici* (Scalliet *et al*. 2012; Fraaije *et al*. 2012).

It is known that SDHIs inhibit the SDH by binding to the Q-site, the ubiquinone binding pocket (Keon *et al*, 1991; Hägerhäll 1997; Horsefield *et al*. 2006). The Q-site is formed by amino acid residues of SDH-B, SDH-C and SDH-D. Whereas SDH-B is the most conserved subunit across different prokaryotic and eukaryotic species (Yankovskaya *et al.* 2003; Sun *et al.* 2005; Horsefield *et al.* 2006), some amino acid residues in SDH-C and SDH-D are also highly conserved (Scalliet *et al*. 2012), e.g. G169 in *E. necator* (this study). Amino acids involved in the binding of ubiquinone, tryptophan at position 224 in SDH-B (*Z. tritici* numbering), serine at position 83 in SDH-C and tyrosine at position 130 in SDH-D are in direct interaction with SDHIs. Compared to the natural substrate, SDHIs were shown to bind deeper into the binding pocket and it seems that they interact with several other positions (Glättli *et al.* 2009; Glättli *et al.* 2011; Sierotzki and Scalliet 2013; Stammler *et al*. 2015). For example, the histidine 267, homologue to H242 in *E. necator*, is located in close proximity to the Q-site and may interact via an H-bond with the SDHIs. The interaction seems to be lost when the histidine is substituted to arginine, tyrosine, leucine or valine (Sierotzki and Scalliet 2013; Stammler *et al*. 2015). The isoleucine at position 269, homologue to B-I244V in *E. necator*, was shown to be in direct vicinity to the binding pocket and seems to build Van-der-Waals interactions with the acid part of carboxamides (Scalliet *et al*. 2012). Interestingly, the glycine 169 in SDH-C is not closely located to the binding pocket and not directly involved in the binding of SDHIs. Aspartic acid and serine are bulkier than glycine and it can be speculated that a substitution may lead indirectly to structural rearrangements in the binding pocket. Different substitutions even at the same position (e.g. B-H272Y/R/L/V in *B. cinerea*) can cause different resistance levels (Stammler *et al*. 2011; Sierotzki and Scalliet 2013; Stammler *et al*. 2015). This suggests, that the sensitivity loss caused by an SDHI mutation is more depended on the amino acid exchange as well as the organism and does not seem to be correlated with the distance to the binding site (Scalliet *et al*. 2012; Stammler *et al*. 2015).

The frequency of mutations conferring a reduced SDHI sensitivity in *E. necator* was not determined before. Based on the sequence aberrations in the *sdh*-genes, it was possible to develop a molecular genetic monitoring method and to identify the most common mutations B-H242R and C-G169D quantitatively. In 2016, the frequency of mutations associated with a reduced sensitivity could be determined and the B-H242R was the most frequent mutation and

represented 92% of the less sensitive isolates analysed (Figure 33). The substitution from histidine to arginine, was also shown to be the most frequent occurring mutation in *B. cinerea* (Veloukas *et al*. 2011; Fernández-Ortuño *et al*. 2012). Although one single isolate with the C-G169D substitution was detected in Italy in 2014, in 2015 and 2016 this type of mutation was only observed in isolates from a single site in Greece. One single isolate with C-G169S mutation was detected in 2015 but not in 2016. In 2016, an isolate with the B-I244V mutation was detected for the first time. Based on this molecular monitoring method, the development and distribution of *sdh*-mutations can be followed up easily.

As a second step, the impact of the mutations identified on the activity of the SDHIs boscalid, fluopyram, fluxapyroxad were assessed. For the most frequent mutations, the same was done with the SDHIs isopyrazam, penthiopyrad and benzovindiflupyr, which are registered for the use in cereals and some fruit crops. Depending on the fungicide and the *sdh*-genotype different sensitivities and levels of cross-resistance between the SDHIs were observed (Figure 36 and Figure 37). Isolates with the B-H242R substitution showed different levels of reduced sensitivity depending the SDHI compound. Growth and sporulation was not or only slightly inhibited by boscalid and isopyrazam. A similar sensitivity loss towards boscalid is also described for various plant pathogens with the homologous mutation, such as *B. cinerea* (Angelini *et al*. 2010; Veloukas *et al*. 2011), *A. alternata* (Avenot and Michailides 2007; Landschoot *et al*. 2017) and *Corynespora cassiicola* (Miyamoto *et al*. 2010a). *E. necator* isolates with this *sdh*-genotype have no or only a weak influence on the effectiveness of fluxapyroxad, fluopyram, benzovindiflupyr and penthiopyrad. Recently, similar observations have been made by Cherrad *et al*. (2017), which confirmed these results. The same was observed for *A. alternata* (Avenot *et al.* 2014) and *B. cinerea* (Amiri *et al.* 2014) isolates carrying this homologous mutation, which were characterized as sensitive against fluopyram and fluxapyroxad. However, the amino acid exchange from histidine to lysine leads to high sensitivity losses to all SDHIs (Stammler *et al*. 2015). This observation suggests that the substituted arginine residue at position 242 does not interact directly with the SDHIs fluxapyroxad, fluopyram, benzovindiflupyr and penthiopyrad. This explains the observation that in trial sites where the H242R was detected, the fluxapyroxad containing fungicide Sercadis® provided high disease control, but Cantus® (boscalid) efficacy was reduced (BASF field trail data). The impact of the the *sdh*-genotype B-I244V on the efficacy of fluopyram was high and much lower for boscalid and fluxapyroxad. Higher resistance levels towards fluopyram compared to boscalid for this *sdh*-genotype were also observed for the homologous mutation in lab mutants of *Z. tritici* (Scalliet *et al*. 2012). Scalliet *et al*. (2012) suggested that the structurally similar valine partially compensate the Van-der Waals interaction carried out by isoleucine. Nevertheless, to confirm these first observations it would be necessary to test several isolates with the same resistance mechanism. Only one isolate carrying the B-I244V

has been identified so far and the impact on the different SDHIs should be verified in further tests. The artificial generation of mutants or stable transformation of powdery mildew species has not been described up to now, most probably due to the biotrophic life style, which makes such experiments difficult to perform. Recently, DNA fragments could be integrated in the *P. xanthii* genome with the help of *A. tumefaciens*-mediated transformation (Martínez-Cruz *et al*. 2017). The integration was shown to be stable under selection pressure and may give the possibility to convert this transformation technique to other powdery mildews, including *E. necator*. This applies also to the C-G169S isolate, conferring a higher sensitivity loss towards boscalid and fluopyram and lower towards fluxapyroxad. In contrast, isolates with the C-G169D genotype had an impact on the effectiveness of all SDHIs. It seems that the substitution to aspartic acid has a stronger influence on the binding of SDHIs compared to the substitution to serine at this position. Serine is less bulky than aspartic acid and the potential structural arrangements influencing the binding pocket have a stronger impact on the binding of boscalid and fluopyram. All SDHIs tested strongly inhibited the germination of sensitive isolates. The germination behaviour of the mutated isolates was more or less affected depending on the SDHI used and the analysed *sdh*-genotype, which reflect the tendencies observed in the leaf disc tests (Table 26). Although all these fungicides belong to the same cross-resistance group, the interaction and affinity to the binding pocket, as well as the biological spectrum, is strongly influenced by the chemical structure of the different SDHIs (Glättli *et al*. 2011; Amiri *et al* 2014).

Table 26: Summary of *sdh*-mutations identified in *E. necator* and their impact on SDHIs. N.A.: Not analysed.

	Boscalid	Fluxapyroxad	Fluopyram	Isopyrazam	Penthiopyrad	Benzovindiflupyr
B-H242R	High	No influence	Low	High	Moderate	No influence
B-I244V	Moderate	Low	High	N.A.	N.A.	N.A.
C-G169D	High	Moderate	Moderate	High	High	High
C-G169S	High	Low	High	N.A.	N.A.	N.A.

Even if the host plant is included by using *in vivo* leaf disc tests, they are set under optimal laboratory conditions and do not necessarily reflect the situation in the field. Boscalid containing products have the longest history and were frequently used before the introduction of other SDHI containing products, which explains the high frequency of the H242R mutation. Considering the impact of the mutations on the different SDHIs, it can be speculated that the more frequent use of fluopyram containing products has led to a selection of isolates carrying the I244V and G169D/S mutations. With the increasing use of fluopyram and fluxapyroxad containing fungicides it can be expected that the frequency of the different SDHI mutations is going to change, as it has been detected in *B. cinerea* in recent years (BASF sensitivity monitoring data; personal communication: Dr. Gerd Stammler, BASF SE). To which extent

isolates with the genotypes B-I244V or C-G169D/S will spread and their impact on the field performance of SDHIs can only be speculated and it will most probably depend on the intensity of use of the different SDHIs.

In general, the structural complexity of SDHIs has led to a distinct resistance development compared with other fungicide classes. A qualitative resistance development is in most cases based on a single mutation in the target gene, e.g. G143A to QoI fungicides, which leads to a split of the population and a sudden loss of the effectiveness of the respective fungicide (Fraaije *et al*. 2005; FRAC 2017). In contrast, if the resistance development is characterised by a gradual shift of the sensitivity, in most cases based on an accumulation of mutations in the target gene, the resistance type is described as quantitative (Brent and Hollomon 2007b). This applies to the development of resistance towards DMI fungicides, where the accumulation of mutations in the *cyp51* gene has led to a stepwise adaption of *Z. tritic*i isolates (Stammler *et al*. 2008; Cools *et al.* 2011). The cross-resistance relationship among SDHIs is more complex and different compared with cross-resistance of other fungicide classes. In contrast to the discrete resistance development towards QoIs (G143A) or the multi-step resistance towards DMIs, several single mutations in *sdh*-genes mediating a low, moderate or high sensitivity loss have been detected. Thus, the development of SDHI resistance shows single features of both resistance types and can therefore be seen as a type of transition from qualitative to quantitative development.

4.4 Persistence and spread of resistance: the impact of competitiveness of resistant *E. necator* isolates

The persistence of resistant isolates is strongly dependent upon the ability to survive and reproduce in the absence of selection pressure, and their ability to compete with the wildtype population. Mutations that confer resistance towards a fungicide may be associated with a lower fitness compared to the wildtype population and negatively interfere physiological and biochemical processes (Anderson 2005). Information about the fitness is of particular relevance in order to define appropriate resistance management practice. Resistant isolates with a restricted fitness should not be able to compete with the wildtype and a restricted usage of this fungicide may allow a reintroduction for occasional use (Fernández-Ortuño *et al*. 2008). In contrast, if the fitness of resistant isolates is comparable with the sensitive population, then the resistant fraction should persist within the population even when the use of the fungicide is stopped (Rallos *et al*. 2014). Many experimental setups for various pathogens and fungicides have been established to determine the stability of fungicide resistance. These indicate a high variation of vegetative and reproductive fitness depending on the species used and the fungicide itself (Rallos *et al*. 2014, Veloukas *et al*. 2014; Fan *et al*. 2015).

Potential fitness costs caused by a resistance mutation may be overcome by genetic changes distinct from the target gene. Further mutations may occur at different loci compensating the cost of resistance (Vallières *et al*. 2011; Wijngaarden *et al*. 2005). Such compensatory mutations may reduce disadvantageous effects of the resistance mutation, but it cannot be ruled out that they are detrimental, especially in the absence of the resistance mutation (Jeger *et al*. 2008). Thus, to precisely assess the fitness of resistant strains, the genetic background of the analysed isolates should be the same except the resistance allele. A similar genetic background can be achieved by the generation of laboratory mutants with the help of selection on chemical substances or transformation. There are more than a few reports of resistant laboratory mutants for several plant pathogens, like *Z. tritici* (Stammler *et al*. 2011; Scalliet *et al*. 2012) and *B. cinerea* (Markoglou *et al*. 2006; Lalève *et al*. 2013). For obligate biotrophic fungi, reports about successful transformations are rarely found and most resulted in transient mutants (Chaure *et al*. 2000; Martínez-Cruz *et al*. 2017). Furthermore, the transformation or selection of mutants can have various side effects, for example an impact on the fitness, especially if the mutagenesis is UV mediated. During the last years, a new gene editing technique has been developed, the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 system. The RNA-based site directed transformation of target organisms, e.g. introduction of mutations, minimizes potential side effects. The CRISPR-Cas9 system could recently be adjusted for several filamentous fungi (Nødvig *et al*. 2015; Liu *et al*. 2015) and further enhancements could give insights into fitness of resistant fungal isolates.

Thus, the use of isogenic *E. necator* isolates, which differ only in the resistance allele, is not yet feasible. To reduce side effects due to a different genetic background, various independent mixtures of wildtype and resistant isolates, as well as different population mixtures, were used for the competitiveness tests. In addition, the isolates used for paired mixtures were analysed with respect to the sensitivity towards different fungicides frequently used in powdery mildew control and only those with similar background were used. The absence of a difference in the germination rate between the sensitive and resistant isolates tested suggests that both, wildtype and less sensitive isolates, have similar preconditions to infect the host surface. Furthermore, experimental approaches with obligate biotrophic fungi are laborious and time consuming, because the host plant must always be included. On this account, the inclusion of high numbers of isolates and replicates is limited, which makes the evaluation of the overall fitness of a resistant population challenging. In addition, the overall fitness of one isolate to another does not only rely on one parameter. Additional information about the growth and infection rate, the number of produced spores and the infection period would provide a more detailed picture about their fitness (Corio-Costet *et al*. 2011; Rallos *et al*. 2014).

It was assumed that metrafenone resistance in *E. necator* is associated with fitness penalties. On the one hand, this suggestion is based on metrafenone resistant *E. necator* isolates, whose

sensitivity were fully restored after several cycles of cultivation on untreated grapevine leaves. It was speculated that this isolate contained small amounts of a sensitive strain, which was more competitive and replaced the resistant strain. On the other hand, there are indications for fitness penalties associated with metrafenone resistance in *B. graminis* f.sp. *tritici* (Stammler *et al*. 2014). A clear conclusion about fitness costs associated with metrafenone resistance in *E. necator* is not possible based on the competitive assays conducted during these studies. The frequency of resistance in the paired mixtures was strongly dependent on the intrinsic viability of the respective sensitive as well as resistant strain. In fact, one sensitive strain seemed to be as competitive as the resistant strains, whereas another sensitive strain, mixed with the same resistant isolates, indicated fitness costs. In conclusion, these results indicate that it is essential to analyse several isolates with sensitive and resistant phenotypes to get an insight in the competitive ability of resistant isolates. With artificial population mixtures, a slight decrease, maybe linked to a reduced competitive ability of the metrafenone resistant fraction, could be observed. Nevertheless, this result could not be confirmed in a further test with the additional stress factor sulphur. In this test, a similar competitive ability was observed for sensitive and metrafenone resistant populations. The exact resistance mechanism of metrafenone is not known and therefore using isolates with the same genetic background is not possible. Furthermore, it should be considered that metrafenone resistance may be linked to various mutations in different genes, even within the same species. The detection of fitness costs associated with metrafenone resistance is therefore problematic and seems to be also dependent on the elucidation of the resistance mechanism.

Several studies about fitness costs associated with SDHI resistance can be found in the literature. Different fitness measures, such as mycelial growth, spore production and competitive ability, have been analysed with laboratory mutants and field isolates of different plant pathogens (Scalliet *et al*. 2012; Veloukas *et al*. 2014; Fan *et al*. 2015; Lalève *et al*. 2014). In addition, the activity of the SDH enzyme was measured and the effect of different mutations was determined (Scalliet *et al*. 2012; Lalève *et al*. 2013). The observed impact on the fitness varied depending on the different target-site mutations and the fungal species (Lalève *et al*. 2014; Veloukas *et al*. 2014), which is consistent with the results obtained during these studies. Isolates with the B-H242R genotype were as competitive as the sensitive isolates, giving strong evidence that there are no or only low fitness cost associated with this resistance mutation. This result is consistent with the fact, that the B-H242R mutation is currently the most frequent observed. Similarly, Veloukas *et al*. (2014) reported that *B. cinerea* isolates with the homologous mutation (H272R) were at least comparable with sensitive isolates and possessed an increased fitness compared to other *sdh*-mutations. In contrast, laboratory mutants of *B. cinerea* showed a strong fitness penalty (Lalève *et al*. 2013; Lalève *et al*. 2014). Considering that the histidine is highly conserved across prokaryotic and eukaryotic species, it is

remarkable that different amino acid substitutions are possible at this position and, at least for the exchange to arginine, without significant fitness costs. Interestingly, all analysed field isolates with a reduced SDHI sensitivity were also QoI resistant and carried the G143A mutation (*E. necator* isolates in this study; Veloukas *et al*. 2014). Since isogenic mutants with H272R show a strong impact on the fitness (Lalève *et al*. 2014), it may be speculated that the accumulation of SDHI and QoI resistance is accompanied by additional compensatory genetic changes which may reduce the negative effects on the fitness. Similar to the observations made with metrafenone resistant isolates, the frequency of C-G169D in mixtures was strongly dependent on the selected sensitive strain. Altogether, a decreasing tendency was visible, which suggests that the substitution from glycine to aspartic acid is leading to a less competitive phenotype and maybe connected with fitness costs. This could explain the low frequencies of this *sdh*-genotype detected during the sensitivity monitoring.

Nowadays more and more plant pathogens, such as *B. cinerea* (Hahn *et al*. 2014; Rupp *et al*. 2016), *Z. tritici* (Lucas *et al*. 2015) and *E. necator* (Dufour *et al*. 2011; Gadoury *et al*. 2012a, b) are known to accumulate resistances against various fungicides. During these studies, *E. necator* field isolates exhibited several resistances towards different fungicide classes, such as QoIs, metrafenone and SDHIs, which led to multiple resistant phenotypes. The knowledge about their fitness would help to understand the evolution and to predict the further spread of such multiple resistant isolates. Based on the competitiveness tests conducted, multiple resistant isolates were able to compete with the sensitive mixing partners, which indicates that there are no fitness costs associated with the multiple resistant phenotypes.

The fitness tests conducted during this study were focused on one single fitness measure, the competitive ability in an environment without selection pressure and with the same portion of sensitive and resistant isolates. Furthermore, all fitness measurements were done under laboratory conditions and do not reflect the natural environment, which includes the formation of overwintering structures, variable weather conditions, competitive effects of microorganisms, different host plant varieties as well as migration and introduction of new genetic material (Jeger *et al*. 2008). The results gained with such experiments show potential fitness costs under optimal conditions. No fitness penalty observed in the experiments does not necessarily mean that there is no fitness cost under field conditions. Field experiments would provide further evidence and include the ecological context. However, such field studies are time and cost consuming and could lead to an additional spread of resistant isolates.

5. Summary

Grape powdery mildew, *Erysiphe necator,* is one of the most significant plant pathogens, which affects grape growing regions world-wide. Because of its short generation time and the production of large amounts of conidia throughout the season, *E. necator* is classified as a moderate to high risk pathogen with respect to the development of fungicide resistance. The number of fungicidal mode of actions available to control powdery mildew is limited and for some of them resistances are already known. Aryl-phenyl-ketones (APKs), represented by metrafenone and pyriofenone, and succinate-dehydrogenase inhibitors (SDHIs), composed of numerous active ingredients, are two important fungicide classes used for the control of *E. necator.* Over the period 2014 to 2016, the emergence and development of metrafenone and SDHI resistant *E. necator* isolates in Europe was followed and evaluated. The distribution of resistant isolates was thereby strongly dependent on the European region. Whereas the north-western part is still predominantly sensitive, samples from east European countries showed higher resistance frequencies.

Classical sensitivity tests with obligate biotrophs can be challenging regarding sampling, transport and especially the maintenance of the living strains. Whenever possible, molecular genetic methods are preferred for a more efficient monitoring. Such methods require the knowledge of the resistance mechanisms. The exact molecular target and the resistance mechanism of metrafenone is still unknown. Whole genome sequencing of metrafenone sensitive and resistant wheat powdery mildew isolates, as well as adapted laboratory mutants of *Aspergillus nidulans*, where performed with the aim to identify proteins potentially linked to the mode of action or which contribute to metrafenone resistance. Based on comparative SNP analysis, four proteins potentially associated with metrafenone resistance were identified, but validation studies could not confirm their role in metrafenone resistance. In contrast to APKs, the mode of action of SDHIs is well understood. Sequencing of the *sdh*-genes of less sensitive *E. necator* isolates identified four different target-site mutations, the B-H242R, B-I244V, C-G169D and C-G169S, in *sdh*B and *sdh*C, respectively. Based on this information it was possible to develop molecular genetic monitoring methods for the mutations B-H242R and C-G169D. In 2016, the B-H242R was thereby identified as by far the most frequent mutation. Depending on the analysed SDH compound and the *sdh*-genotype, different sensitivities were observed and revealed a complex cross-resistance pattern.

Growth competition assays without selection pressure, with mixtures of sensitive and resistant *E. necator* isolates, were performed to determine potential fitness costs associated with fungicide resistance. With the experimental setups used, a clear fitness disadvantage associated with metrafenone resistance was not identified, although a strong variability of fitness was observed among the tested resistant *E. necator* isolates. For isolates with a

reduced sensitivity towards SDHIs, associated fitness costs were dependent on the *sdh*-genotype analysed. Competition tests with the B-H242R genotypes gave evidence that there are no fitness costs associated with this mutation. In contrast, the C-G169D genotypes were less competitive, indicating a restricted fitness compared to the tested sensitive partners. Competition assays of field isolates, which exhibited several resistances towards different fungicide classes, indicated that there are no fitness costs associated with a multiple resistant phenotype in *E. necator*. Overall, these results clearly indicate the importance to analyse a representative number of isolates with sensitive and resistant phenotypes.

6. Zusammenfassung

Der echte Rebenmehltau, *Erysiphe necator*, ist eines der bedeutendsten Pflanzenpathogene in Rebenanbaugebieten weltweit. Die Kombination einer kurzen Generationszeit und die Produktion einer Vielzahl an Konidien führen dabei zu einem mittleren bis hohen Risiko hinsichtlich der Entstehung von Fungizid-Resistenz. Die Anzahl an verfügbaren Fungizid-Klassen zur Bekämpfung von Echtem Mehltau ist begrenzt, wobei für einige ein gewisser Resistenz-Hintergrund bekannt ist. Aryl-phenyl-ketone (APKs) sowie Succinat dehydrogenase inhibitoren (SDHIs) stellen zwei wichtige Fungizid-Klassen zur Bekämpfung von *E. necator* dar. Basierend auf den Jahren 2014 bis 2016 wurde die Entstehung und Entwicklung von Metrafenon- und SDHI-resistenten Isolaten untersucht. Die Frequenz resistenter Isolate zeigte dabei Schwankungen und eine starke Abhängigkeit von der untersuchten Region. Osteuropäische Länder zeigten dabei ein höheres Resistenzaufkommen, während der nordwestliche Teil Europas als sensitiv eingestuft werden konnte.

Die Durchführung klassischer Sensitivitätstests mit obligat biotrophen Pathogenen ist aufwendig, welches insbesondere in Bezug auf die Probennahme, den Transport und die Kultivierung der lebenden Isolate zu Schwierigkeiten führen kann. Um diese Schwierigkeiten zu umgehen, werden molekular genetische Methoden bevorzugt um ein effizienteres Monitoring zu ermöglichen. Die Entwicklung solcher molekularbiologischen Nachweismethoden basieren auf der Kenntnis des spezifischen Resistenzmechanismus. Das Zielgen, sowie der zugrundeliegende Resistenzmechanismus von Metrafenon ist noch unbekannt, weshalb Gesamtgenomsequenzierungen mit sensitiven und resistenten Weizenmehltau Isolaten sowie adaptierten *Aspergillus nidulans* Labormutanten durchgeführt wurden. Basierend auf einer vergleichenden SNP Analyse konnten vier Proteine identifiziert werden, welche möglicherweise im Zusammenhang mit Metrafenone Resistenz stehen. Die Ergebnisse weiterführender Untersuchungen, konnten Mutationen in diesen Proteinen als ursächliche Resistenzmechanismen bisher nicht bestätigen. Im Gegensatz zu APKs ist das Zielgen der SDHIs bekannt, weshalb durch die Sequenzierung der *sdh* Gene von *E. necator* Isolaten mit einer geringeren Sensitivität, vier verschiedene Mutationen, B-H242R, B-I244V, C-G169D und C-G169S, in *sdhB* und *sdhC* identifiziert werden konnten. Basierend auf der Sequenzinformation konnte im Folgenden eine molekulargenetische Monitoring-Methode entwickelt werden, wodurch B-H242R als die häufigste Mutation identifiziert werden konnte. Die ermittelten Sensitivitätseinbußen waren dabei stark von der jeweiligen *sdh* Mutation und dem untersuchten SDHI abhängig und zeigten ein komplexes Kreuzresistenz-Muster.

In den vorliegenden Untersuchungen wurden Konkurrenztests ohne Selektionsdruck durchgeführt, um Fitness Nachteile welche möglicherweise mit Fungizid Resistenz verbunden sind, zu identifizieren. Es konnte kein klarer Fitnessnachteil im Zusammenhang mit

Metrafenone Resistenz gezeigt werden. Fitnessnachteile von Isolaten mit einer verringerten SDHI Sensitivität zeigten eine starke Abhängigkeit von der zugrundeliegenden Mutation. Im Gegensatz zu Isolaten mit der C-G169D, welche einen leichten Nachteil in der Fitness vermuten lassen, scheinen Isolate mit der B-H242R keine oder nur geringe Fitnessnachteile aufzuweisen. Einige der untersuchten Feldisolate wiesen mehrere Resistenzen gegenüber verschiedener Fungizid-Klassen auf. Die Ergebnisse der Konkurrenz basierten Versuche weisen darauf hin, dass für Isolate mit multiplen resistenten Phänotypen keine Fitnessnachteile im genutzten Testsystem vorliegen. Die durchgeführten Experimente zeigten eindeutig, dass es wichtig ist, eine repräsentative Anzahl an Isolaten, insbesondere sensitive, in solche Untersuchungen einzubeziehen.

7. Literature

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8. Supplementary material

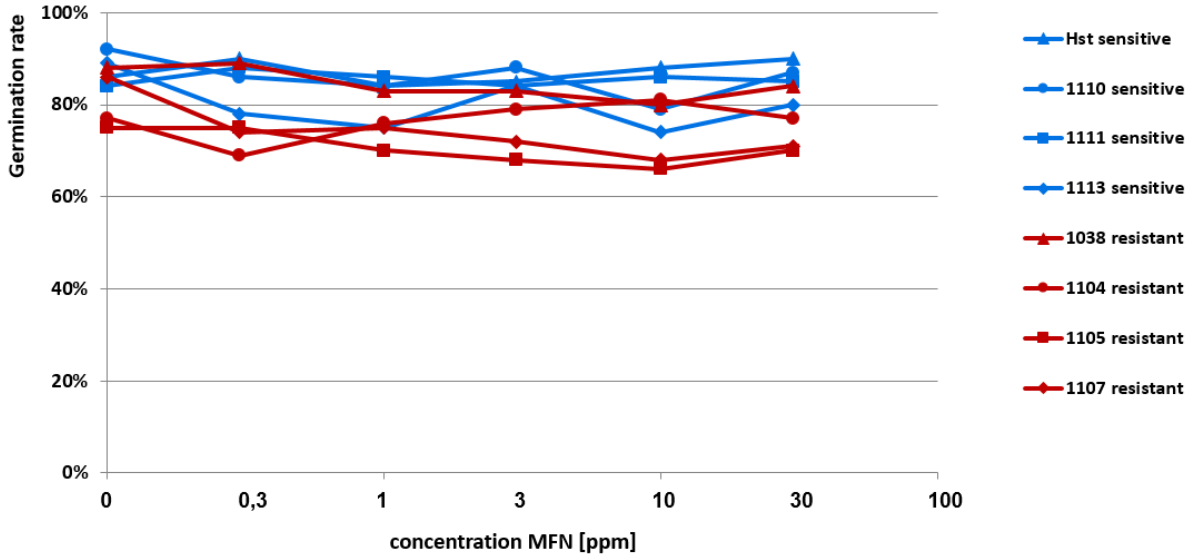

Figure 41: Germination rate of *E. necator* isolates under metrafenone treatment. Shown is the percentage of germinated spores for sensitive (blue) and resistant (red) isolates on different metrafenone dilutions.

Table 27: *E. necator* isolates used in this study. Isolates were gained and preanalysed by EpiLogic company or were isolated from infected plant material. Isolates were used for further sensitivity characterisation in leaf disc assays, the molecular SDHI monitoring 2016 or the conducted competitiveness tests (used isolates are indicted in the respective chapters). MFN: Sensitivity towards metrafenone; SDHI: Sensitivity towards succinate dehydrogenase Inhibitor; QoI: Sensitivity towards Quinone outside inhibitors; N.A.: Not analysed.

Strain ID	MFN	SDHI	QoI	Origin
		***E. necator* isolates**		
1038	resistant	sensitive	sensitive	
1050	sensitive	sensitive	G143A	HU
1051	sensitive	sensitive	G143A	HU
1052	sensitive	sensitive	G143A	HU
1053	sensitive	sensitive	G143A	HU
1054	resistant	sensitive	G143A	HU
1055	resistant	sensitive	G143A	HU
1056	resistant	sensitive	G143A	HU
1057	resistant	sensitive	G143A	HU
1058	resistant	sensitive	G143A	HU
1059	sensitive	sensitive	G143A	HU
1030	sensitive	sensitive	sensitive	FR
1061	sensitive	sensitive	N.A.	DE

Strain ID	MFN	SDHI	QoI	Origin
1064	resistant	sensitive	N.A.	DE
1065	sensitive	sensitive	N.A.	DE
1062	sensitive	sensitive	N.A.	DE
1063	sensitive	sensitive	N.A.	DE
1066	sensitive	sensitive	N.A.	DE
1067	sensitive	sensitive	N.A.	DE
1068	sensitive	sensitive	N.A.	DE
1069	sensitive	sensitive	N.A.	DE
1070	sensitive	sensitive	N.A.	DE
1071	sensitive	sensitive	N.A.	DE
1072	sensitive	sensitive	N.A.	DE
1073	sensitive	sensitive	N.A.	DE
1074	sensitive	sensitive	N.A.	DE
1075	sensitive	sensitive	N.A.	DE
1076	resistant	N.A.	N.A.	HU
1077	resistant	N.A.	N.A.	HU
1078	resistant	N.A.	N.A.	HU
1079	resistant	N.A.	N.A.	HU
1080	resistant	N.A.	N.A.	HU
1081	resistant	N.A.	N.A.	HU
1082	resistant	N.A.	N.A.	HU
1083	resistant	N.A.	N.A.	HU
1084	resistant	N.A.	N.A.	HU
1085	resistant	N.A.	N.A.	HU
1086	resistant	N.A.	N.A.	HU
1087	resistant	N.A.	N.A.	HU
1088	resistant	N.A.	N.A.	HU
1104	resistant	less sensitive	G143A	HU
1105	resistant	sensitive	sensitive	IT
1106	resistant	sensitive	G143A	IT
1107	resistant	sensitive	G143A	AU
1108	resistant	sensitive	G143A	AU
1109	sensitive	sensitive	100	AU

Strain ID	MFN	SDHI	QoI	Origin
1110	sensitive	sensitive	G143A	AU
1111	sensitive	sensitive	sensitive	IT
1112	sensitive	sensitive	G143A	IT
1113	sensitive	less sensitive	G143A	IT
1114	sensitive	sensitive	G143A	DE
1115	sensitive	sensitive	G143A	DE
1116	sensitive	sensitive	N.A.	DE
1117	resistant	sensitive	sensitive	IT
1118	resistant	sensitive	N.A.	IT
1119	resistant	sensitive	N.A.	IT
1120	resistant	sensitive	G143A	IT
1121	resistant	sensitive	N.A.	IT
1122	resistant	H242R	G143A	SK
1123	resistant	H242R	N.A.	SK
1124	resistant	H242R	N.A.	SK
1125	resistant	H242R	G143A	SK
1129	resistant	H242R	G143A	SK
1130	resistant	H242R	N.A.	SK
1131	resistant	G169S	G143A	SK
1133	sensitive	G169D	G143A	GR
1134	sensitive	G169D	G143A	GR
1135	sensitive	G169D	G143A	GR
1136	sensitive	G169D	G143A	GR
1137	N.A.	H242R	N.A.	HU
1138	N.A.	H242R	N.A.	HU
1139	N.A.	H242R	N.A.	HU
1140	N.A.	H242R	G143A	HU
1143	N.A.	G169D	G143A	SL
1144	N.A.	G169D	G143A	SL
1152	sensitive	H242R	G143A	HU
1158	sensitive	sensitive	sensitive	FR
1159	sensitive	H242R	G143A	FR
1160	sensitive	H242R	G143A	FR

Strain ID	MFN	SDHI	QoI	Origin
1161	sensitive	H242R	G143A	FR
1162	sensitive	H242R	G143A	FR
1163	sensitive	H242R	G143A	FR
1164	sensitive	H242R	G143A	FR
1165	N.A.	H242R	G143A	HU
1166	N.A.	H242R	G143A	HU
***E. necator* molecular SDHI monitoring 2016**				
1167	resistant	H242R	G143A	CZ
1168	resistant	H242R	G143A	CZ
1169	resistant	H242R	G143A	CZ
1170	resistant	H242R	G143A	CZ
1171	resistant	H242R	G143A	CZ
1172	resistant	H242R	G143A	CZ
1173	resistant	H242R	G143A	CZ
1174	resistant	H242R	G143A	CZ
1175	sensitive	H242R	G143A	CZ
1176	sensitive	H242R	G143A	CZ
1177	sensitive	H242R	G143A	CZ
1182	sensitive	H242R	G143A	AT
1183	sensitive	H242R	G143A	HU
1184	resistant	H242R	G143A	SK
1185	resistant	H242R	G143A	SK
1186	resistant	H242R	G143A	SK
1187	resistant	H242R	G143A	SK
1188	resistant	H242R	G143A	SK
1189	resistant	H242R	G143A	SK
1190	resistant	H242R	G143A	SK
1191	sensitive	H242R	G143A	SK
1192	resistant	sensitive	G143A	SK
1193	sensitive	H242R	G143A	SK
1194	resistant	H242R	G143A	SK
1195	resistant	H242R	G143A	SK
1196	resistant	H242R	G143A	SK

Strain ID	MFN	SDHI	QoI	Origin
1197	resistant	H242R	G143A	SK
1198	resistant	H242R	G143A	HU
1199	resistant	H242R	G143A	HU
1200	resistant	sensitive	G143A	HU
1201	resistant	sensitive	G143A	HU
1202	resistant	H242R	G143A	HU
1203	resistant	H242R	G143A	AT
1204	sensitive	H242R	G143A	AT
1205	sensitive	H242R	G143A	AT
1206	resistant	H242R	G143A	AT
1218	resistant	H242R	G143A	SK
1219	resistant	sensitive	G143A	AT
1220	resistant	H242R	G143A	AT
1221	resistant	H242R	G143A	AT
1222	resistant	H242R	G143A	AT
1223	resistant	G169D	G143A	TR
1224	resistant	H242R	G143A	TR
1225	sensitive	G169D	G143A	TR
1226	sensitive	G169D	G143A	TR
1227	sensitive	H242R	G143A	AT
1228	resistant	H242R	G143A	AT
1229	resistant	H242R	G143A	AT
1230	N.A.	H242R	G143A	AT
1231	N.A.	H242R	G143A	IT
1232	N.A.	I244V	G143A	DE

Table 28: Mutations of genes in the four resequenced adapted (moderately adapted and resistant) *B. graminis* f.sp. *tritici* isolates compared with both reference genomes (96224 and 2588).

Annotation (e-value)	Predicted function	Strains	Amino acid exchange	Position
Pyridoxine kinase (7.5e-107)	Vitamin B metabolism	C, D, E,F	V => I	124
Rsc complex subunit (0)	Chromatin remodelling	C, D, E,F	R => K	152
Di-trans.poly-cis-decaprenylcistransferase (2.3e-122)	Terpenoid biosynthesis	C, D, E,F	I => M	235
Nitrogen catabolic enzyme regulatory protein (1.9e-131)	Nitrate assimilation	C, D, E,F	V => E	215
WGS project CABT00000000 data. contig 2.43 (9.1e-10)	No function found	C, D, E,F	F => L	11
O-acyltransferase (1.1e-228)	Phospholipid remodelling	C, D, E,F	V => I	415

Table 29: Presence and absence of mutations in the o-acyltransferase gene of further *B. graminis* f.sp *tritici* isolates

ID	Resistance type	H218D	P298L	V415I
2592	sensitive	-	-	-
2611	sensitive	-	+	+
2616	sensitive	-	-	-
2595	moderately adapted	-	-	-
2613	moderately adapted	-	-	-
2624	moderately adapted	-	-	-
2602	resistant	-	-	-
2609	resistant	-	-	-
2622	resistant	-	-	-
2626	resistant	+	-	+

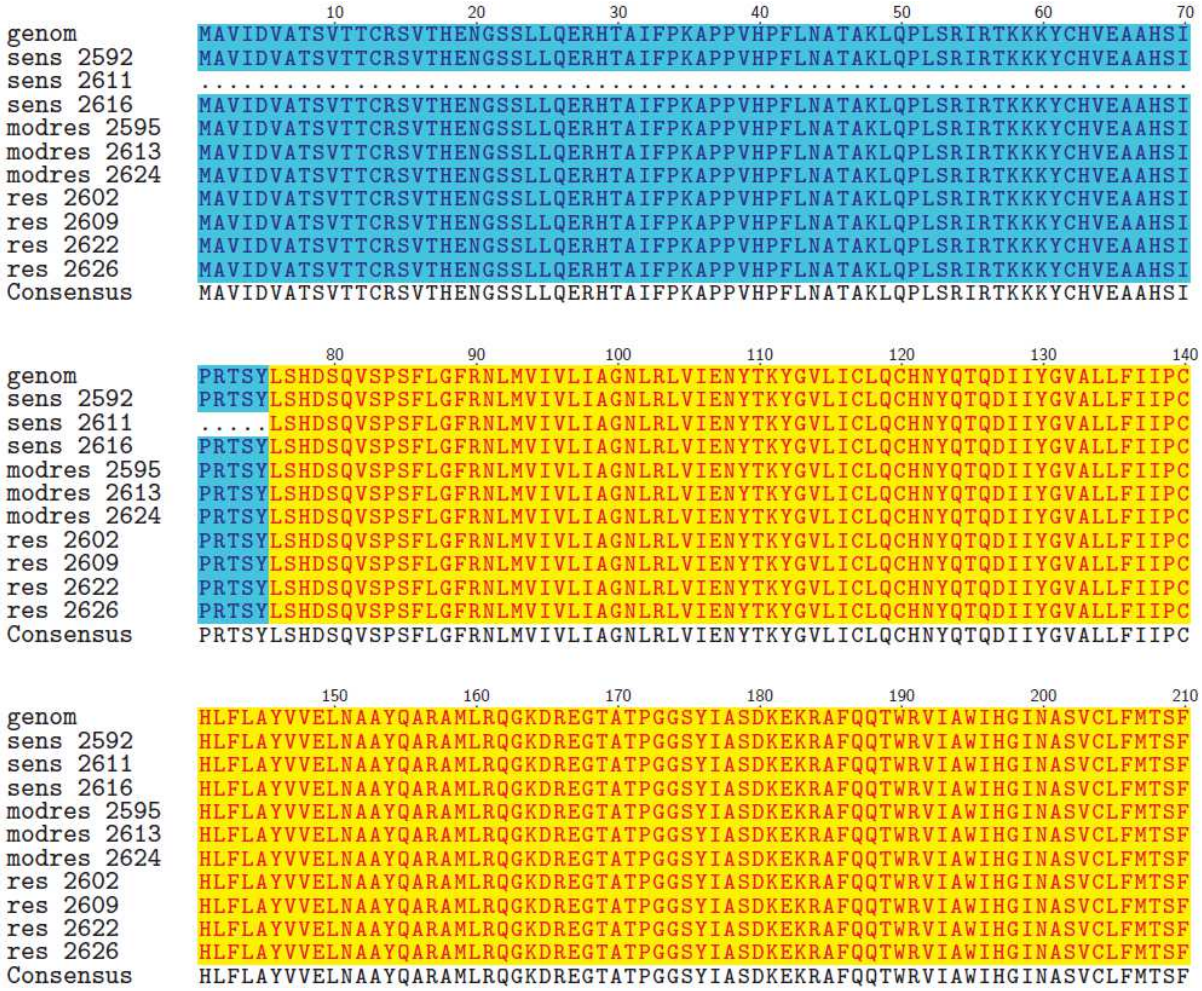

Figure 42: O-acyltransferase amino acid sequence alignment of sensitive, moderately adapted and metrafenone resistant *B. graminis* f.sp. *tritici* isolates. Further description is given on page 141.

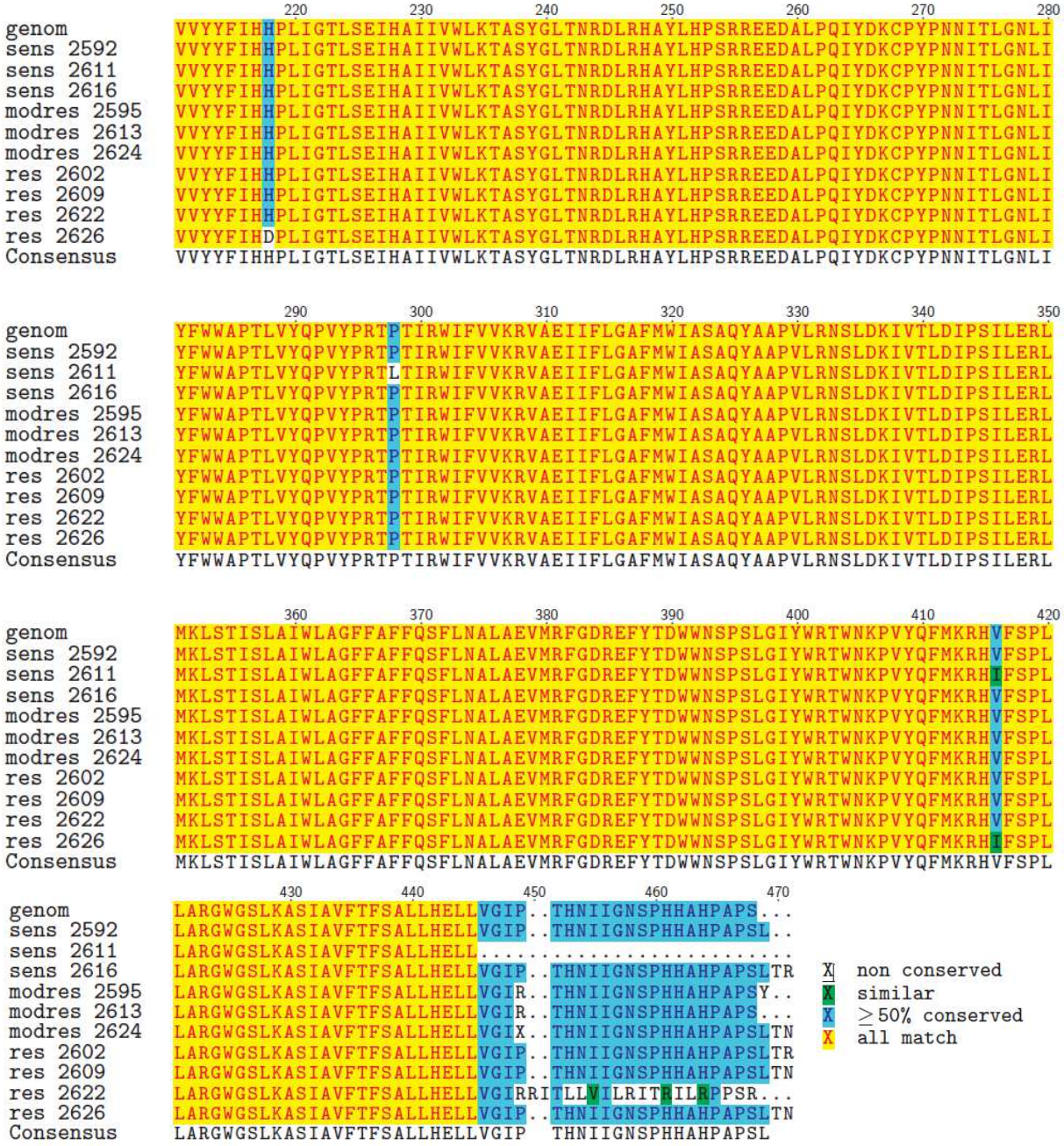

Figure 42 1: O-acyltransferase amino acid sequence alignment of sensitive, moderately adapted and metrafenone resistant *B. graminis* f.sp. *tritici* isolates. Genom: sequence of the reference genome 96224; sens: metrafenone sensitive; modres: metrafenone moderately adapted; res: metrafenone resistant; All isolates were obtained during the EpiLogic airborne monitoring in 2014.

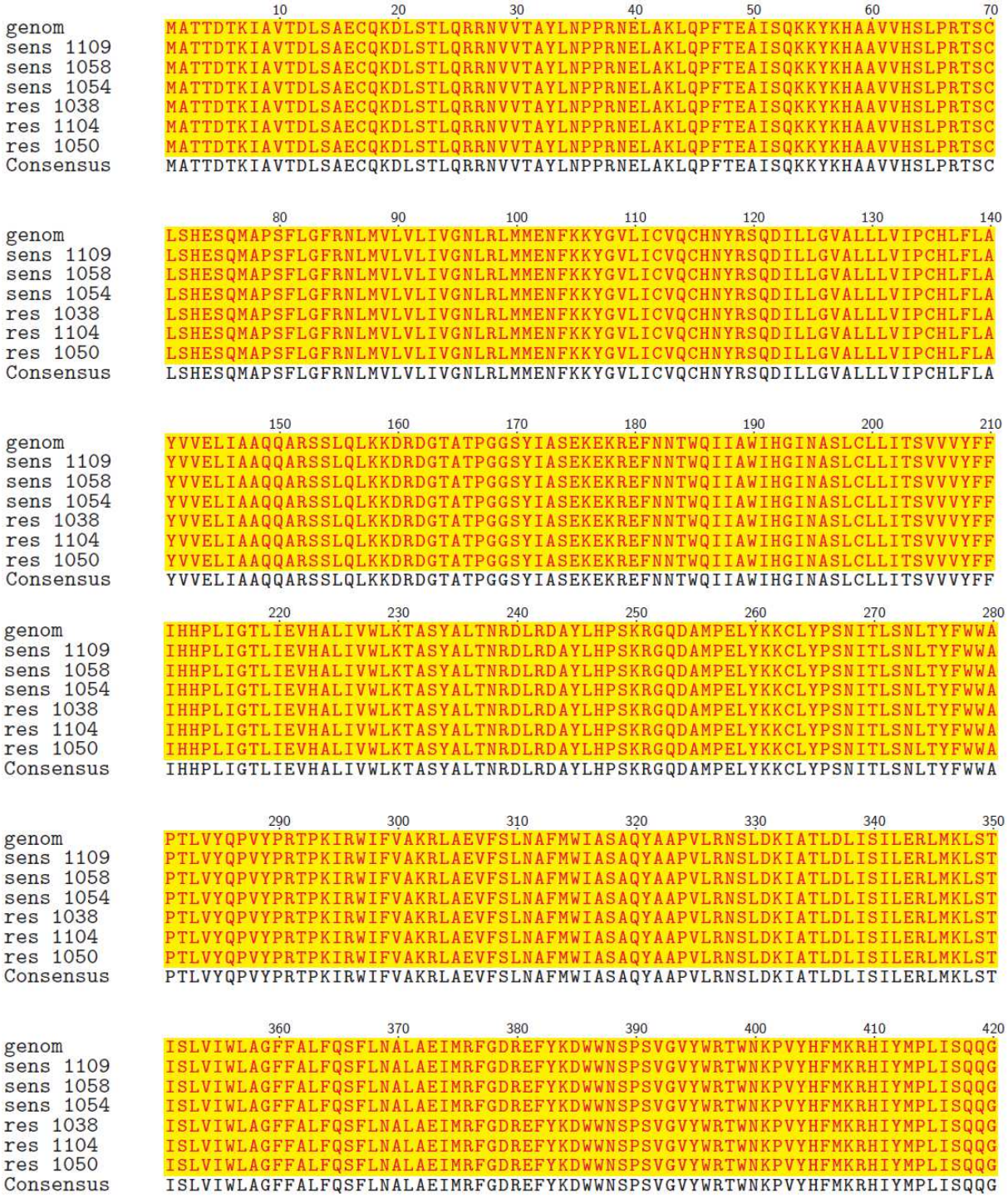

Figure 43: Alignment of the o-acyltransferase amino acid sequence ortholog of sensitive and metrafenone resistant *E. necator* isolates. Further description is given on page 143.

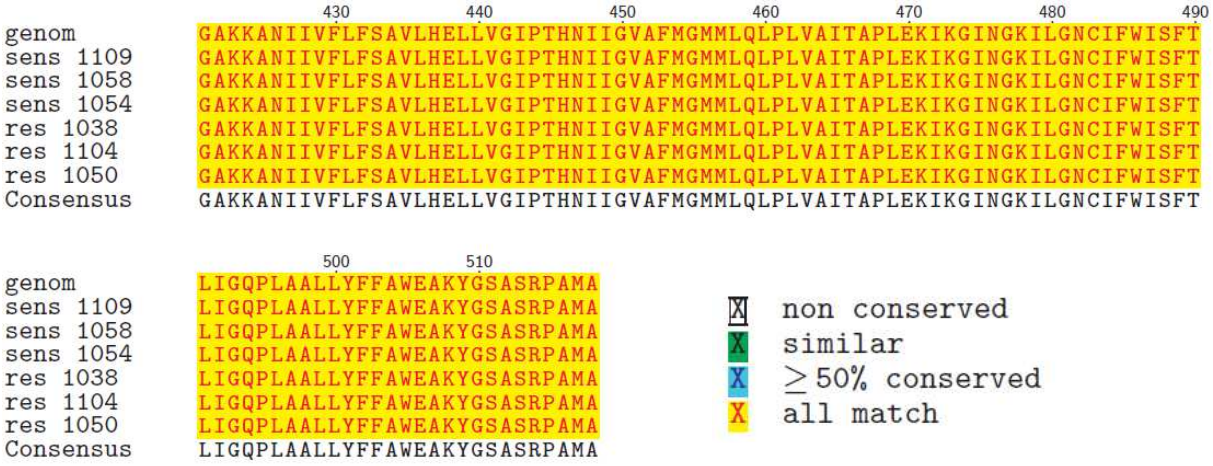

Figure 43 1: Alignment of the o-acyltransferase amino acid sequence ortholog of sensitive and metrafenone resistant *E. necator* isolates. Genom: sequence of the reference genome (Jones *et al.* 2014); sens: metrafenone sensitive; modres: metrafenone moderately adapted; res: metrafenone resistant.

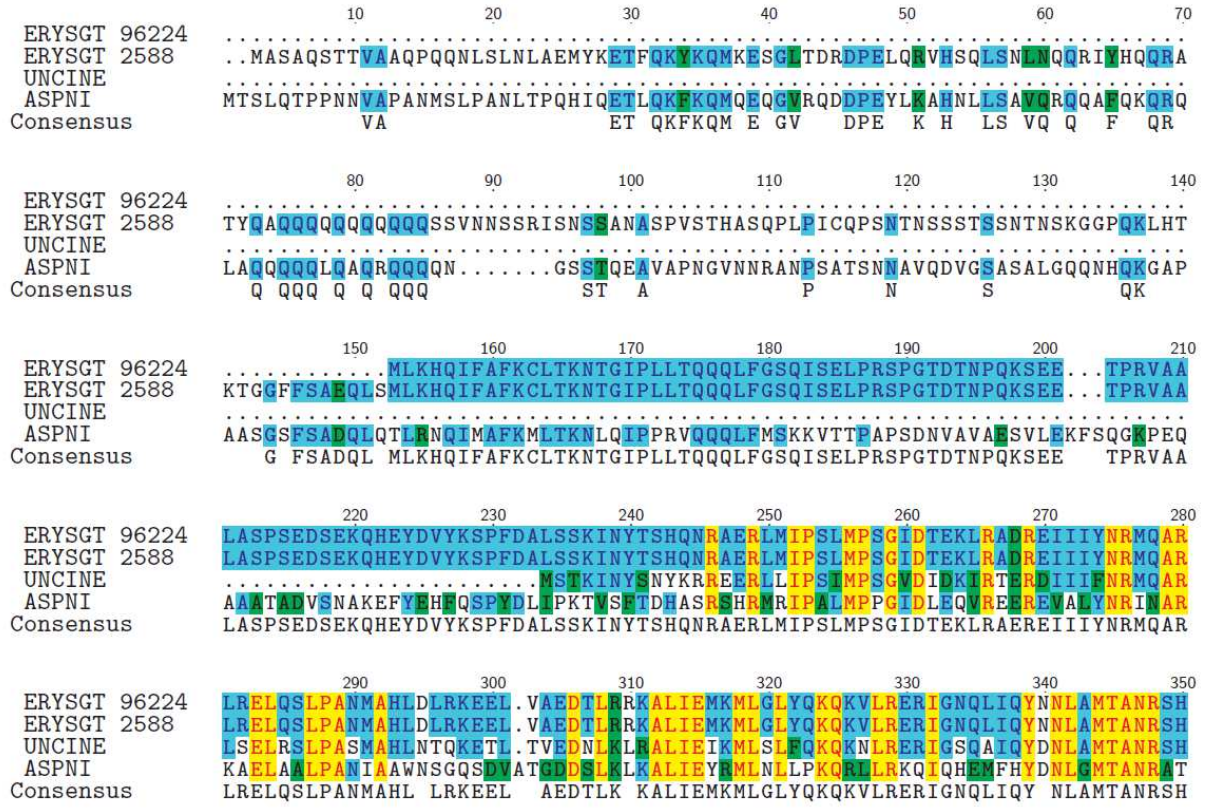

Figure 44: Alignment of the Rsc complex subunit amino acid sequence homologues of *B. graminis* f.sp. *tritici*, *E. necator* and *A. nidulans*. Further description is given on page 145.

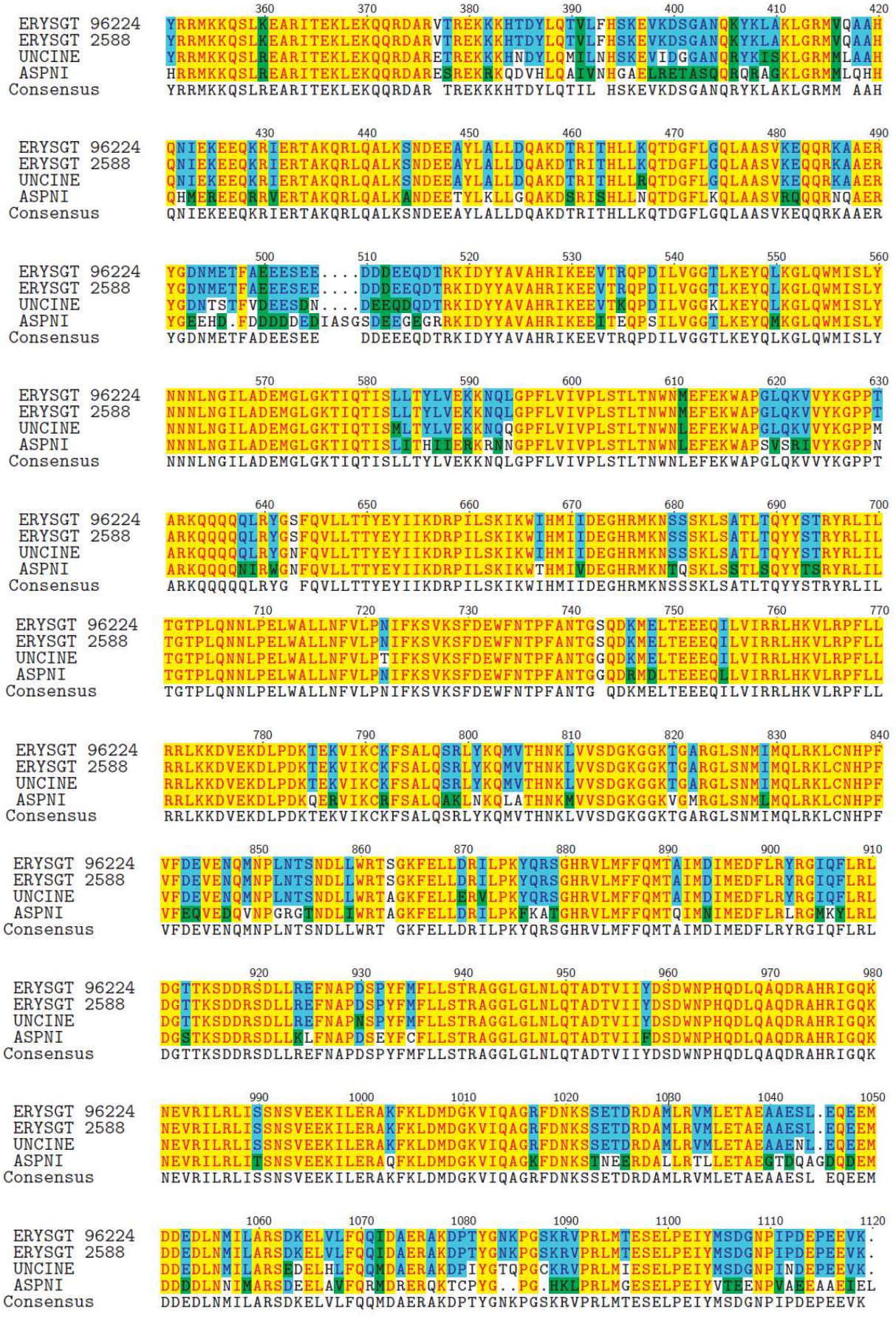

Figure 44 1: Alignment of the Rsc complex subunit amino acid sequence homologues of *B. graminis* f.sp. *tritici*, *E. necator* and *A. nidulans*. Further description is given on page 145.

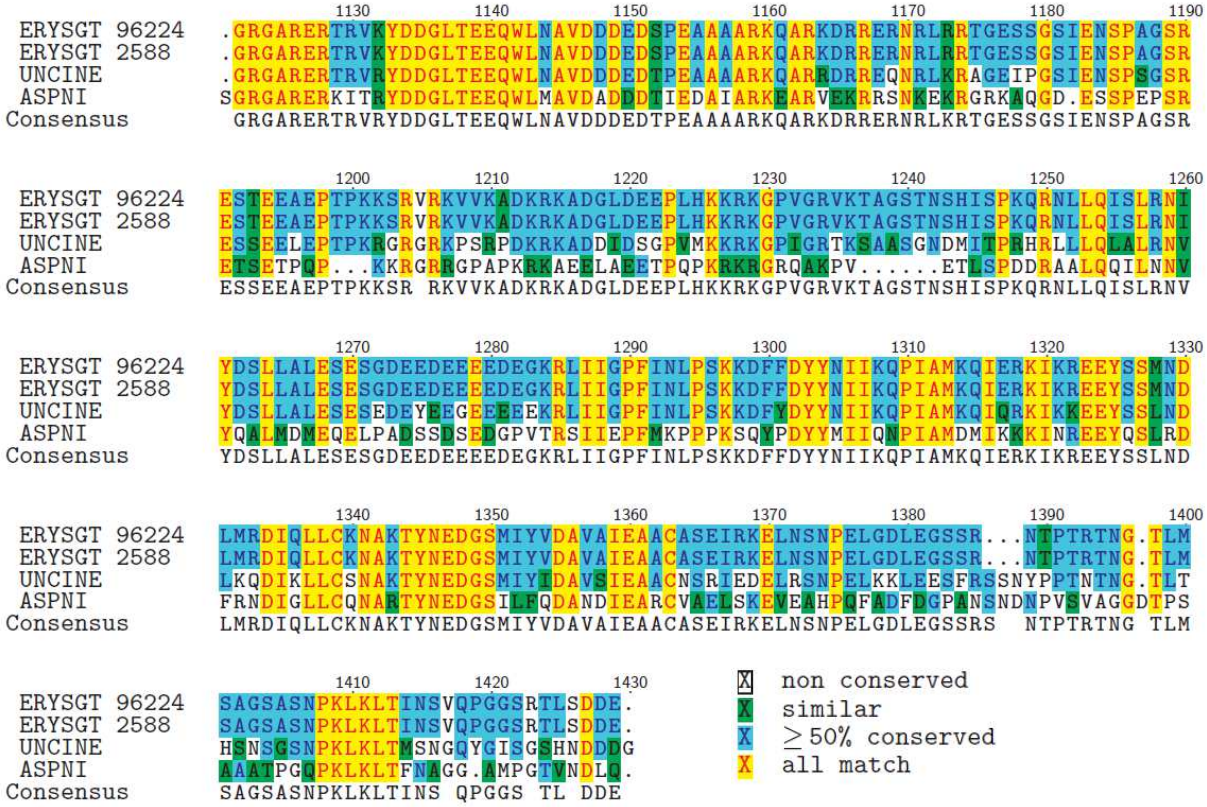

Figure 44 2: Alignment of the Rsc complex subunit amino acid sequence homologues of *B. graminis* f.sp*. tritici*, *E. necator* and *A. nidulans*. ERYSGT: *B. graminis* f.sp. *tritici*; UNCINE: *E. necator*; ASPNI: *A. nidulans*.

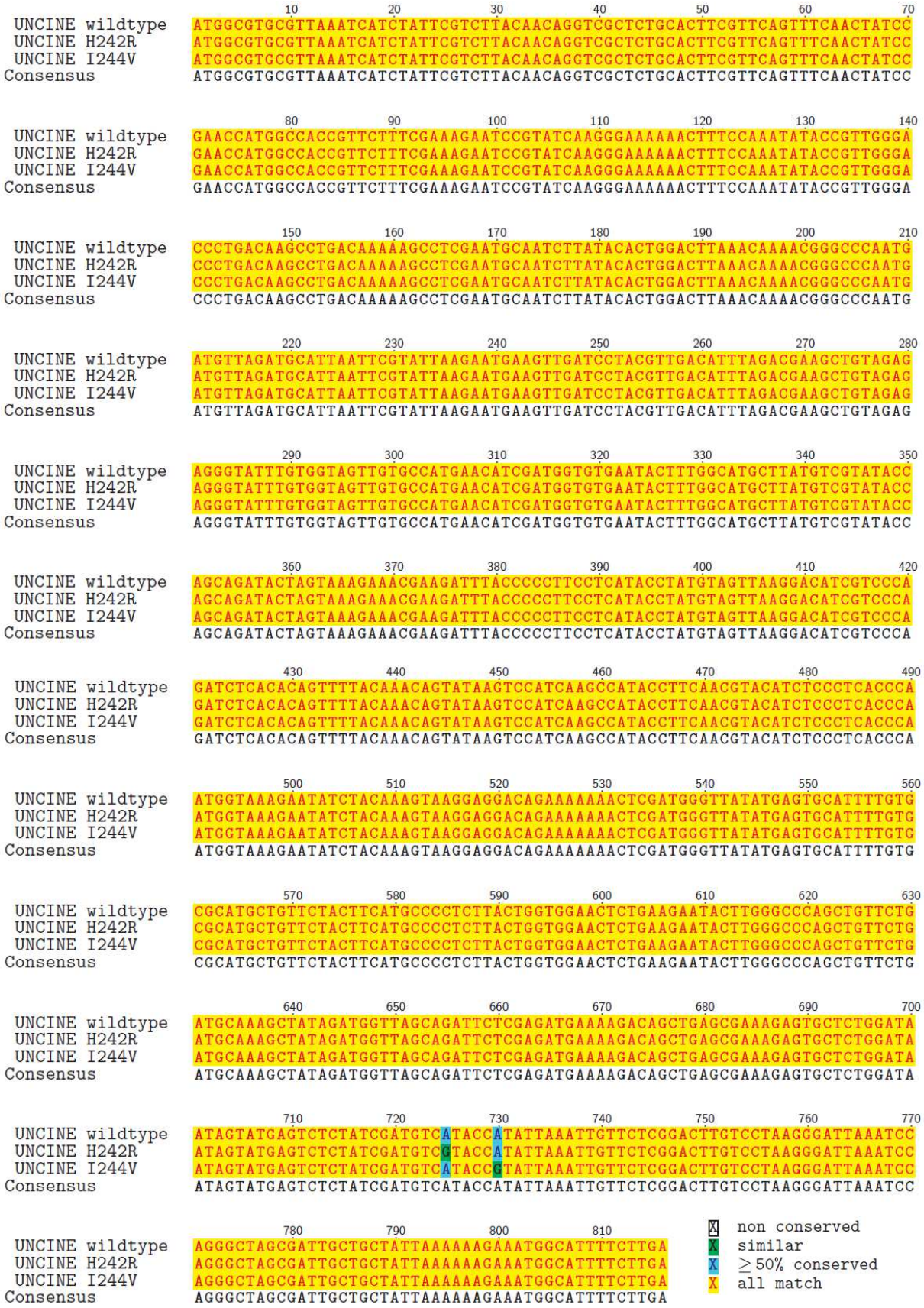

Figure 45: *SdhB* DNA sequence alignment of *E. necator*. Comparative *SdhB* DNA sequence alignment of a sensitive wildtype isolate, isolate 1104 with the H242R mutation and isolate 1232 with the I244V mutation; UNCINE: *E. necator*.

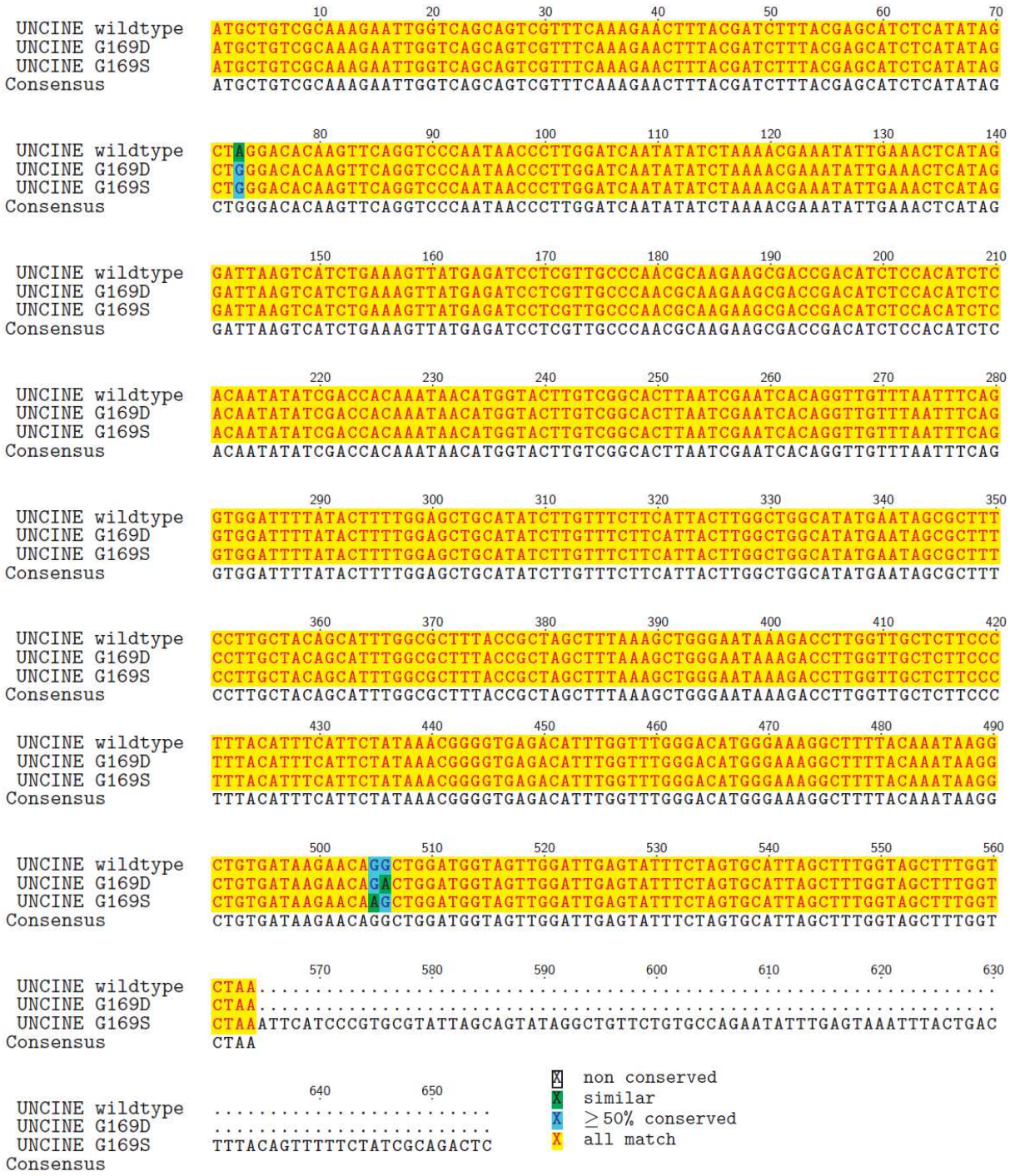

Figure 46: *SdhC* DNA sequence alignment of *E. necator*. Comparative *SdhC* DNA sequence alignment of a sensitive wildtype isolate, isolate 1113 with the G169D mutation and isolate 1131 with the G169S mutation; UNCINE: *E. necator*.

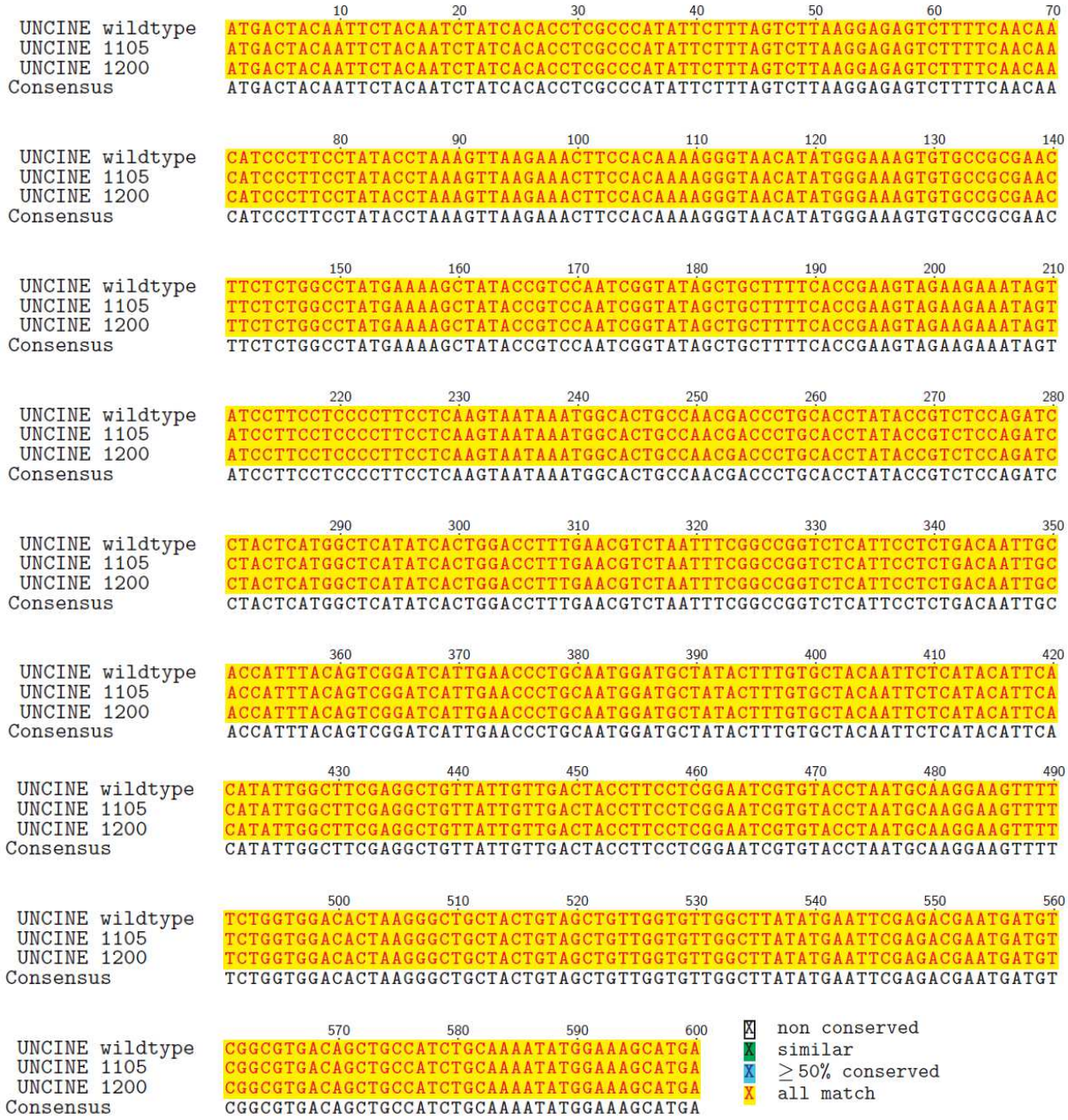

Figure 47: *SdhD* DNA sequence alignment of *E. necator*. Comparative *SdhD* DNA sequence alignment of the sensitive wildtype isolate and two further sensitive isolates 1105 and 1200; UNCINE: *E. necator*.

Statistical analysis of paired mixtures:

```
Linear model tree

Model formula:
Y_MFNres ~ X_cycle | Test_code

Fitted party:
[1] root
|   [2] Test_code in Ic, Id, IIa, IIe
|   |   [3] Test_code in Ic, Id: n = 24
|   |       (Intercept)     X_cycle
|   |         77.003788    2.305507
|   |   [4] Test_code in IIa, IIe: n = 24
|   |       (Intercept)     X_cycle
|   |         51.020833    4.274038
|   [5] Test_code in Ia, Ib, IIb, IIc, IId, IIf
|   |   [6] Test_code in Ia, Ib, IId: n = 35
|   |       (Intercept)     X_cycle
|   |         43.638300   -4.247043
|   |   [7] Test_code in IIb, IIc, IIf: n = 36
|   |       (Intercept)     X_cycle
|   |         59.194444   -2.967949

Number of inner nodes:    3
Number of terminal nodes: 4
Number of parameters per node: 2
Objective function (residual sum of squares): 9133.987
```

Scenario 1		MFN sensitive / without G143A	
		1030	1111
MFN resistant / with G143A	1050	Ia	Ic
	1106	Ib	Id

Scenario 2		MFN sensitive / with G143A		
		1054	1057	1112
MFN resistant / without G143A	1038	IIa	IIc	IIe
	1105	IIb	IId	IIf

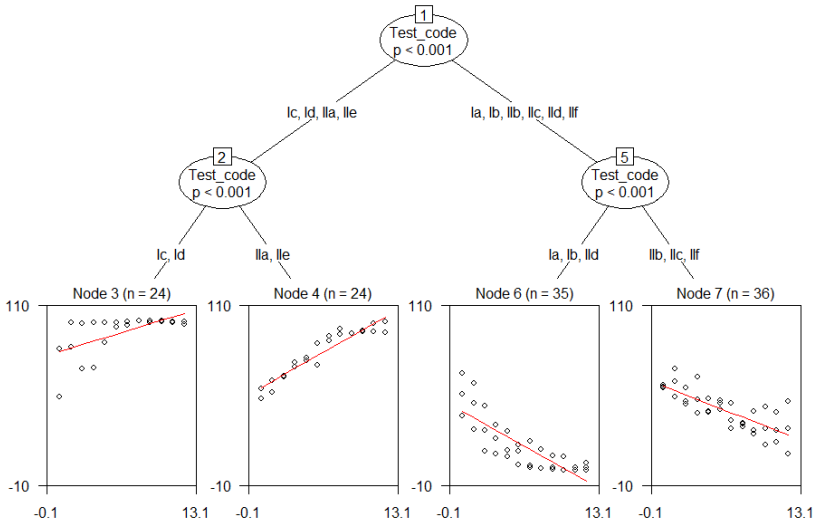

Figure 48: Detailed results and visualisation of the statistical analysis of paired mixtures (MFN -G143A test scenario 1 and 2). Green (regression coefficient > 0: no reduced fitness of resistant isolate; Orange = regression coefficient < 0: significant reduced fitness of resistant isolate.

```
Linear model tree

Model formula:
Y_MFNres ~ X_cycle | Test_code

Fitted party:
[1] root
|   [2] Test_code in Ia, Ib, Ic, Id: n = 28
|       (Intercept)     X_cycle
|         57.941964    6.066964
|   [3] Test_code in IIa, IIb, IIc, IId: n = 28
|       (Intercept)     X_cycle
|         76.214286    3.928571

Number of inner nodes:    1
Number of terminal nodes: 2
Number of parameters per node: 2
Objective function (residual sum of squares): 4620.355
```

A		SDHI sensitive	
		1108	1120
Reduced SDHI sensitivity	1104	Ia	IIa
	1125	Ib	IIb
	1129	Ic	IIc
	1152	Id	IId

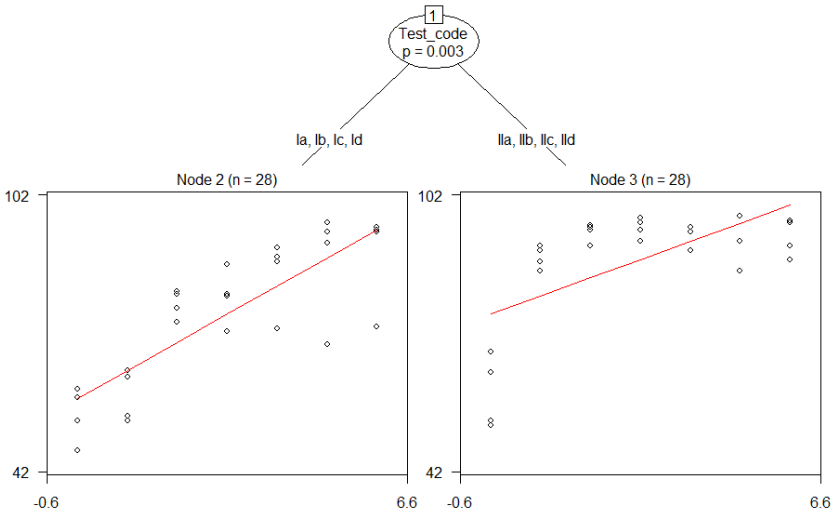

Figure 49: Detailed results and visualisation of the statistical analysis of paired mixtures (SDHI -H242R). Green (regression coefficient > 0: no reduced fitness of resistant isolate; Orange = regression coefficient < 0: significant reduced fitness of resistant isolate.

```
Linear model tree

Model formula:
Y_MFNres ~ X_cycle | Test_code

Fitted party:
[1] root
|   [2] Test_code in IIIa, IIIb, IIIc, IIId: n = 28
|       (Intercept)     X_cycle
|         65.683036   -9.995536
|   [3] Test_code in IVa, IVb, IVc, IVd: n = 28
|       (Intercept)     X_cycle
|          51.46429     2.12500

Number of inner nodes:    1
Number of terminal nodes: 2
Number of parameters per node: 2
Objective function (residual sum of squares): 4937.944
```

B		SDHI sensitive	
		1012	1109
Reduced SDHI sensitivity	1113	IIIa	IVa
	1133	IIIb	IVb
	1143	IIIc	IVc
	1144	IIId	IVd

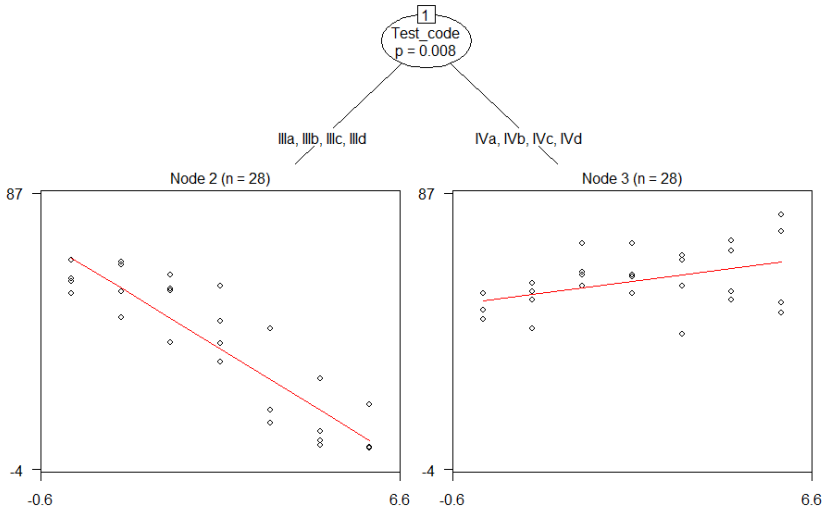

Figure 50: Detailed results and visualisation of the statistical analysis of paired mixtures (SDHI -G169D). Green (regression coefficient > 0: no reduced fitness of resistant isolate; Orange = regression coefficient < 0: significant reduced fitness of resistant isolate.

```
Linear model tree

Model formula:
Y_MFNres ~ X_cycle | Test_code

Fitted party:
[1] root
|   [2] Test_code in Vb, Vc, VIa: n = 21
|       (Intercept)     X_cycle
|         73.869048    2.440476
|   [3] Test_code in Va, Vd, VIb, VIc, VId: n = 35
|       (Intercept)     X_cycle
|         80.503571    3.160714

Number of inner nodes:    1
Number of terminal nodes: 2
Number of parameters per node: 2
Objective function (residual sum of squares): 2068.567
```

Mixture		wildtype	
		1030	1111
Multiple resistant	1104	Va	VIa
	1122	Vb	VIb
	1125	Vc	VIc
	1152	Vd	VId

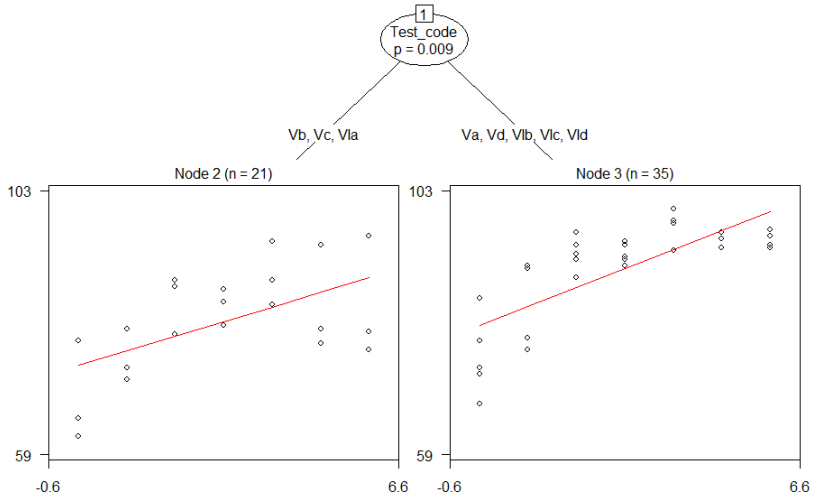

Figure 51: Detailed results and visualisation of the statistical analysis of paired mixtures (multiple resistant isolates (H242R)). Green (regression coefficient > 0: no reduced fitness of resistant isolate; Orange = regression coefficient < 0: significant reduced fitness of resistant isolate.

Lebenslauf

PERSÖNLICHE DATEN

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AUSBILDUNG

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2014 - 2017	Promotion bei BASF SE, Fungizid Resistenzforschung
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	Voraussichtlicher Abschluss: Doktor der Naturwissenschaften
	Beschreibung: Characterisation of SDHI and metrafenone resistance and effects on fitness in the grape powdery mildew pathogen *Erysiphe necator.*
2012–2014	Technische Universität Kaiserslautern
	Master-Studiengang: Microbial and Plant Biotechnology
	Abschluss: Master of Science (1,3)
	Masterarbeit bei BASF SE, Fungizid Resistenzforschung
	Masterarbeit: Differenzierung von *Stemphylium*-Arten an Spargel.
	Die Arbeit war Teil eines vom BLE geförderten Projektes unter der Koordination des DLR Rheinland-Pfalz
2009 – 2012	Technische Universität Kaiserslautern
	Bachelor-Studiengang: Biowissenschaften
	Abschluss: Bachelor of Science (2,3)
	Bachelorarbeit: Promotoranalyse eines durch die Oberflächenhärte in Keimlingen von *Botrytis cinerea* induzierten Gens.

2007 – 2009	Naturwissenschaftliches Technikum Dr. Künkele Abschluss: Staatlich geprüfte Biologisch Technische Assistentin (1,9)
1998 – 2006	Nikolaus von Weis Gymnasium Speyer Abschluss: Allgemeine Hochschulreife

BERUFLICHE ERFAHRUNGEN

2012 – 2013 Wissenschaftliche Hilfskraft in der Abteilung Phytopathologie der Technischen Universität Kaiserslautern

- Mikrobiologische, molekularbiologische und phylogenetische Arbeitsmethoden zur Untersuchung von *Botrytis cinerea*
- Resistenz von *Botrytis cinerea* in Bezug auf verschiedene Fungizide

2012 Dreimonatiges Betriebspraktikum bei BASF SE in der Abteilung Fungizid Resistenzforschung

- Mikrobiologische Arbeitsmethoden im Bereich phytopathogener Pilze
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PUBLIKATIONEN

Graf S., Zito R., Gold R.E. and Stammler G. (2017). Status of *In Vivo* and Molecular Diagnosis of Fungicide Resistance in Powdery Mildews. In: Deising HB; Fraaije B; Mehl A; Oerke EC; Sierotzki H; Stammler G (Eds), "Modern Fungicides and Antifungal Compounds", Vol. VIII, pp. 243-248. 2016 Deutsche Phytomedizinische Gesellschaft eV Verlag, Braunschweig.

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Taher K., **Graf S.**, Fakhfakh M. M., Salah H. B., Yahyaoui A., Rezgui S., and Stammler G. (2014). Sensitivity of *Zymoseptoria tritici* isolates from Tunisia to pyraclostrobin, fluxapyroxad, epoxiconazole, metconazole, prochloraz and tebuconazole. *Journal of Phytopathology*, *162*(7-8), 442-448.

BEITRÄGE KONFERENZEN

Präsentation bei der 60. Deutschen Pflanzenschutztagung, 20.-23. September 2016, Martin-Luther-Universität Halle-Wittenberg, Halle (Saale), Deutschland: Status of *In vivo* and Molecular Diagnosis of Fungicide Resistance in Powdery Mildews

Präsentation beim 18. Internationalen Reinhardbrunn Symposium, 24.-28. April 2016, Friedrichroda, Deutschland: Status of *In vivo* and Molecular Diagnosis of Fungicide Resistance in Powdery Mildews

Präsentation bei der DPG-Arbeitskreistagung Mykologie und Wirt-Parasit-Beziehungen, 17.-18. März 2014, Justus-Liebig-Universität Gießen, Gießen, Deutschland: Sensitivity Status and Diagnosis of Fungicide Resistance in Powdery Mildews.

Poster-Präsentation beim XVIII. International Plant Protection Congress, 24.-27. August 2015, Freie Universität Berlin, Berlin-Dahlem, Deutschland: Cytochrome b gene is a reliable tool for detection of fungal species in plant tissue.

Poster-Präsentation bei der DPG-Arbeitskreistagung Mykologie und Wirt-Parasit-Beziehungen, 19.-20. März 2015, Martin-Luther-Universität Halle-Wittenberg, Halle (Saale), Deutschland: Metrafenone – a powdery mildew fungicide with unknown mode of action.

Präsentation bei der DPG-Arbeitskreistagung Mykologie und Wirt-Parasit-Beziehungen, 20.-21. März 2014, Rheinisch-Westfälische Technische Hochschule Aachen, Aachen, Deutschland: Species specific identification of Stemphylium spp. on asparagus.

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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.

Kaiserslautern, im August 2017

Sarah Graf