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SDHI cross-resistance pattern of *Alternaria solani* field mutants and consequences for Early Blight control

JUERGEN DERPMANN[1], ANDREAS MEHL[1]

[1] Bayer AG, Division CropScience, Alfred-Nobel-Strasse 50, D-40789 Monheim, Germany

SUMMARY

Alternaria solani is a fungal pathogen, which can infect commercially important *Solanaceae* crops such as potato and tomato. After first infection, dark-brown leaf spot symptoms with characteristic concentric rings appear on leaves. From there, asexually produced conidia are dispersed leading to further infections in the canopy, in case weather conditions are favorable. To stop such infections, fungicides are widely used. However, *A. solani* strains, which are adapted to selection pressures from commonly used respiration inhibitors such as quinone outside inhibitors (QoIs) and succinate dehydrogenase inhibitors (SDHIs), were reported by various researchers over the last years.

To monitor the spread of such phenotypes, samples were collected in important potato growing regions in Netherlands, Belgium, Germany and Great Britain from 2016 to 2018. Molecular tools were applied to identify the mutations responsible for detected phenotypes. A cross-resistance study was conducted to demonstrate the impact of detected mutations on selected compounds from the group of SDHIs. Finally, greenhouse experiments were conducted to elucidate the impact of the detected mutations on commercially available solutions and its' implication on fungicide resistance management is discussed.

KEYWORDS

Alternaria solani, early blight, fungicide resistance, SDHI, QoI, DMI, efficacy

INTRODUCTION

Alternaria solani is a highly destructive pathogen causing the Early Blight disease of potatoes and tomatoes, which leads worldwide to significant yield losses. Different factors, such as environmental conditions and plant physiology seem to have an influence on the disease progress of Early Blight disease. Epidemics primarily caused by *Alternaria solani* mainly occur when the weather is warm and dry with short periods of high moisture (Chaerani and Voorrips, 2006). First symptoms occur on lower leaflets at favourable weather conditions. Subsequently, the pathogen spreads onto higher leaf levels within the crop. At high disease severity, whole

plants can get defoliated. Thus, heavy infections can cause considerable quantitative as well as qualitative yield losses (Chaerani and Voorrips, 2006).

The control of this polycyclic diseases requires multiple, targeted applications of fungicides (Leiminger and Hausladen, 2009). Amongst the fungicides registered, respiration inhibitors such as quinone outside inhibitors (QoIs) and succinate dehydrogenase inhibitors (SDHIs) play a key role in the management of Early Blight disease. Also, demethylation inhibitors (DMIs) have been registered to control Early Blight disease for several years. Up until now, no cases of field resistance were reported in *A. solani* for DMI fungicides (FRAC, 2018). Even though both sub-groups of respiration inhibitors show no cross-resistance, in recent years resistance cases were reported for SDHI as well as QoI fungicides from the US and Europe (FRAC, 2018; Landschoot *et al.*, 2017; Baukse *et al.*, 2018a). Reduction in QoI fungicide sensitivity can be attributed to point mutations in the mitochondrial target gene, cytochrome b (*cyt-b*). In *A. solani*, the substitution of phenylalanine (F) to leucine (L) at position 129 (F129L) has been observed. This F129L-genotypes leads to low resistance factors towards different QoI fungicides (Pasche *et al.*, 2005). In contrast to QoIs, reduction of SDHI-sensitivity is caused by several mutations in the four subunits of the succinate dehydrogenase (*sdh*) genes leading to a more complex cross-resistance pattern within the group of SDHIs. Sequencing of boscalid-resistant field isolates of *A. solani* showed in subunit *sdh*B an amino acid exchange from histidine (H) to trypsine (Y) at amino acid position 278, which lead to the *sdh*B-H278Y-genotype. Another mutation in the same base-triplet conferred an amino acid exchange from H to arginine (R), resulting in the *sdh*B-H278Y-genotype (Mallik *et al.*, 2014). In subunit *sdh*C a mutation at amino acid position 134 caused an exchange from H to R leading to the *sdh*C-H134R-genotype. A similar amino acid substitution was found in subunit *sdh*D at position 133 resulting in *sdh*D-H133R-genotype. In field isolates originating from the US, another amino acid substitution was found in subunit *sdh*D. At amino acid position 123 aspartic acid (D) was exchanged to glutamic acid (E) leading to the *sdh*D-D123E-genotype (Mallik *et al.*, 2014), which was to the authors knowledge up until now not reported to be present in Europe (FRAC, 2018).

Cross-resistance patterns across SDHI fungicides are complex due to the fact that the impact of reported genotypes on SDHI-sensitivity varies between the mutation and SDHI tested. Field isolates of the *sdh*B genotypes displayed *in-vitro* low resistance factors towards penthiopyrad, whereas *sdh*C or *sdh*D-genotypes showed high resistance factors to the same fungicide. Boscalid showed for all genotypes high to very high resistance factors. In contrast, both *sdh*B mutants were sensitive to fluopyram, while *sdh*C and *sdh*D mutants showed low to moderate resistance to the fungicide (Gudmestad *et al.*, 2013). Such an incomplete cross-resistance pattern was also observed by conducting *in-vivo* greenhouse (Gudmestad *et al.*, 2013; Bauske *et al.*, 2018b). To elucidate the consequences of the reported genotypes on commercially available SDHI containing products, following experiments were conducted:

- Sensitivity testing and molecular analysis of European field isolates collected from 2016 to 2018
- *In-vitro* SDHI cross-resistance study testing identified genotypes
- *In-vivo* study to evaluate impact of genotypes on efficacy of solo SDHIs as well as SDHI containing products

MATERIALS AND METHODS

Sampling, cultivation and sensitivity testing of Alternaria solani
Infected leaves were collected randomly from commercial fields. Isolation was done similar to the methodology described by FRAC (2006). In most cases, five isolates per sample were generated. Fungicide sensitivity was determined for selected DMI, QoI and SDHI-fungicides in a microtiter assay similar to that described by Hu et al (2011), but using a conidial suspension adjusted to 10^4 conidia per milliliter. Two replicates were tested per isolate. EC_{50} values were calculated from the blank-corrected extinction values using the ABASE software package.

DNA isolation and pyrosequencing
Isolates with an EC_{50} value of more than 1 ppm of boscalid and/or fluopyram or more than 0.1 ppm of azoxystrobin were selected for further molecular analysis. For each of the isolates, DNA isolation and pyrosequencing were performed similar to that described by Weber *et al.* (2015).

SDHI cross-resistance study
When possible, twelve isolates from each genotype were tested together with twelve isolates showing a wild-type sensitivity in a microtiter assay as described in paragraph "Sampling, cultivation and sensitivity testing". In total seven active ingredients belonging to the mode of action group of succinate dehydrogenase inhibitors (SDHIs) were tested in this cross-resistance study. Two replicates were tested per isolate. The experiment was repeated twice.

Greenhouse experiment
Potato plants (*Solanum tuberosum*, var. Rambo) were grown until the growth stage of four fully expanded leaves in a greenhouse at 18°C. Subsequently, plants were treated with commercial fungicidal products as listed in Table 1 at a water rate of 400 L/ha in a calibrated spray cabin. After drying, treated plants were brought back into the greenhouse.

Table 1. *SDHI containing products used for protective treatment of potato plants one day prior to infection at 400 L/ha in a calibrated spray cabin*

Product name	Full Product label rate	Fungicide(s)	Fungicide rate(s)
Cantus WG 500 g/kg	0.134* kg/ha	Boscalid	67 g/ha
Sercadis SC 300 g/L	0.25 L/ha	Fluxapyroxad	75 g/ha
Luna Privilege SC 500 g/L	0.125 L/ha	Fluopyram	62.5 g/ha
Perseus SC 75+50 g/L	0.75 L/ha	Fluoxapyroxad + Difenoconazole	56 g/ha + 38 g/ha
Propulse SE 125+125 g/L	0.5 L/ha	Fluopyram + Prothioconazole	62.5 g/ha + 62.5 g/ha

**Adjusted boscalid dose-rate to match the full label rate of 0.25 L/ha Signum SC (containing 267g of boscalid)*

One isolate of *A. solani* per detected SNPs from paragraph "DNA isolation and pyrosequencing" was selected for the greenhouse experiment. Spore suspensions were generated for the selected five isolates (four genotypes and a wildtype isolate) and adjusted to 10^4 spores per milliliter.

Plants were inoculated with *A. solani* by spraying of treated plants with spore suspension until plants were wet. After drying, treated plants were brought back into the greenhouse and placed under plastic covers to create 100% relative humidity for the first three days. Subsequently, covers were removed and plants were incubated at 18°C in the greenhouse.

Disease severity was scored from 0-100% according to Duarte *et al.* (2013). Analysis for difference in treatments was done for each genotype by performing an one-factorial ANOVA and a posteriori Tukey's HSD test ($p \leq 0.05$). Analysis for difference in each genotype to the genotype a Students' *t*-test ($p \leq 0.05$) for independent samples was performed. Efficacy expressed as percent ABBOTT was calculated for each product by using means of treatments and comparing it to the untreated control. Two experiments were conducted testing solo SDHIs and mixture concepts (see Table 1).

RESULTS AND DISCUSSION

Sensitivity status of Alternaria solani towards DMIs, QoIs and SDHIs

In 2016, in total 203 isolates of *A. solani* were generated from Germany, France, Netherlands, Belgium, Denmark and Sweden. The monitoring was initiated in 2016, continued in 2017 with a smaller number of samples (n=9) and was expanded in 2018 to include also Nordic countries (n=20). Even though the number of isolates was moderate compared to monitoring procedures for other pathogens, results were in line with those reported by other companies in those years (FRAC, 2018).

The mEC_{50} values for the DMI fungicides prothioconazole and difenoconazole ranged from 0.28 to 0.98ppm and from 0.004 to 0.009ppm, respectively (see Table 2) in 2018. This was in an acceptable range compared to the mEC_{50} values of the reference isolates for prothioconazole (0.17 – 0.35ppm) and difenoconazole (0.003 – 0.006ppm). The highest individual EC_{50} value were 1.38 and 1.43ppm of prothioconazole for two isolates detected in the same German sample in 2017. However, a mix of both isolates could be controlled on the same level as an isolate showing wild-type sensitivity by a foliar dose of 62.5 g/ha prothioconazole in an *in-vivo* experiment (data not shown). In general, shifting of DMI fungicides leading to reduced field efficacy was reported for other plant pathogens. Therefore, *A. solani* needs to be monitored continuously towards prothioconazole in order to follow the evolution of the pathogen in the future.

Table 2. *Results of Alternaria solani sensitivity monitoring conducted from 2016 to 2018 testing the DMI-fungicides prothioconazole and difenoconazole in an in-vitro microtiter assay*

		Year	2016		2017			2018				
		Country	BE	NL	BE	DE	NL	BE	DE	NL	DK	SE
	no. of	**sites**	2	7	2	4	3	6	5	4	2	3
		isolates	13	34	14	18	21	26	26	24	12	15
mEC50 [mg/L]	**Prothio-conazole**	**mean**	0.36	0.33	0.36	0.43	0.35	0.72	0.45	0.35	0.52	0.31
		site-range	0.35-0.36	0.24-0.41	0.30-0.43	0.27-1.12	0.29-0.43	0.56-0.98	0.39-0.51	0.32-0.40	0.48-0.54	0.28-0.41
	Difeno-conazole	**mean**						0.006	0.004	0.007	0.005	0.007
		site-range	n.t.*	n.t.	n.t.	n.t.	n.t.	0.004-0.009	0.004-0.004	0.006-0.009	0004-0.005	0.005-0.007

**n.t.: difenoconazole was not tested in 2016 and 2017*

With exception of Denmark, moderate to high frequencies of isolates showing the F192L substitution in the *cyt-b*-gene were detected (36% – 100%). The F129L-genotype causing moderate resistance factors for QoI-fungicides, such as azoxystrobin. F129L-genotypes were reported to be controlled by a protective application at full dose rate (Pasche *et al.*, 2005; Hausladen *et al.* 2015). However, dependency on long term efficacy or curative application timing lead to the erosion of efficacy of QoI-fungicides in artificially infected field trials or greenhouse trials (Adolf *et al.*, 2017; Liljeroth *et al.*, 2015). Most isolates detected carrying the F129L-mutation also carried a mutation in one of the *sdh*-genes.

The mEC_{50} values of reference isolates for the SDHI fungicides boscalid and fluopyram ranged from 0.03 – 0.06ppm and 0.06 – 0.1ppm, respectively, showing only a slight difference in intrinsic activity of the two SDHI fungicides. In contrast, EC_{50} values of suspicious isolates increased to 1.18 – >30ppm of boscalid or 0.007 – 1.55ppm of fluopyram. Therefore, pyrosequencing assays were performed in order to identify mutations in the *sdh*-genes. With exception of Denmark, moderate to high frequencies of *sdh*B-H278Y and *sdh*C-H134R genotypes were detected (9%-38% and 17%-80%, respectively). Only few isolates showed the *sdh*B-H278R or *sdh*D-H133R genotype (in total 5% and one single isolate, respectively). All SNPs reported in this study were reported previously at similar frequencies (Hausladen *et al.*, 2017; Landschoot *et al.*, 2017; FRAC, 2018). The *sdh*D-D123E mutation reported by Baukse *et al.* (2018b) originating from the US was not detected in any of the European isolates tested in this study, which was also not mentioned by other European researchers up until now (Hausladen *et al.*, 2017; Landschoot *et al.*, 2017; FRAC, 2018). Also, in agreement with most other researchers, multiple mutations in *sdh*-genes did not occur simultaneously in one isolate, but in every case one mutation in one *sdh*-gene was responsible for each of the respective phenotypes of suspicious isolates. Isolates showing none of the mutations tested showed a sensitivity comparable to the mEC_{50} value of sensitive reference strains.

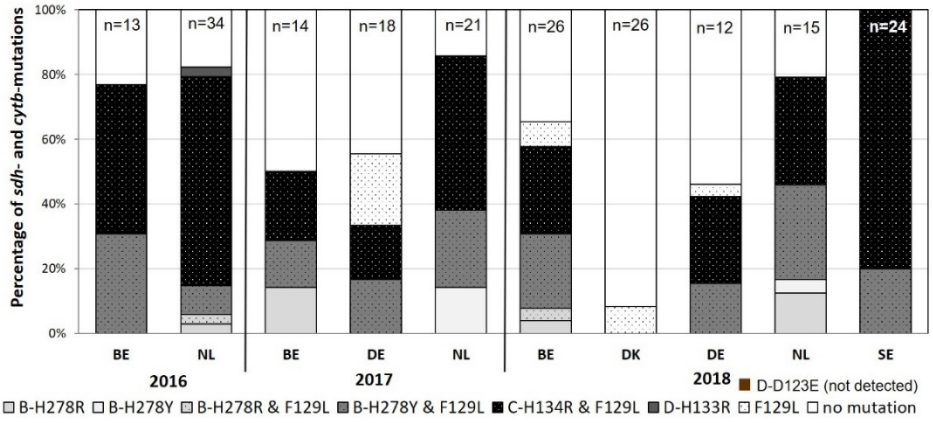


Figure 1. *Percentage of sdh- and cyt-b-genotypes in populations of Alternaria solani collected in different countries from 2016 to 2018*

SDHI cross-resistance study

To characterize the impact of detected genotypes on the activity of SDHI fungicides, in total seven SDHI fungicides from different chemical classes were tested in an *in-vitro* cross-resistance study.

Boscalid clearly showed high resistance factors of more than 100 for all genotypes tested indicating a full *in-vitro* resistance (see Table 3). Interestingly, genotype *sdh*B-H278Y as well as *sdh*B-H278R showed a hypersensitivity to fluopyram. All other SDHIs from the pyrazole-4-carboxamide class, such as bixafen and fluxapyroxad showed low resistance factors. For genotype *sdh*C-H134R as well as *sdh*D-H133R genotypes moderate resistance factors were measured, whereas moderate to high resistance factors were observed for all other SDHI fungicides. This difference is especially evident for the frequently occurring *sdh*C-H134R genotype. Such an incomplete cross-resistance between *in-vitro* tested SDHIs was reported for *A. solani* previously by Gudmestad *et al.* (2013) and for other plant pathogens by Sierotzki and Scalliet (2013).

Table 3. *Mean resistance factors caused by four genotypes of Alternaria solani towards seven different fungicides of the SDHI group determined in an in-vitro cross-resistance study in relation to the mEC_{50} value of wildtype isolates*

		mean Resistance Factor [mEC_{50} mutation / mEC_{50} wildtype]						
		pyridine-carboxamide	pyridinyl-ethyl-benzamide	pyrazole-4-carboxamides				
Genotypes	**n**	**Boscalid**	**Fluopyram**	**Bixafen**	**Fluxa-pyroxad**	**Iso-pyrazam**	**Penthio-pyrad**	**Benzovindi-flupyr**
B-H278R	9	137	0.8	2	2	2	4	2
B-H278Y	12	169	0.7	13	19	14	62	13
C-H134R	12	**>1200***	32	95	99	608	**>229**	406
D-H133R	1*	**>283**	39	48	51	384	**>173**	229
mEC50 value of wildtype	12	0.025	0.033	<0.004	0.009	0.008	0.131	0.005

**The highest concentration tested in the microtiter assay was 30ppm of active ingredient. In case the EC_{50} value of at least one isolates was higher than 30ppm, the mean Resistance Factor caused by the genotype is marked by the ">"-sign and printed in bold-face*

In-vivo efficacy of SDHI-containing products

However, detection of mutations or *in-vitro* measurement of increased EC_{50} values do not necessarily lead to field resistance. Therefore, an *in-vivo* greenhouse study was conducted to determine the impact of the described genotypes on the efficacy of a protective application of commercially available product concepts at their recommended field rates at realistic spray conditions one day prior to inoculation. Under such more realistic test conditions, a significant loss of efficacy of a solo applied boscalid as present in the full label rate of 0.25 L/ha Signum® was observed for all genotypes tested (7% – 63%) compared to an efficacy of 86% of boscalid in controlling the wildtype isolate (see Figure 1). Disease severity on untreated plants was significantly lower at seven days after inoculation for the selected *sdh*B-H278Y and *sdh*B-H278R

isolates compared to the wildtype isolate and significantly higher compared to the *sdh*C-H134R, *sdh*D-H133R isolates. An interaction between aggressiveness of isolates and efficacy of products cannot be excluded, but the less aggressive *sdh*B-H278Y and *sdh*B-H278R isolates showed after ten days similar losses of boscalid-efficacy as for the other genotypes after four to seven dates (data not shown). These results show the impact of high resistance factors caused by the detected genotypes as observed in the *in-vitro* cross resistance study (see Table 3) and leads to a complete loss of efficacy under field conditions as reported in by Metz and Hausladen (2019) in an artificially infected field trial.

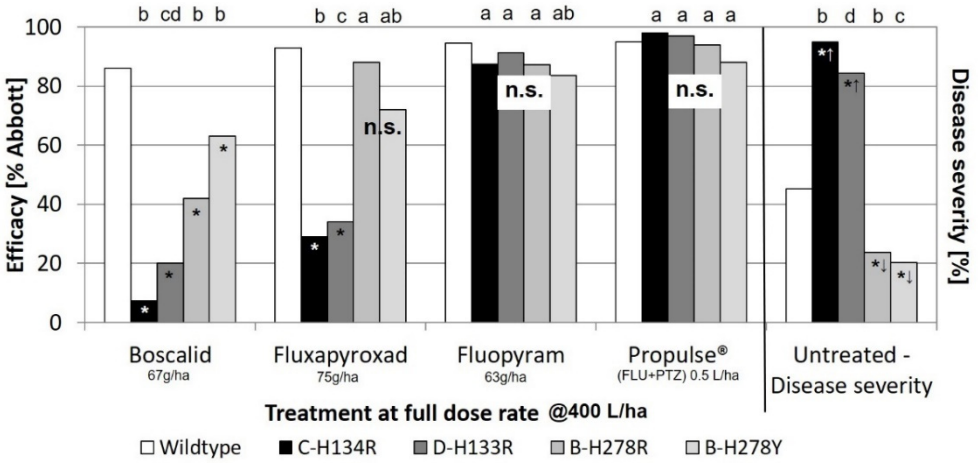


Figure 2. *Efficacy expressed as %ABBOTT of different solo SDHIs and SDHI containing products in controlling four genotypes of Alternaria solani carrying different mutations in sdh-genes as well as the wildtype tested in greenhouse experiments. Disease severity of the tested isolates is given for the untreated control. Different letters indicate significant differences in disease severity between treatments of one genotype according to Tukey's HSD test. An asterisks indicates a significant difference in disease severity between a given genotype and the wildtype isolates within the given treatment according to Student's t-test*

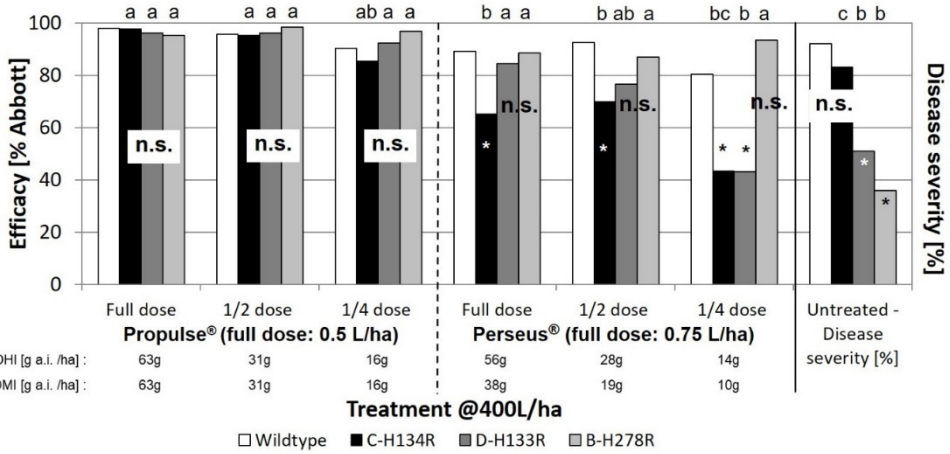
Also, the solo application of fluxapyroxad showed a significant decrease in efficacy from 93% for the wild-type isolate to 29 – 34% for *sdh*C-H134R and *sdh*D-H133R genotypes, respectively (see Figure 1). Such results show the impact of moderate to high resistance factors observed in the *in-vitro* cross resistance study (see Table 3) and would probably lead to recognizable loss of efficacy under field conditions. But for *sdh*B-H278Y and *sdh*B-H278R isolates, the efficacy was nearly on the level of the wild-type isolate (72 – 88%), not only seven but also after ten days of incubation (data not shown). This reveals that no or only a minor impact on field efficacy of a protective application of fluxapyroxad is to be expected for control of *sdh*B-H278Y and *sdh*B-H278R mutations with a low *in-vitro* measure resistance factors of 2-19 (see Table 3) In contrast, more severe losses in efficacy are to be expected for *sdh*C-H134R and *sdh*D-H133R genotypes under field conditions, thus a strong mixing partner is needed. Difenoconazole is used at rate of 125-150 g a.i. per hectare, e.g. in the product Revus Top®. However, in the product

Perseus® at full label rate of 0.75 liter per hectare the difenoconazole rate is reduced to 38 g a.i. per hectare. At such a low rate of difenoconazole, the aggressive *sdh*C-H134R isolate cannot be fully controlled (65%) on the level of the wildtype isolate (89%), since fluxapyroxad is not contributing in controlling of that genotype (see Figure 2). This lack of efficacy gets even more obvious in case the Perseus® dose-rate is reduced to ½ or ¼ of the recommended dose rate (to 70% or 43% efficacy, respectively), whereas the level of control of the wildtype as well as the *sdh*B-H278R-genotype stays on a high level (80% or 93%, see Figure 2). This result shows that the high intrinsic activity leading to a difenoconazole mEC_{50} value for the sensitive reference strains of 0.004ppm as observed in the *in-vitro* test-system (see Table 2) does not translate directly into a high *in-vivo* control efficacy of a reduced rate of difenoconazole in case the mixing partner fluxapyroxad is compromised.

The solo application of fluopyram showed a control efficacy of 84 and 87% for the genotypes *sdh*B-H278Y and *sdh*B-H278R on the same level as the control efficacy for the wildtype (95%, see Figure 1). This observation in this *in-vivo* study was in line with the observation of resistance factors lower than 1 in the *in-vitro* study (see Table 3). Genotypes *sdh*C-H134R and *sdh*D-H133R showed for a one day protective application a similarly high efficacy of 88 and 91%. However, first small spots of chlorosis were visible on the leaves, especially for the *sdh*C-H134R isolate. However, increase of incubation time to 10 days under constant optimal conditions for the fungus did not lead to formations of lesions, whereas the untreated and boscalid-treated plants were completely defoliated (data not shown). In general, it cannot be excluded, that the long-term or curative efficacy of a solo fluopyram application to control *sdh*C-H134R and *sdh*D-H133R genotypes could be reduced. Gudmestad *et al.* (2013) Bauske *et al.* (2018b) showed, that a reduction of dose-rate of 100ppm of fluopyram to 10ppm resulted in small reduction of mean efficacy for *sdh*C-H134R (from 99% to 83%) and for *sdh*D-H133R (from 100% to 86%) as well as a stronger reduction for *sdh*D-D123E (from 95% to 66%). A further reduction by a factor of 10 to 1ppm of fluopyram does not only lead to further reduction of efficacy for the genotypes tested (63%, 37%, 33%), but also to reduction of efficacy to control wildtype isolates (from 100% over 99% to 84%). Metz and Hausladen (2019) reported, that efficacy of a spray program testing only solo fluopyram-applications in a 14 day spray interval was reduced from about 70% in plots artificially inoculated with a wildtype isolate to about 40% in plots inoculated with the *sdh*C-H134R-genotype. This level of control efficacy was on the level of that of solo boscalid-treatments in plots artificially inoculated with the wildtype isolate (about 45%). In contrast, the efficacy of boscalid was reduced to less than 10% in the artificially inoculated plots with the *sdh*C-H134R-genotype of the same field trial. In the present *in-vivo* greenhouse study, the mixture concept Propulse®, containing additionally the DMI prothioconazole, showed a high efficacy to control all tested genotypes of 88 – 98% (see Figure 1 and 2). This was on the same level as that observed for the wildtype isolate (95 – 98%). Also, no spots of chlorosis were visible even after 10 days indicating, that all genotypes were fully controlled. Even at a not recommended reduction to ½ or ¼ of the full Propulse® label rate, the protective efficacy to control the *sdh*C-H134R-genotype stays on the same level (98% or 85%) as that of the wildtype (96% or 90%, see Figure 2). These results are in agreement with reports of very good field efficacy of Propulse® also in regions from which previously the *sdh*C-H134R genotype was reported (Evenhuis *et al.*, 2018).

The results of the *in-vitro* and *in-vivo* studies reported here as well as reports from experiments by other researchers highlight the need for product concepts containing SDHIs to have a mixing partner, which is able to fully control the disease on its' own, as recommended in the resistance

management guidelines annually published by the FRAC SDHI Working group (FRAC, 2018). Together with a better knowledge on disease identification, disease prediction models as well as utilization of biocontrol agents, such as *Bacillus* spp. or *Trichoderma* spp. (Metz et al, 2019), all available tools could be combined in an integrated Early Blight disease management including an effective fungicide resistance management to keep a sufficient number of effective disease management tools available for farmers in the future.



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