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Status of *In Vivo* and Molecular Diagnosis of Fungicide Resistance in Powdery Mildews

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ABSTRACT

Powdery mildew fungi are important plant pathogens in many crop plants. Examples of agronomically important species include wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*), cucurbit powdery mildew (*Podosphaera xanthii* and *Golovinomyces cichoracearum*), and grape powdery mildew (*Erysiphe necator*). Concerning the development of fungicide resistance, they are classified as moderate to high risk pathogens. Besides SBIs (sterol biosynthesis inhibitors), the aryl-phenyl-ketones with metrafenone and pyriofenone and the SDHIs (succinate dehydrogenase inhibitors) are important modes of action for effective disease management of powdery mildews. Whereas the SDHIs inhibit the respiration chain at complex II, the mode of action of the aryl-phenyl-ketones is still unknown. A regular sensitivity monitoring is therefore recommended for several species and different modes of action. Classical sensitivity tests with living fungal material can be challenging regarding sampling, transport, and maintenance of living strains. Whenever possible, molecular genetic methods such as qPCR or pyrosequencing are preferred for a more efficient monitoring. Such methods require the knowledge of the genetic background of the resistance mechanisms, which are typically target site mutations.

In this study, a summary on the current knowledge on the sensitivity status of wheat, cucurbit, and grape powdery mildew for European populations towards SDHIs and aryl-phenyl-ketones is provided. In particular, the genetic background of grape powdery mildew isolates with a reduced sensitivity towards SDHIs was investigated.

MONITORING METHODS

For the analyses of the sensitivity status of powdery mildew populations, intensive monitoring programs in European growing regions were carried out for several years. Samples were obtained with two different sampling methods: field sampling and air borne sampling. For field sampling infected leaves were collected directly within trial or commercial sites. For air

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borne sampling spores were sampled with a spore trap mounted on a car driving through different regions in Europe (performed by EpiLogic; Freising-Weihenstephan, Germany).

From each sample a defined number of isolates was obtained, which were analysed in a following detached leaf test. The sensitivity of these isolates was determined with adequate discriminatory doses of the analysed fungicide.

RESULTS AND DISCUSSION OF SENSITIVITY MONITORING

Wheat powdery mildew

Aryl-phenyl-ketones

Several years of sensitivity monitoring of *B. graminis* f.sp. *tritici* to metrafenone using *in vivo* methods have shown that two different resistance phenotypes (moderately adapted, resistant) occur at low levels in wheat in commercial practice (Felsenstein et *al.*, 2010).

Since 2012, the frequency of both phenotypes remained stable (Figure 1). Between 2012 and 2015, the frequencies were on average 27% for the moderately adapted and 1% for the resistant phenotype.

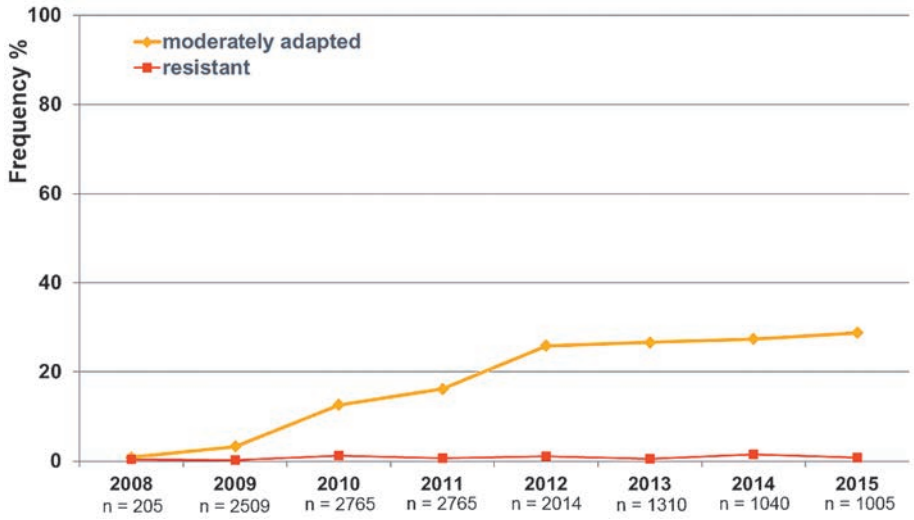

Figure 1 Development of the frequency of metrafenone adaptation in *B. graminis* f.sp. *tritici* from 2008 to 2015 using a detached leaf test.

SDHIs

European fluxapyroxad monitoring performed with detached leaf tests since 2012 showed an overall sensitive situation. Also in 2015 all isolates (n=100) were within a previously established baseline. No isolates with reduced sensitivity against SDHIs were observed.

Persistent Identifier: urn:nbn:de: 0294-sp-2017-Reinh-41-2

Cucurbit powdery mildew

Aryl-phenyl-ketones

Sensitivity monitoring of the "high risk" cucurbit powdery mildews (*Podosphaera xanthii* and *Golovinomyces cichoracearum*) is currently done using *in vivo* detached leaf assays (FRAC 2016). No isolates with reduced sensitivity against aryl-phenyl-ketones were detected in extensive European monitoring studies.

SDHIs

Extensive monitoring programs have been performed since 2005. At some commercial sites in Europe, single isolates with reduced sensitivity towards SDHIs could be detected since 2012 (FRAC 2016, minutes of the SDHI meeting, 2012-2015). Studies using a detached leaf test showed that all tested SDHIs are affected by the adapted isolates (Figure 2). The ED_{50} values increased by 2 ppm to 20 ppm above the respective sensitive reference strain depending on the used SDHI.

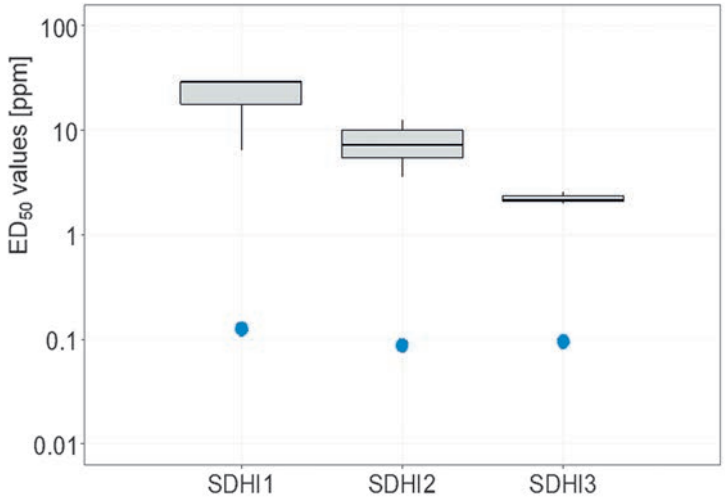

Figure 2 ED_{50} values of isolates of *Podosphaera xanthii* and *Golovinomyces cichoracearum* with reduced sensitivity against SDHIs determined with a detached leaf test. ED_{50} values of adapted isolates are shown as boxplots (adapted isolates n=3), the sensitive reference strain is shown as solid circles in blue (n=1).

Grape powdery mildew

Aryl-phenyl-ketones

The "moderate risk" pathogen grape powdery mildew (*Erysiphe necator*) is currently monitored using *in vivo* assays. European monitoring studies in 2015 using a detached leaf test identified some samples containing aryl-phenyl-ketone resistant isolates, similar to studies reported earlier (Kunova et *al.* 2015). In contrast to wheat powdery mildew, only one

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resistance phenotype was detected. No moderately adapted phenotype was observed. The resistance mechanism is currently under investigation.

SDHIs

Extensive monitoring programs were carried out since 2003. Single isolates with a reduced SDHI sensitivity were identified for the first time in European monitoring studies in 2014 and 2015 (FRAC 2016, minutes of the SDHI Meeting 2015).

Development of molecular genetic methods

To identify potential target site mutations leading to a reduced sensitivity against SDHIs, the genes for the SDH subunits of these isolates were sequenced and analysed. Two different amino acid substitutions in conserved regions of the SDH enzyme could be identified (Figure 3).

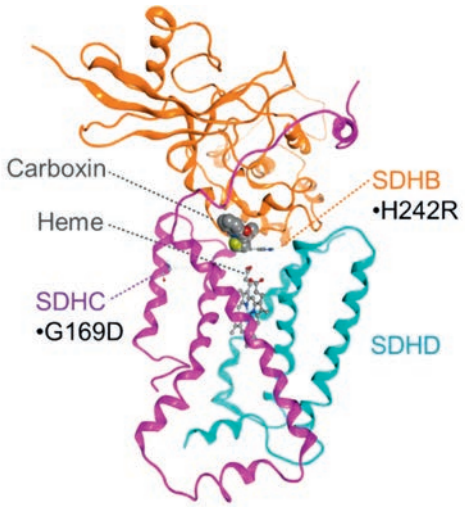

Figure 3 Homology protein model of the succinate dehydrogenase subunits B, C and D from *Erysiphe necator* based on X-ray from *Gallus gallus* (PDB 2WQY) with the amino acid substitutions B-H242R and C-G169D.

The sequence of *sdh*B revealed a point mutation leading to an amino acid substitution at position 242 from histidine to arginine. This amino acid exchange is homologous to known substitutions in other plant pathogenic fungi (e.g. H272R/Y/L in *Botrytis cinerea* or H277Y in *Alternaria alternata* and *Pyrenophora teres*, Stammler et *al.* 2015). Sequence analysis of *sdh*C showed a point mutation causing an amino acid exchange from glycine to aspartic acid at position 169. For a more efficient and rapid monitoring pyrosequencing assays were developed for both amino acid substitutions. The impact of these substitutions was analysed in a germination test (Figure 4) and in a leaf disc assay (Figure 5) using single spore isolates of *E. necator*. Spore germination of sensitive isolates is inhibited at about 0.3 ppm for fluxapyroxad and about 1 ppm for fluopyram. For isolates carrying the B-H242R substitution

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germination was fully inhibited at 30 ppm, which was observed for both SDHIs. Isolates with the C-G169D substitution showed germination at 30 ppm, but less pronounced compared to the untreated control.

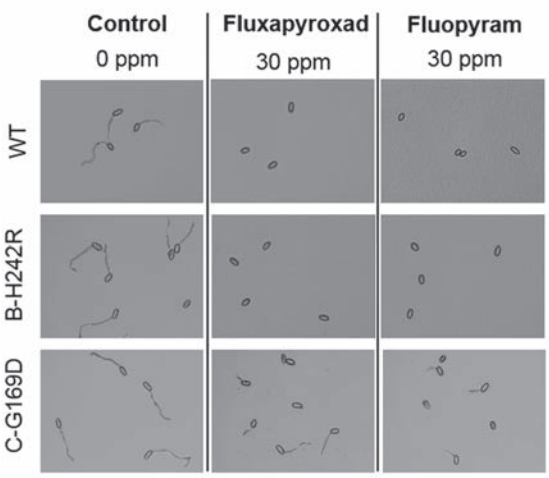

Figure 4 Characterization of amino acid substitutions B-H242R and C-G169D in a spore germination test on *E. necator*.

The ED_{50} values of fluxapyroxad and fluopyram for isolates carrying the amino acid substitution B-H242R or C-G169D were determined in a leaf disc assay.

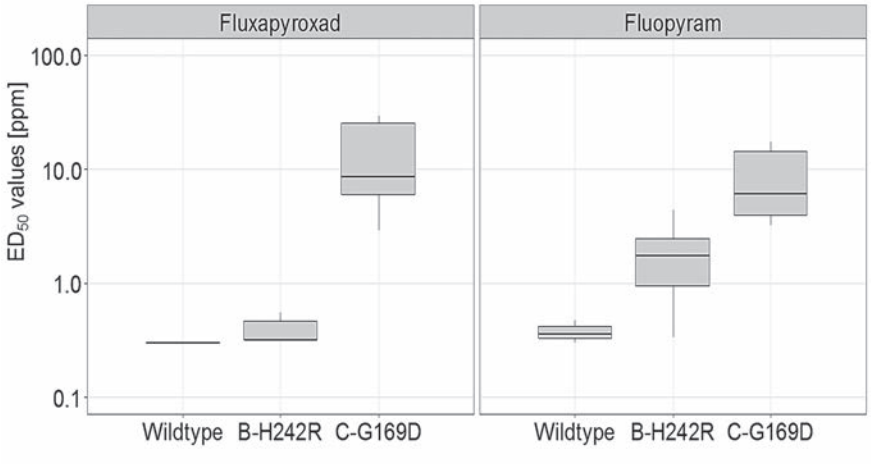

Figure 5 Characterization of amino acid substitutions B-H242R and C-G169D in *E. necator* in a leaf disc assay. Mean ED_{50} values, Boxplots, sensitive n=3, B-H242R n=6, C-G169D n=7.

Persistent Identifier: urn:nbn:de: 0294-sp-2017-Reinh-41-2

Isolates with the B-H242R substitution have no or weak influence on the effectiveness of fluxapyroxad and fluopyram. Cross-resistance could be shown for isolates carrying the C-G169D substitution, whereby the efficacy of both SDHIs was moderately affected.

CONCLUSION

Aryl-phenyl-ketones: Wide-scale European resistance monitoring showed a stable resistance situation for wheat powdery mildew. All monitored cucurbit powdery mildew locations were fully sensitive and first findings of resistant grape powdery mildew isolates is reported.

SDHIs: An overall sensitive situation was observed for all analysed powdery mildews. Single isolates of cucurbit powdery mildew and grape powdery mildew with a reduced sensitivity were observed.

To keep these modes of action as effective tools for the disease management of powdery mildews the monitoring of the sensitivity over the years is essential. Aryl-phenyl-ketones and SDHIs remain valuable tools for disease management in powdery mildews. It is important to follow the FRAC guidelines to ensure that these modes of action remain effective for a long time.

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