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Succinate-Dehydrogenase Inhibitor (SDHI) Resistance Evolution in Plant Pathogens

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SCOPE OF RESISTANCE RESEARCH

Resistance is an evolutionary process, based on mutations, gene flow, genetic drift and selection enabling certain organisms to survive the exposure to pesticides and increase their frequency in pest populations. Individuals carrying genetic mutations or natural variations confering a shift in sensitivity towards one our multiple chemical components can be selected by the use of pesticides. Repeated rounds of selection (treatments) can lead to an increase of resistant individuals in populations and when the frequency of resistant individuals becomes predominant compared to the sensitive fraction the efficacy of the pesticide can be impaired. Resistance research includes studies aimed to 1) determine the baseline sensitivity of target pathogen species, determine the genetic origin of adaptation (mode of resistance), 2) perform forced selection or mutagenesis experiments if no naturally resistant strains are available, 3) understand the inheritance of resistance, 4) elucidate the cross resistance pattern to other fungicides of the same class or of different classes, 5) describe and model the evolutionary potential in a field population and 6) study and elucidate mititgation procedures, 7) propose anti-resistance strategies aimed at reducing the selection pressure. Resistance management cannot prevent the evolution of resistance, but it can significantly lower the occurence of resistance and delay its development within populations and therefore preserve the effectiveness of pesticide treatments (FAO 2012). Resistance reseach is not only an essential tool to manage commercialized products, but it is crucial in all stages of a pesticide life starting from the early discovery (Torriani et *al.* 2015) through the process of registration. During the early stages of pesticide development the specific mode of action and possible mechanisms of resistance for a new molecule needs be characterized. Moreover, the intrinsic activity (potency) and the spectrum of activity need to be elucidated for the different target pests. Since resistance management is a regulatory requirement for registration, resistance risk evaluation and field sensitivity baselines are integral parts of the registration dossier (OEPP/EPPO 2015). In this contribution we will focus to the research aimed to better characterize the resistance evolution of the fungicide class Succinate DeHydrogenase Inhibitors (SDHIs).

SUCCINATE DEHYDROGENASE INHIBITORS RESISTANCE RESEARCH

The fungicide class of SDHI was first described more than 50 years ago with the earliest compound named carboxin (von Schmeling & Kulka 1966). The first generation of SDHI molecules showed to control a narrow spectrum of plant pathogens. Recently, this group has been enlarged with novel broad spectrum fungicides controlling a range of diseases in various crops (Sierotzki & Scalliet 2013). According to the Fungicide Resistance Action Committee (FRAC, www.frac.info) the SDHI group contained in 2016 a total of 19 different active ingredients from nine different chemical groups. All fungicides belonging to SDHI are considered cross resistant. SDHIs block the TCA cycle at the level of succinate to fumarate oxidation, leading to an inhibition of mitochondrial respiration (Sierotzki & Scalliet 2013). Resistance has been reported in about 15 fungal pathogens. SDH enzyme consists of four subunits (Cecchini 2003). SDHI fungicides specifically interrupt fungal respiration by blocking the electron transport from the heme group to ubiquinone at regions overlapping with the ubiquinone sites. SDHI resistance is multi-monogenic and several target site mutations have been described targeting sdhB, sdhC and sdhD subunits within or close to ubiquinone-binding site. Each mutation could lead to different resistance factors between the SDHIs. Distinct species can co-evolve a similar panel of core resistant alleles associated to decreased SDHI sensitivity, e.g. *Pyrenophora teres* (Rehfus et *al.* 2016)*, Alternaria* species (Avenot & Michailides 2007). Other species such as *Zymoseptoria tritici* (Torriani et *al.* 2015; Dooley et *al.* 2016) and *Venturia inaequalis* (FRAC www.frac.info) evolved another set of resistant alleles.

Case study 1: *Pyrenophora teres* (barley pathogen)

The plant pathogenic fungus *Pyrenophora teres* is the causal agent of Net Blotch of barley. Under favorable conditions this disease can lead to 10 to 40% of yield reduction (Mathre 1997; Minarikova & Polisenska 1999). According to Pathogen Risk List from FRAC *P. teres* was ranked as a medium risk pathogen to evolve fungicide resistance. Applications including fungicide belonging to SDHIs, quinone outside inhibitors (QoIs), demethylation inhibitors (DMIs) and aniline-pyrimidines (APs) are used to control Net Blotch. QoI resistance in *P. teres* is likely only possibly based by the mutation F129L in the mitochondrial encoded cytb (Sierotzki et *al.* 2005,). As observed in other pathogens, resistance factors associated to this mutation are significantly lower in comparison to G143A mutation. The frequency of F129L in Europe is variable between countries with a mean around 25% for 2013 and 2014 (Rehfus et *al.* 2016). Situation in 2015 was similar if compared to 2014 with average frequency below 30% and highest frequency of resistance detected in UK and Northern Germany with an average of 65% (FRAC, www.frac.info). However, due to the moderate resistance mediated by F129L and the moderate frequency full rates of QoI are supposed to provide disease control. Internal European fungicide sensitivity monitoring from 2015 highlighted a stable situation for the DMIs since more than ten years and a sensitive situation for APs with resistance being detected below 0.1%.

The first SDHI shifted genotype was monitored in 2012 in the Northern Germany carring the mutation H277Y in *sdhB* (Figure 1A), associated to low resistance factors (Figure 1B). In the following two seasons (2013-2014) other mutations affecting the SDHI activity evolved and the frequency of SDHI resistance increased in the northern parts of Germany and France. In 2015 the SDHI sensitivity stabilized on the levels of 2014. Among the R-alleles evolved from 2013 onwards, R64K, G79R, H134R and S135R in *sdhC* and H134R in *sdhD* were associated to moderate resistance factors (Figure 1B; Rehfus et *al.* 2016). As reported in Rehfus et *al.* (2016), our quantitative molecular analysis from bulk samples identified the mutation G79R in *sdhC* as the most frequent SDH mutant in field populations. The C-G79R frequency was moderate in Germany, France and Belgium for both 2014 and 2015. A similar set of R-alleles including homologs of H277Y in *sdhB,* H134R and S135R in *sdhC* and H134R in *sdhD* evolved in *Alternaria* species and *Sclerotinia scleotiorum* (Figure 3), but in both cases with much lower frequency in the populations. For the complete list of SDHI R-alleles evolved in *P. teres* and resistance distribution refers to the latest FRAC SDHI minutes online.

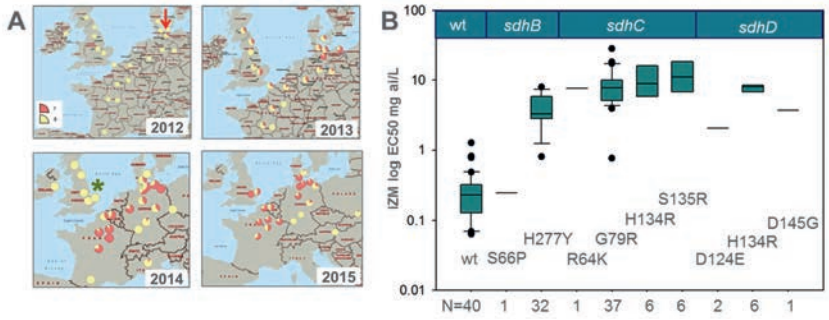

Figure 1 A: Geographic distribution of SDHI shifted phenotypes (air monitoring). The percentage of sensitive (yellow) vs less sensitive isolates (red) per location is presented. Less sensitive strains show growth >= 50% at 3.0 mg/L and >= 10 % at 10.0 mg/L isopyrazam (IZM) relative to control strain (Data generated by EpiLogic GmbH). B: Correlation between R-alleles in sdhB, sdhC and sdhD subunits and sensitivity to IZM.

Case study 2: *Zymoseptoria tritici* (wheat pathogen)

Zymoseptoria tritici (aka *Mycosphaerella graminicola*) is the causal agent of Septoria blotch in wheat. According to FRAC *Z. tritici* was ranked as a medium risk pathogen to evolve fungicide resistance. The control of this disease is critical for the cereal production in Europe (O'Driscoll et *al.* 2014) and approximately 1.2 billion dollars are spent on fungicides annually (Torriani et *al.* 2015). Septoria leaf blotch is, after the wide spread of QoI resistance mainly managed through applications of SDHIs, DMIs and multisites such as chlorothalonil in many wheat growing areas in Europe. In Eastern and Southern Europe QoIs still contribute to the spray program. Syngenta tests within its fungicide sensitivity monitoring program hundreds of single spore isolates of *Z. tritici* from different European populations every year. More than 5000 isolates were phenotyped in the last 11 years. DMIs are used since the 1970s and in the recent decades European populations of *Z. tritici* evolved a reduced sensitivity (Torriani et *al.* 2015). Overall, in 2015 the DMI sensitivity was similar as in 2014. SDHI sensitivity was

monitored since 2004 and overall no sensitivity shift was monitored in European populations being largely sensitive (Figure 2) as represented by the median. Forward genetics studies were conduced to elucidate the evolutionary potential of SDHI resistance (Scalliet et *al.* 2012). Through UV mutagenesis approach 27 amino acid changes at 18 positions in sdhB, sdhD and sdhD were identified. Five of the six R-alleles positions naturally evolved in field populations (FRAC, www.frac.info) were predicted in the lab, highlightening forward genetics as an useful tool to better understand possible evolutionary changes associated to resistance. Field isolates showing decreased SDHI sensitivity were monitored since 2012 at low frequency, harbouring the following R-alleles: N225T, T268I in sdhB and T89N, W80S, N86S in sdhC. These mutations are associated to low resistance factors. In 2015, the R-allele H152R in sdhC was monitored at very low frequency in populations from Ireland and UK (Dooley et *al.* 2016; FRAC, www.frac.info). This mutation showed moderate resistance factors. The same mutation was identified through UV mutagenesis to impair the SDH activity. Mutant sdhC-H152R showed a residual enzymatic activity of 22% if compared to the wild type (Scalliet et *al.* 2012). Whether the sdhC-H152R mutation selected in the field is similarly associated to reduced enzymatic activity still needs to be elucidated. Quantitative analysis of historical monitoring samples showed the absence of this mutation before 2013. Our first detection of sdhC-H152R occured in 2014 in a bulk sample from Germany, the mutation was found at a frequency of 3.5%. In 2015, quantitative analysis of 270 field samples detected the presence of sdhC-H152R mutation in 7 samples only, again at a low frequency which was comprised between 1 and 10%. The mutation was monitored in Northern Germany, UK and Ireland. Preliminary data from 2016 show that the frequency of sdhC-H152R remains at similar level as found in 2015. This data suggest that *Z. tritici* SDHI resistance is evolving slower compared to the case of *P. teres.* Homolog R-alleles to sdhC-H152R of *Z. tritici* were recently described in *Venturia inaequalis* and *Ramularia collo-cygni* (Figure 3; FRAC, www.frac.info).

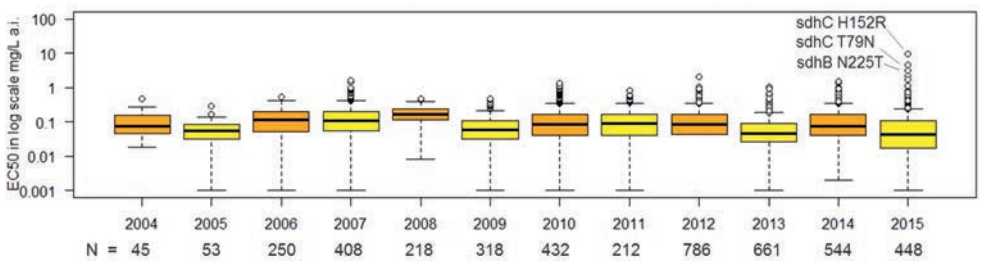

Figure 2 *Z. tritici* isopyrazam sensitivity from 2004. The sampling size (N) is marked below the years.

Case study 3: *Ramularia collo-cygni* (barley pathogen)

Ramularia collo-cygni is a causal agent of Ramularia Leaf Spot of barley. The economic relevance of this pathogen is increasing in Europe (Havis et *al.* 2015) and according to the FRAC it was ranked as a high risk pathogen to evolve fungicide resistance. Today, *R. collo-cygni* is mainly controlled by the used of fungicides belonging to SDHI, DMI and multisites as chloro-

thalonil. Similarly as for *Z. tritici*, Piotrowska (2014) generated UV mutants to get a better understanding of the possible SDHI resistance evolution in *R. collo-cygni.* Through extensive monitoring in Germany we identified in 2015 the first genotypes showing strongly decreased sensitivity to SDHI, carring either the mutation H142R or H149R in sdhC. Mutation sdhC-H142R is homologous to the R-allele sdhC-H134R described in *P. teres* and *Alternaria* species and the mutation sdhC-H146R of *S. sclerotiorum.* Instead, sdhC-H149R is homologous to the mutations sdhC-H152R and H151R described in *Z. tritici* and *V. inaequalis* respectively (Figure 3).

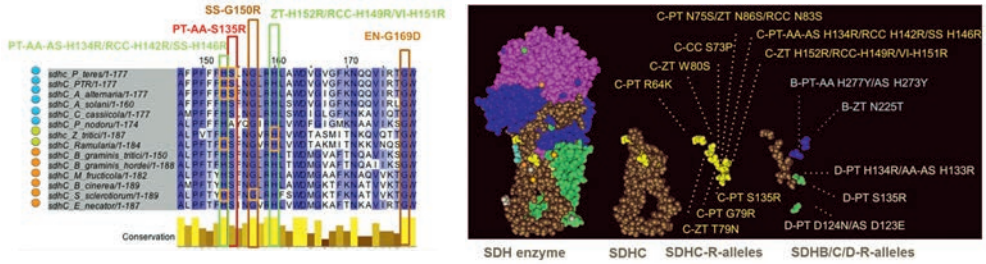

Figure 3 Partial sdhC amino acid alignment highlightening homolog resistance alleles between species belonging to the Pleosporales (blue circles), Capnodiales (green circles) and Leotiomycetes (orange circles) (PT=*P.teres,* AA=*A.alternata,* AS=*A.solani*, RCC=*R.collo-cygni*, SS=*S.sclerotiorum*, ZT=*Z. tritici* VI=*V. inaequalis*, EN=*Erysiphe necator*). Frequency of aa conservation is marked as bars (bottom left). In the left panel the tridimentional location of R-alleles in SDHenzyme is presented (right panel).

DISCUSSION

The three case studies presented above highlight the importance of resistance research to understand fungicide resistance evolution. To understand SDHI resistance evolution in different pathogens there is the need of solid data from the lab (e.g. possible evolutions, fitness assessment, strength of resistance) and from field monitoring (biotest and molecular, establishing frequency and spread). As described above for *P. teres, Z. tritici* and *R. collo-cygni* each pathogen evolves SDHI resistance at different speed and with a certain set of different R-alleles. The relevance of these mutations is difficult to be predicted into general patterns. Although homologs R-alleles exist between species, these might have different resistance factor associated to different compounds and species. The position of a given R-allele in the tridimentional structure of SDH enzyme can differently alter the enzymatic activity and the binding property of different SDHIs. It is therefore challenging to forecast resistance evolution in sensitive species. However it seems that certain mutations, especially those targeting amino acids located in the proximity of the SDH catalytic domain and largely conserved between species, are associated to higher resistance factors (Figure 3). For most pathogens further research is needed to understand possible fitness cost associated to the resistance and the relevance for SDHI field performance. In additon, the resistance management for one fungicide class also

requires sound knowledge about the sensitivity/resistance situation of the putative partner fungicides. The data generated are used for establishing sound resistance mitigation tactics, which accordingly need to be agreed and followed among all partners involved in crop protection.

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