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SDHI Sensitivity Status of *Zymoseptoria tritici* and *Botrytis cinerea*

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INTRODUCTION

Inhibitors of the complex II of the fungal respiratory chain, also known as succinate dehydrogenase inhibitors (SDHI), are of prime importance for crop protection worldwide. This calls for sound resistance monitoring and management, in order to sustain the efficacy of this important chemical class. The molecular basis for the resistance is complex: numerous different *sdh* mutations in various plant pathogenic fungi lead to different levels of SDHI sensitivity, which is, at least for few mutations, even dependent on the chemical structure of the active ingredient. To understand if and how two important SDHIs, Bixafen and Fluopyram, are affected by resistance, we perform annual sensitivity monitoring for several major pathogens. This paper describes latest monitoring data based on standard *in vitro* bioassays, complemented by sequencing of *sdh* mutations, and conclusions regarding resistance management for two of the economically most important pathogens, *Zymoseptoria tritici* and *Botrytis cinerea*.

MATERIAL AND METHODS

Sampling, cultivation and sensitivity testing of *Zymoseptoria tritici*:
Approx. 20 wheat leaves were randomly sampled from a commercial field showing typical *Z. tritici* lesions and visible pycnidia were taken per sample. Lesions of several leaves were cut out and placed upside down onto water agar. After an incubation period of 24 h at room temperature, spores originating from one single pycnidium and lesion were suspended in 100 µL sterile water, scattered on Czapek-Dox vegetable juice agar and incubated at 18-20°C. After 6 days, spores originating from a single colony were suspended in 3 mL sterile glucose peptone medium and incubated 24 h at 150 rpm on a shaker. At least three isolates per sample were analyzed (exception: a sample from the French region Lorraine yielded only one isolate). All isolates were tested twice with the following final Bixafen concentrations: 0; 0.0064, 0.032; 0.16; 0.8; 4; 20 and 100 µg active ingredient per mL. Microtiter plates were prepared as follows: 10 µL methanol, containing the amount of active ingredient required per concentration, was added to the wells. After evaporation of the solvent and one day before inoculation, 140 µL dextrose-peptone medium was added to each well and the plate was kept at 150 rpm overnight on an orbital shaker. Then, 60 µL of the spore suspension, prepared as described

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above, were added to each well. The plates were incubated on a shaker for 7 days at 20°C and 90 % relative humidity. Growth was monitored photometrically at 620 nm. EC_{50} values were calculated from the blank-corrected extinction values using an in-house software.

Sampling, cultivation and sensitivity testing of *Botrytis cinerea*

Conidia were removed from the surface of heavily sporulating berries using sterile cotton swabs. One cotton swab was used per berry. Conidia from swabs were tapped onto PDA containing 25 mg/L Enrofloxacin to limit bacterial contamination. Following incubation in darkness at 20 °C for 2-3 days, plates were inspected for the absence of contaminating *Mucor* or *Penicillium* spp. A plug of *Botrytis cinerea* mycelium was transferred onto Last-and-Hamley medium (Last & Hamley 1956) and incubated under black light at 20 °C for 10-12 days until massive sporulation. Conidial suspensions were prepared by washing the plates with sterile water, filtering through four layers of gauze and adjusting the spore concentration to $2x10^5$ conidia/mL. The following final SDHI concentrations were used to determine EC_{50} values in duplicates: 0.00192; 0.0096; 0.048; 0.24; 1.2; 6 and 30 mg/L. After evaporation of the solvent, 100 µL succinate medium (K_2HPO_4 3.0 g/L, KH_2PO_4 4.0 g/L, $(NH_4)SO_4$ 1.5 g/L, $MgSO_4$ heptahydrate 0.75 g/L, sodium succinate hexahydrate 7.5 g/L, yeast extract 3.0 g/L, Enrofloxacin 40 mg/L) was added to each well and the plate was placed on an orbital shaker for at least 2 h. After adding 100 µL spore suspension, microtiter plates were incubated in darkness at 150 rpm on an orbital shaker at 21°C for 5 days. EC_{50} values were determined as described above.

DNA isolation and pyrosequencing

DNA isolation and pyrosequencing were performed as described by Weber and Co. (2015).

RESULTS AND DISCUSSION

Bixafen sensitivity status of *Zymoseptoria tritici*

To investigate the Bixafen sensitivity status of *Zymoseptoria tritici,* microtiter plate tests were conducted with a total of 1372 fungal isolates from 287 field samples collected in 2015. Sampling was performed in several European countries, with a strong focus on UK (232 isolates), Ireland (90 isolates), France (125 isolates) and Germany (855 isolates). Similar to the previous years, we observed an overall homogeneous sensitivity distribution within the sampled populations, with a mean EC_{50} of 0.034 ppm (2014: 0.039 ppm). For most regions in the analyzed countries we determined a relatively narrow sensitivity range of the samples, within the order of magnitude of the sensitive reference isolates (Table 1), indicating an overall Bixafen-sensitive pathogen population. Out of the 1372 isolates analyzed, 19 (1.4 %) isolates had increased EC_{50} values (0.2-0.6 ppm), resulting in resistance factors of merely 8-20 for Bixafen. In 5 of these isolates, sequencing of the subunits B, C and D of the succinate dehydrogenase resulted in the detection of the following mutations: B-N225I (Scalliet et *al.* 2012) found in two isolates from a single sample from UK, C-T79N (FRAC 2015) in a single strain from Ireland and C-N86S (FRAC 2015) in one strain from Germany and one from Ireland. The mutation C-H152R (Scalliet et *al.* 2012; Dooley et *al.* 2016) was not found.

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Table 1 Bixafen sensitivity of European *Zymoseptoria tritici* populations.

Country / Region	Samples / Isolates	mEC_{50}min - mEC_{50}max [mg/l] [1)]
Germany: 111 samples / 855 isolates		
Bavaria	1 / 3	0,04
Lower Saxony	1 / 3	0,04
North-Rhine Westphalia	1 / 3	0,03
Mecklenburg-Vorpommern	30 / 241	0.03 - 0.11
Saxony-Anhalt [2)]	51 / 410	0.02 - 0.09
Saxony	26 / 175	0.03 - 0.09
Schleswig-Holstein	1 / 20	0,04
France: 43 samples / 125 isolates		
Nord-Pas de Calais	9 / 27	0.02 - 0.06
Midi Pyrenées	2 / 6	0.02 - 0.03
Picardy	2 / 6	0.03 - 0.05
Île de France	2 / 6	0.03 - 0.13
Champagne-Ardenne	3 / 9	0.05 - 0.07
Brittany	2 / 6	0.02 - 0.03
Centre	3 / 9	0.03 - 0.03
Burgundy	1 / 3	0,02
Poitou-Charentes	3 / 9	0.01 - 0.05
Lorraine	1 / 1	0,05
Normandie	13 / 37	0.02 - 0.06
Aquitaine	2 / 6	0.02 - 0.03
Great Britain: 79 samples / 232 isolates		
Scotland	7 / 21	0.02 - 0.08
West Midlands	11 / 32	0.03 - 0.07
East Midlands [3)]	14 / 42	0.02 - 0.21
East of England	38 / 110	0.02 - 0.12
South West England	2 / 6	0.02 - 0.03
South East England	7 / 21	0.02 - 0.05
Ireland: 30 samples / 90 isolates		
Leinster [2)]	6 / 18	0.02 - 0.27
Munster [2)]	24 / 72	0.02 - 0.09
Denmark:	3 / 9	0.02 - 0.03
Sweden:	5 / 15	0.02 - 0.04
Austria:	12 / 34	0.02 - 0.03
Baltic states:	4 / 12	0.02 - 0.03
Reference isolates	5	0.02 - 0.03

[1)] mEC_{50}: geometric mean calculated from the EC_{50} values of all isolates from a sample
[2)] A single isolate with a *sdh* mutation was found in one sample
[3)] Two isolates with a *sdh* mutation were found in one sample

Fluopyram sensitivity status of *Botrytis cinerea*

In 2015, 591 *Botrytis cinerea* strains isolated from infested grapes from France, Germany, Italy and Chile were analyzed with respect to their sensitivity to Fluopyram. No resistance was found in Italian strains, whereas in France, Germany and Chile the proportion of Fluopyram-

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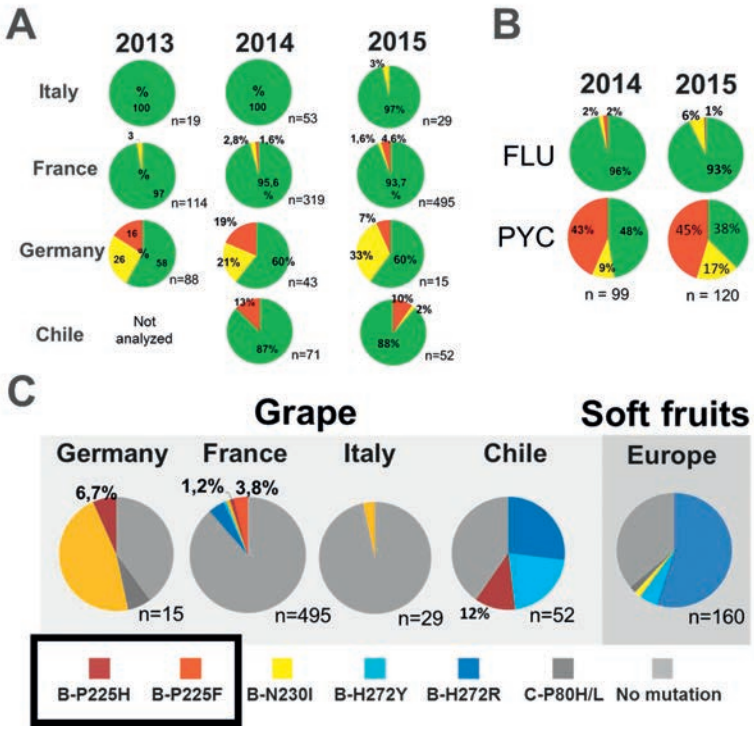

Figure 1 Sensitivity status of *Botrytis cinerea* isolated from infested grapes towards Fluopyram (A) and from European soft fruits towards Fluopyram (FLU) and a pyridine-carboxamide (PYC) (B). (C) *sdh* mutation spectra found in the corresponding 2015 samples. Mutations mediating Fluopyram resistance are surrounded by a frame in the legend. Colour code in (A) and (B): green represents the proportion of fully sensitive isolates (within baseline, EC_{50} <4 ppm), yellow represents the proportion of isolates with reduced sensitivity but still reacting to the fungicide in a dose-response manner (EC_{50} 4-30 ppm) and red represents the proportion of resistant isolates with EC_{50} >30 ppm.

resistant strains was between 5 and 10%. A comparison with the results of previous years revealed that Fluopyram resistance did not increase in Germany and Chile, but instead appears to be in a steady-state at around 10-20% resistant strains (Figure 1 A), probably as a result of a tradeoff between selection and fitness penalty (Veloukas et *al.* 2014; Lalève et *al.* 2014; Bayer internal data). The lack of any reported Fluopyram efficacy failures until now suggests that the proportion of less than 20% Fluopyram-resistant strains observed since several years on a population level (Figure 1 A, B) is of limited practical relevance.

Of the 120 *Botrytis cinerea* isolates from infested soft fruits, only one (1%) Fluopyram-resistant isolate was identified, indicating a population of overall very high Fluopyram sensitivity (Figure 1B). However, in contrast, resistance towards other chemical SDHI sub-classes, e.g. pyridine-carboxamides was found at a much higher proportion (>40%). The reason for this is the high prevalence of B-H272R and B-H272Y mutations in *Botrytis cinerea* isolates from European soft fruits (Figure 1 C, Weber et *al.* 2015, Veloukas et *al.* 2011). Strains carrying these

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sdh mutations are resistant to pyridine-carboxamides, but remain sensitive to Fluopyram due to incomplete SDHI cross-resistance (Table 2, Lalève et *al.* 2014). Interestingly, B-H272R/Y appear also to be the dominating mutant alleles in isolates from Chilean grapes (Figure 1 C).

Table 2 *sdh* mutation spectrum of the 1999 *Botrytis cinerea* isolates analyzed between 2012 (introduction of Fluopyram) and 2015 and the corresponding average EC_{50} values for Fluopyram and a pyridine-carboxamide (PYC) determined *in vitro*.

sdh allele [1)]	Frequency		Average EC_{50} (ppm)	
	[n]	%	PYC	Fluopyram
wild-type	1467	73,4	0,7	1,1
B-P225H	32	1,6	>30	> 29,2 [2)]
B-P225F	38	1,9	>30	>30
B-P225L	3	0,2	>30	>30
B-N230I	63	3,2	13,9	10,6
B-H272Y	66	3,3	>30	0,3
B-H272R	317	15,9	> 27,9 [2)]	1,0
C-P80H	9	0,5	14,1	8,0
C-P80L	3	0,2	3,1	6,5
Unknown	1	0,7	5,1	6,8

[1)] Mutations B-P225T, B-H272L/V, D-H132R and C-A85V reported previously (FRAC 2015) were not identified in our monitoring program.
[2)] Very most strains of this *sdh* genotype have an EC_{50} value of >30 ppm.

Table 2 summarizes all *sdh* mutations identified in our *Botrytis cinerea* monitoring program since the introduction of Fluopyram in 2012. Most abundant mutations were the above-mentioned B-H272R and B-H272Y alleles (15.9% and 3.3%), as well as the mutation B-N230I (3.2%). Strains carrying mutation B-N230I displayed a reduced sensitivity towards Fluopyram, but still were controlled by higher doses of the fungicide. Alleles that mediate Fluopyram resistance were sparse: strains carrying B-P225H, B-P225F and B-P225L were identified at a frequency of 1.6%; 1.9% and 0.2%, respectively. We did not find the previously reported B-P225T, B-H272L/V, D-H132R and C-A85V mutations (FRAC 2015). Interestingly, for 13 out of 1999 isolates we determined slightly increased EC_{50} values for Fluopyram, although our pyrosequencing approach failed to detect any of the previously reported mutations, suggesting that either novel *sdh* mutant alleles or alternative mechanisms not based on target site mutations may account for this reduction of Fluopyram sensitivity. However, full sequencing of *sdh* subunits revealed new mutant alleles for 12 of these isolates, carrying either mutation C-P80H or C-P80L, which, to our knowledge, were not reported previously. Taken together, this survey of *sdh* mutations suggests a rather limited spectrum of mutant alleles present in current *Botrytis cinerea* populations from grapes and soft fruits that are of relevance for Fluopyram.

CONCLUSION

We observed a stable Bixafen sensitivity of *Zymoseptoria tritici* in Europe. Exceptionally, single strains (5 out of 1372; 0.4%) with *sdh* mutations B-N225I, C-T79N and C-N86S were found in 2015, leading to resistance factors for Bixafen of less than 20. Mutation C-H152R

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was not found. Given the low abundance of the identified mutants and their relatively low impact on Bixafen sensitivity, their practical relevance remains to be determined. Thus, further intensive monitoring of SDHI sensitivity of *Zymoseptoria tritici* is indispensable.

In 2015, Fluopyram resistance remained stable on a relatively low level for *Botrytis cinerea* populations from commercial vineyards and soft fruit fields. In fact, mutations B-P225H, B-P225F and B-P225L causing Fluopyram resistance were the rarest amongst all the identified *sdh* mutant alleles. The majority of the identified *sdh* mutant strains (B-H272Y/R) were not resistant to Fluopyram, but to another chemical SDHI sub-class, due to an incomplete cross-resistance. A comprehensive survey of all previously reported *sdh* mutations in the fungal isolate collection analyzed to date in our monitoring program suggests a rather limited spectrum of *sdh* mutant alleles with relevance for Fluopyram.

Taken together, Bixafen and Fluopyram currently encounter highly sensitive *Zymoseptoria tritici* and *Botrytis cinerea* populations, respectively, and therefore are very valuable tools for resistance management, allowing protection of other fungicides with different modes of action.

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