# V Fungicide resistance-related investigations

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**Fungicide resistance of *Zymoseptoria tritici* in Denmark and Sweden**

The resistance level of the wheat pathogen *Zymoseptoria tritici* (*Z. tritici*) against the azoles epoxiconazole and prothioconazole and the SDHI fluxapyroxad was tested *in vitro* to survey the sensitivity of the Danish-Swedish *Z. tritici* population. Each year, leaf samples with apparent symptoms of *Z. tritici* are collected at growth stage 73-77 in collaboration with SEGES, Jordbruksverket in Sweden and local advisors. The resistance testing is carried out at AU Flakkebjerg. In 2019, a total of 209 Danish isolates from 21 sites and 341 Swedish isolates from 31 sites were investigated for sensitivity to prothioconazole-desthio and fluxapyroxad (Tables 1 and 4). The disease pressure was medium to high in 2019.

The sensitivity testing was carried out on microtitre plates. Single pycnidium isolates were used to produce spore suspensions by scraping off six-day-old *Z. tritici* spores and transferring them into Milli-Q water. Spore suspensions were homogenised and adjusted to a spore concentration of $2.4 \times 10^4$ spores $ml^{-1}$. Technical duplicates of each isolate were included in the study. Stock solutions of all three fungicides were made by dissolving the active ingredients (Sigma) in 80% ethanol. Those stock solutions were then utilised to prepare 2 x potato dextrose broth (PDB) mixtures to obtain the following final microtitre plate fungicide concentrations (ppm): 30, 10, 3.3, 1.0, 0.3, 0.1, 0.33, 0 (epoxiconazole), 6.0, 2.0, 0.6, 0.2, 0.07, 0.008, 0.002, 0 (prothioconazole-desthio) and 3.0, 1.0, 0.3, 0.1, 0.03, 0.01, 0.0033, 0 (fluxapyroxad). A total of 100 µl of spore suspension and 100 µl of fungicide solution were added to a 96-deep well microtitre plate. Microtitre plates were wrapped in tinfoil and incubated at 20°C for six days in the dark. Plates were visually analysed in an Elisa reader at 620 nm. Fungicide sensitivities were calculated as the concentration of a fungicidal compound, at which fungal growth *in vitro* is inhibited by 50% ($EC_{50}$) by a non-linear regression (curve fit) using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The isolates IPO323 and OP15.1 were used as reference isolates.

**Results - *Denmark***

Prothioconazole-desthio has been included in the testing since 2016 to replace prothioconazole. In 2019, the average $EC_{50}$ value for the Danish *Z. tritici* isolates with 0.26 ppm was slightly lower than in 2018 (0.33 ppm) (Figure 1; Table 2). The resistance factor (RF; $EC_{50}$ value isolate/$EC_{50}$ value reference isolate) for prothioconazole-desthio was 26 compared to 35 and 32 in the years before. It is difficult to compare results for prothioconazole from previous years, as there are no clear correlations between those two chemical compounds. Furthermore, there was no clear cross-resistance between epoxiconazole and prothioconazole-desthio in previous years. From 2017 to 2018, a significant shift in azole sensitivity took place for epoxiconazole ($EC_{50}$ in 2016: 1.39 ppm; 2017: 1.81 ppm; 2018: 4.52 ppm; Table 2). Only a subset of 18 Danish *Z. tritici* isolates was tested for sensitivity towards epoxiconazole in 2019. No further shift has occurred, and the average $EC_{50}$ for epoxiconazole was lower than in 2018 (2.03 ppm). However, all isolates tested still had an $EC_{50}$ value of > 1 ppm.

The resistance levels of the SDHI fluxapyroxad were at the same low level in 2019 as in 2018 with an average resistance factor of 2, indicating that the Danish *Z. tritici* population remains sensitive towards SDHI fungicides (Table 1; Figure 2).

**Table 1.** Mean $EC_{50}$ values and resistance factors (RF) for prothioconazole-desthio and fluxapyroxad from different sites in 2019 for *Z. tritici* screened.

| Location | | | $EC_{50}$ (ppm) | | | | Number |
|---|---|---|---|---|---|---|---|
| | | | Prothio-desthio | RF | Fluxa | RF | |
| 19-ZT-DK- | 1 | Horsens,LMO | 0.37 | 37 | 0.20 | 1 | 18 |
| 19-ZT-DK- | 2 | Flakkebjerg | 0.14 | 14 | 0.15 | 1 | 20 |
| 19-ZT-DK- | 3 | Sejet | 0.77 | 77 | 0.23 | 1 | 10 |
| 19-ZT-DK- | 4 | Falster | 0.24 | 24 | 0.63 | 4 | 1 |
| 19-ZT-DK- | 5 | Skive | 0.22 | 22 | 0.15 | 1 | 10 |
| 19-ZT-DK- | 6 | Djursland | 0.38 | 38 | 0.06 | 0 | 10 |
| 19-ZT-DK- | 7 | Ringsted | 0.11 | 11 | 0.08 | 1 | 8 |
| 19-ZT-DK- | 8 | Brønderslev | 0.71 | 71 | 0.51 | 3 | 10 |
| 19-ZT-DK- | 9 | Vollerup | 0.11 | 11 | 0.07 | 0 | 2 |
| 19-ZT-DK- | 10 | Odense | 0.23 | 23 | 0.06 | 0 | 10 |
| 19-ZT-DK- | 11 | Vojens | 0.09 | 9 | 0.15 | 1 | 10 |
| 19-ZT-DK- | 12 | Odense | 0.20 | 20 | 0.64 | 4 | 8 |
| 19-ZT-DK- | 13 | Odense | 0.21 | 21 | 0.25 | 2 | 9 |
| 19-ZT-DK- | 14 | Åbenrå | 0.26 | 26 | 1.52 | 9 | 10 |
| 19-ZT-DK- | 15 | Rønnede | 0.09 | 9 | 0.08 | 0 | 9 |
| 19-ZT-DK- | 16 | Ålborg | 0.12 | 12 | 0.27 | 2 | 9 |
| 19-ZT-DK- | 17 | Rønde | 0.13 | 13 | 0.07 | 0 | 10 |
| 19-ZT-DK- | 18 | Rønne | 0.40 | 40 | 0.09 | 1 | 9 |
| 19-ZT-DK- | 19 | Rønne | 0.27 | 27 | 0.21 | 1 | 10 |
| 19-ZT-DK- | 20 | Horsens | 0.10 | 10 | 0.20 | 1 | 10 |
| 19-ZT-DK- | 21 | Spøttrup | 0.44 | 44 | 0.23 | 1 | 7 |
| 19-ZT-DK- | 22 | Vojens | 0.20 | 20 | 0.15 | 1 | 9 |
| Average | | | 0.26 | 26 | 0.27 | 2 | 209 |

**Table 2.** Summary of mean $EC_{50}$ (ppm) values and resistance factors (RF) for epoxiconazole, prothioconazole-desthio and fluxapyroxad assessed for *Z. tritici* in Denmark. The total numbers of isolates tested are given in brackets.

| Year | Epoxiconazole | RF | Prothio-desthio | RF | Fluxapyroxad | RF |
|---|---|---|---|---|---|---|
| 2012 | 0.30 (40) | 15 | - | - | - | - |
| 2013 | 0.36 (133) | 18 | - | - | - | - |
| 2014 | 0.50 (290) | 25 | - | - | - | - |
| 2015 | 0.45 (262) | 17 | - | - | - | - |
| 2016 | 1.39 (220) | 66 | 0.13 (26) | 17 | - | - |
| 2017 | 1.81 (272) | 94 | 0.32 (263) | 32 | - | - |
| 2018 | 4.52 (155) | 212 | 0.33 (155) | 35 | 0.26 (155) | 2 |
| 2019 | 2.03 (18) | 102 | 0.26 (209) | 26 | 0.27 (209) | 2 |
| Ref. IPO323 | 0.02 - 0.03 | - | 0.01 | - | 0.10 - 0.20 | - |

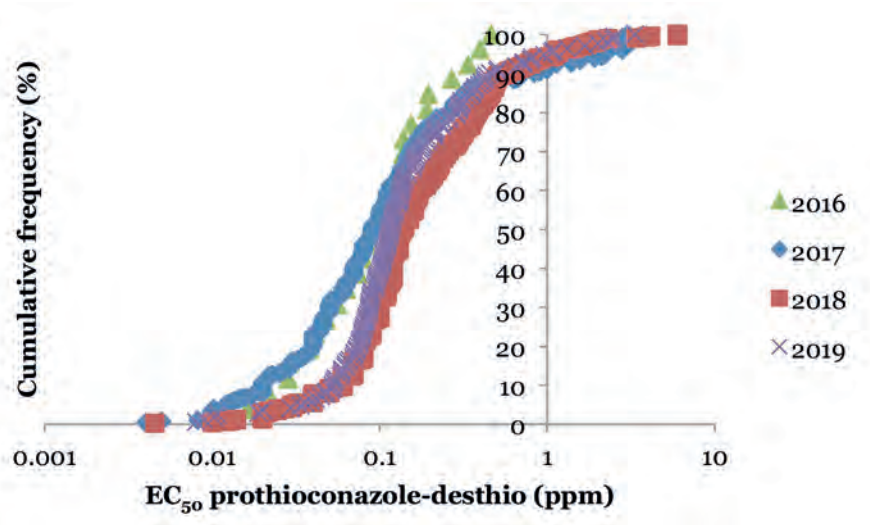


**Figure 1.** Cumulative frequencies of $EC_{50}$ values of prothioconazole-desthio (ppm) for Danish *Z. tritici* populations 2016-2019. Each point of the curve represents a single *Z. tritici* isolate.

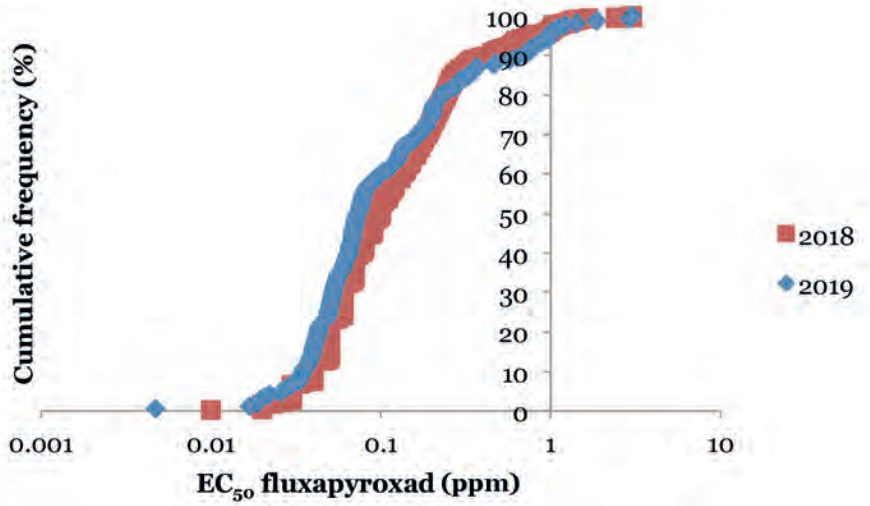


**Figure 2.** Cumulative frequencies of $EC_{50}$ values of fluxapyroxad (ppm) for *Z. tritici* populations in Denmark in 2018 and 2019.

**Table 3.** Summary of measured $EC_{50}$ (ppm) values and resistance factors (RF) for epoxiconazole, prothioconazole-desthio and fluxapyroxad assessed for *Z. tritici* in Sweden. The total numbers of isolates tested are shown in brackets.

| Year | Epoxiconazole | RF | Prothio-desthio | RF | Fluxapyroxad | RF |
|---|---|---|---|---|---|---|
| 2012 | 0.36 (211) | 18 | - | - | - | - |
| 2013 | 0.65 (170) | 33 | - | - | - | - |
| 2014 | 0.27 (337) | 35* | - | - | - | - |
| 2015 | 0.33 (227) | 12 | - | - | - | - |
| 2016 | 0.52 (212) | 24 | - | - | - | - |
| 2017 | 3.17 (163) | 170 | 0.58 (150) | 71 | - | - |
| 2018 | 4.53 (127) | 181 | 0.35 (127) | 35 | 0.19 (127) | 2 |
| 2019 | 1.15 (25) | 58 | 0.17 (341) | 17 | 0.09 (341) | 1 |
| Ref. IPO323 | 0.02 - 0.03 | - | 0.01 | - | 0.10 - 0.20 | - |

**Table 4.** Results from individual sites in Sweden with data from sensitivity testing for *Z. tritici* tested for prothioconazole-desthio and fluxapyroxad.

| Location | | | $EC_{50}$ | | | | |
|---|---|---|---|---|---|---|---|
| | | | Prothio-desthio | R factor | Fluxa | R factor | Number |
| 19-ZT-SWE- | 1 | Skövde | 0.03 | 3 | 0.06 | 0 | 20 |
| 19-ZT-SWE- | 2 | Motala | 0.06 | 6 | 0.19 | 1 | 20 |
| 19-ZT-SWE- | 3 | Simrishamn | 0.21 | 21 | 0.04 | 0 | 20 |
| 19-ZT-SWE- | 4 | Smedby, Kalmar | 0.04 | 4 | 0.03 | 0 | 10 |
| 19-ZT-SWE- | 5 | Vickleby, Färjestaden | 0.06 | 6 | 0.03 | 0 | 10 |
| 19-ZT-SWE- | 6 | Albrunna, Degerhamn | 0.07 | 7 | 0.06 | 0 | 8 |
| 19-ZT-SWE- | 7 | Nybble, Örebro | 0.06 | 6 | 0.08 | 0 | 9 |
| 19-ZT-SWE- | 8 | Julita, Äsköping | 0.11 | 11 | 0.04 | 0 | 10 |
| 19-ZT-SWE- | 9 | Skrukeby, Mjölby | 0.03 | 3 | 0.04 | 0 | 10 |
| 19-ZT-SWE- | 10 | St. Åby, Ödeshög | 0.59 | 59 | 0.09 | 1 | 9 |
| 19-ZT-SWE- | 11 | Glyttinge, Linköping | 0.11 | 11 | 0.12 | 1 | 10 |
| 19-ZT-SWE- | 12 | Förråd, Linghem | 0.08 | 8 | 0.10 | 1 | 10 |
| 19-ZT-SWE- | 13 | Skälsund, Norrköping | 0.21 | 21 | 0.14 | 1 | 10 |
| 19-ZT-SWE- | 14 | Ullekalv, Skänninge | 0.04 | 4 | 0.14 | 1 | 10 |
| 19-ZT-SWE- | 15 | Germundsgård, Nossebro | 0.06 | 6 | 0.17 | 1 | 10 |
| 19-ZT-SWE- | 16 | Baggård, Grästorp | 0.36 | 36 | 0.09 | 1 | 8 |
| 19-ZT-SWE- | 17 | Emtunga Gård, Vara | 0.32 | 32 | 0.10 | 1 | 10 |
| 19-ZT-SWE- | 18 | Heljerud, Brålanda | 0.60 | 60 | 0.13 | 1 | 10 |
| 19-ZT-SWE- | 19 | Hedegård, Mellerud | 0.49 | 49 | 0.12 | 1 | 8 |
| 19-ZT-SWE- | 20 | Forsby, Skövde | 0.42 | 42 | 0.17 | 1 | 6 |
| 19-ZT-SWE- | 21 | Lilla Vallskog, Uppsala | 0.03 | 3 | 0.04 | 0 | 10 |
| 19-ZT-SWE- | 22 | Hagby | 0.02 | 2 | 0.02 | 0 | 10 |
| 19-ZT-SWE- | 23 | Sigtuna, Stockholm | 0.03 | 3 | 0.03 | 0 | 10 |
| 19-ZT-SWE- | 24 | Folingbo, Visby | 0.03 | 3 | 0.05 | 0 | 10 |
| 19-ZT-SWE- | 25 | Kattarp, Helsingborg | 0.11 | 11 | 0.36 | 2 | 10 |
| 19-ZT-SWE- | 26 | Vallby, Trelleborg 1 | 0.60 | 60 | 0.16 | 1 | 10 |
| 19-ZT-SWE- | 27 | Löderup, Ystad | 0.16 | 16 | 0.09 | 1 | 9 |
| 19-ZT-SWE- | 28 | Hviderup, Eslöv | 0.12 | 12 | 0.06 | 0 | 8 |
| 19-ZT-SWE- | 29 | Smedstrorp, Tomelilla | 0.14 | 14 | 0.04 | 0 | 8 |
| 19-ZT-SWE- | 30 | Bösild, Halmstad | 0.07 | 7 | 0.06 | 0 | 10 |
| 19-ZT-SWE- | 31 | Brunnby, Västerås | 0.05 | 5 | 0.02 | 0 | 9 |
| 19-ZT-SWE- | 32 | Väsby, Tierp | 0.03 | 3 | 0.03 | 0 | 9 |
| 19-ZT-SWE- | 33 | Haga, Enköping | 0.27 | 27 | 0.02 | 0 | 10 |
| Average | | | 0.17 | 18 | 0.09 | 1 | 341 |

**Results - *Sweden***

As in Denmark, a significant shift in $EC_{50}$ values for epoxiconazole took place in 2017. In 2018, the sensitivity towards this active ingredient continued to decrease ($EC_{50}$ in 2018: 4.53 ppm; 2017: 3.17 ppm; Tables 3-4). In 2019, the resistance level was lower; however, the mean $EC_{50}$ value was still above 1 ppm. $EC_{50}$ values for prothioconazole-desthio were with an average of 0.17 ppm slightly lower in Sweden in 2019 than in previous years (Figure 3; Table 3) and lower than Danish populations in 2019 (0.26 ppm). The results varied among sites (0.03-0.60 ppm). However, in 2019 the $EC_{50}$ were more similar across the country compared to previous years (Table 4). The results for fluxapyroxad were in line with the Danish results (Figure 4) with an average resistance factor of 1.

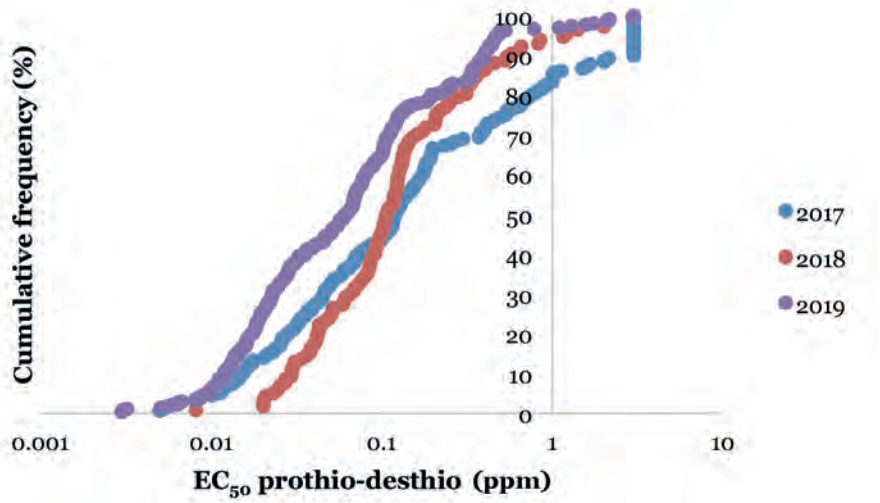


**Figure 3.** Cumulative frequencies of $EC_{50}$ values of prothioconazole-desthio (ppm) for *Z. tritici* populations in Sweden in 2017-2019.

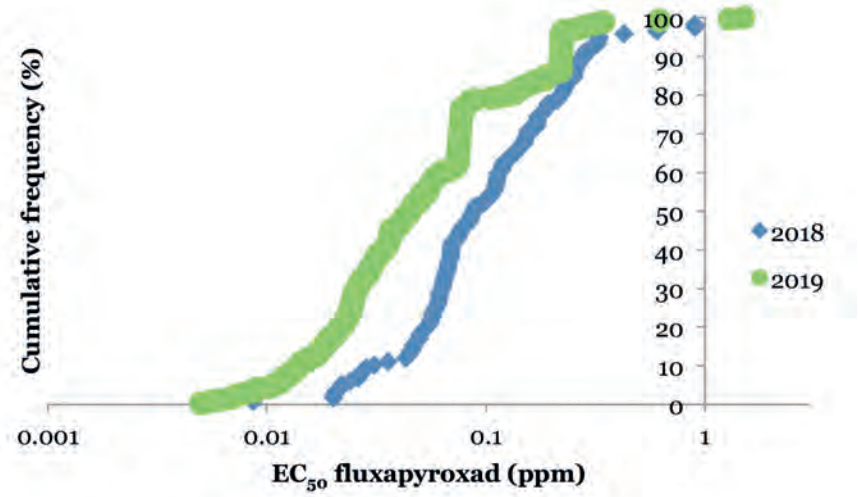


**Figure 4.** Cumulative frequencies of $EC_{50}$ values of fluxapyroxad (ppm) for *Z. tritici* populations in Sweden in 2018 and 2019.

### The sensitivity of difenoconazole and tebuconazole

A subset of 50 *Z. tritici* isolates from Denmark and Sweden was tested for sensitivity to the azoles tebuconazole and difenoconazole. The resistance level for tebuconazole has been at a high level for many years. In 2019, the average $EC_{50}$ value was 6.79 ppm (2018: 6.21 ppm) with single isolates ranging from 0.15 to 30.00 ppm. The average $EC_{50}$ was higher in Denmark (8.20 ppm) than in Sweden (5.91 ppm). The average RF for tebuconazole was > 1000 (reference isolate IPO323: 0.006 ppm). Those values were in line with results from 2018 where the average $EC_{50}$ for *Z. tritici* from Denmark and Sweden was 6.21 ppm with an average RF of > 1000. $EC_{50}$ values for difenoconazole ranged from 0.01 to 0.50 ppm, with an average $EC_{50}$ value of 0.08 ppm and a resistance factor of 10, indicating the presence of a few slightly adapted isolates in the Scandinavian *Z. tritici* population.

### *CYP51* mutations in the Danish-Swedish *Z. tritici* populations 2019

The decline of azole effectivity has been linked to molecular changes in the target gene *CYP51*. In 2019, single isolates from Denmark and Sweden were analysed by Sanger sequencing and qPCR (KASP) for the frequency of the essential *CYP51* mutations in *Z. tritici*: D134G, V136A/C, I381V and S524T (Figure 5). Mutation I381V continued to dominate throughout the region and was present in frequencies of

90-100%. The frequencies for mutations D134G, V136A/C and S524T, all of which have emerged in the past ten years in the Northern European *Z. tritici* population, varied from 6% to 66%. The evolution of *CYP51* mutations in Denmark is illustrated in Figure 5.

Compared to 2018 and in recent years, the frequencies in 2019 remain more and less at the same level. *Z. tritici* populations in the Baltic countries and Finland begin to resemble those in Denmark and Sweden, indicating that the evolution in the *CYP51* gene has reached the north-eastern parts of Europe (data not shown).

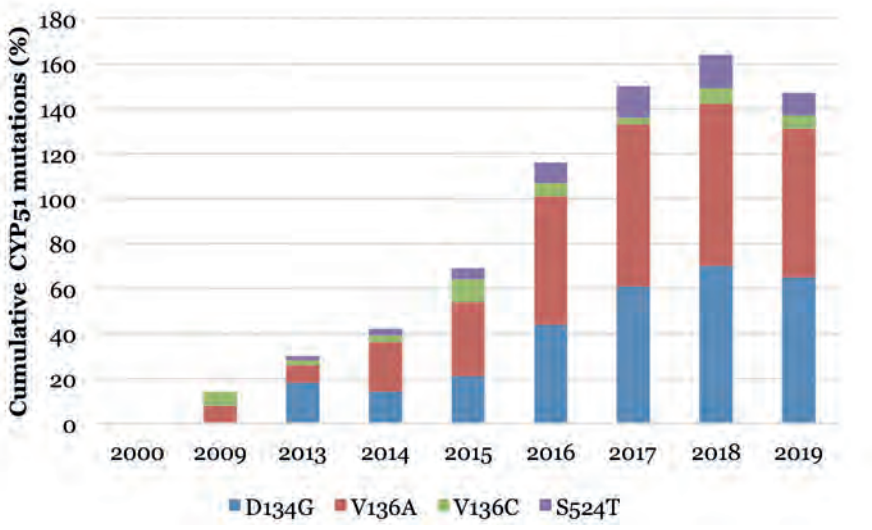


**Figure 5.** Cumulative frequencies of CYP51 mutations D134G, V136A/C, and S524T for the Danish *Z. tritici* populations 2000-2019.

### *Sdh* mutations conferring resistance to SDHI fungicides

Several point mutations in the *Sdh* subunits have been associated with high $EC_{50}$ values. In 2017 and 2018, the first isolates harbouring the C-T79N mutation were found in Denmark. In Sweden, both in 2017 and 2018, a few isolates were tested positive for the presence of the C-N86S mutation. Again in 2019, single isolates were found with C-T79N in Denmark. It must be stated that *Sdh* mutations exist in the Danish-Swedish *Z. tritici* populations; however, at very low frequencies, and with no field impact yet.

### Strobilurin and SDHI resistance in net blotch

In 2019, a total of 19 leaf samples with net blotch (*Pyrenophora teres*) symptoms were collected. The samples were collected by AU Flakkebjerg, SEGES and Jordbruksverket and originated from untreated field trials and farmers' fields. Twelve samples came from Danish fields, seven samples from Swedish fields.

As in previous years, BASF carried out an investigation for point mutation, associated with fungicide resistance. The effect of the F129L mutation on strobilurin field performance is only a partial effect. The data from Denmark show that the level of F129L has remained stable and has not changed. Overall, F129L was found in 83% of all Danish samples. The majority harboured the mutation with < 60%. Furthermore, seven Swedish samples were investigated, three of which were tested negative for F129L, two with < 20% and three with a frequency between 20 and 60% (Table 5).

No *Sdh* mutations were found, with the exception of one locality in Denmark where D-D145G was found.

**Table 5.** Incidence of the F129L mutation in Danish net blotch samples.

| Year | No. of samples | No. without F129L | No. with 1-20% | No. > 20-60% | No. > 60% | No. of samples with F129L % |
|---|---|---|---|---|---|---|
| 2008 | 20 | 9 | 5 | 3 | 3 | 55 |
| 2009 | 44 | 18 | 7 | 13 | 6 | 59 |
| 2010 | 16 | 5 | 3 | 7 | 1 | 69 |
| 2011 | 34 | 13 | 4 | 12 | 5 | 62 |
| 2012 | 19 | 14 | 1 | 2 | 2 | 24 |
| 2013 | 25 | 17 | 2 | 4 | 2 | 32 |
| 2014 | 20 | 13 | 2 | 3 | 2 | 35 |
| 2015 | 8 | 3 | 0 | 3 | 0 | 38 |
| 2016 | 20 | 9 | 3 | 8 | 0 | 55 |
| 2017 | 20 | 2 | 4 | 2 | 2 | 80 |
| 2019 | 12 | 2 | 3 | 3 | 4 | 83 |

### Genetic analysis of QoI-resistance of *P. tritici-repentis* 2019

Two *P. tritici-repentis* samples from Flakkebjerg were tested for QoI mutations. The cytb G143A mutation was present in both samples with frequencies of approx. 80%.