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Sensitivity of *Sclerotinia sclerotiorum* to strobilurin fungicides in Slovakia

Monika Tóthová, Kamil Hudec, Peter Tóth*

Department of Plant Protection, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture in Nitra, Nitra, Slovakia
**Corresponding author: monika.tothova@uniag.sk*

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Abstract: Rapeseed isolates of *Sclerotinia sclerotiorum* (Lib.) de Bary 1884 from the Nitra Region of Slovakia were investigated for their *in vitro* sensitivity to azoxystrobin and picoxystrobin; and for determining their EC_{50} value. The growth of *S. sclerotiorum* was evaluated on a PDA medium amended with the selected fungicide´s active ingredient at 4 different concentrations – 0.08, 0.83, 8.33, and 83.30 ppm. The overall mean EC_{50} values for azoxystrobin and picoxystrobin were 2.73 ppm and 3.12 ppm, respectively. The majority of the isolates had resistance factors up to 20, which suggests a shift in the *S. sclerotiorum* population sensitivity towards the resistance.

Keywords: azoxystrobin; EC_{50}; mycelial growth inhibition; rapeseed; picoxystrobin; *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary 1884, the causal agent of the sclerotinia stem rot in rapeseed, is one of the most economically damaging pathogens (Huszár 2011). The current control of *S. sclerotiorum* in rapeseed fields is carried out by fungicides during the flowering phase (BBCH 61–69) when no infection is visible (Bečka *et al*. 2011). An application date at the growth stage of the flowering did not play an important role in the case of the fungicides with a combination of active ingredients from the newly synthesised triazoles and strobilurins. The older fungicides based on older triazoles and Sterol biosynthesis inhibitor (SBI) fungicides had a greater influence on the yield when applied during BBCH 65–69 (Spitzer *et al*. 2012).

Azoxystrobin and picoxystrobin are strobilurin quinoe outside inhibitors (Qol) fungicides belonging to the same chemical class – methoxy-acrylates (FRAC 2018). These fungicides only exhibit a single site activity, and their propensity to develop resistance is relatively high (Derbyshire & Denton-Giles 2016). Up to now, 42 pathogens species have been reported to already have developed resistance to strobilurins from fungal classes such as Ascomycetes, Deuteromycetes, Basidiomycetes and Oomycetes (Ishii & Holloman 2015). The resistance of *S. sclerotiorum* or any other pathogens to strobilurins in rapeseeds has not been reported (FRAC 2013).

Monitoring of the pathogen sensitivity to the fungicides has an important value in resistance management strategies. Thus, the objective of this study was to determine the sensitivity of *S. sclerotiorum* isolates from rapeseed fields of the Nitra Region in Slovakia based on the mycelium growth inhibition rate and the EC_{50} (the half maximal effective concentration) calculation. This study is the first one dealing with the monitoring of *S. sclerotiorum's* sensitivity to two single component strobilurin fungicides in Slovakia.

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MATERIAL AND METHODS

Sampling and isolation of the fungus. During the growing season in 2015, rapeseed fields were sampled at five different localities in the Nitra Region of Slovakia. The samplings were conducted during the flowering and ripening stages. The petals and infected stem tissues were transferred to Petri dishes with a potato-dextrose agar medium (PDA). *S. sclerotiorum* purification was carried out by using the hyphal tip culture technique. Six strains (3 from the petals and 3 from the stems) per each sampling site were selected for investigation of the *S. sclerotiorum in vitro* sensitivity to 2 different active ingredients.

Sensitivity of S. sclerotiorum to the selected active ingredients. The effect of azoxystrobin (Amistar 250 g/l SC, Syngenta) and picoxystrobin (Acanto 250 g/l SC, DuPont™) on the growth of *S. sclerotiorum* was evaluated on a PDA medium amended with the selected fungicide's active ingredient at 4 different concentrations 0.08, 0.83, 8.33, and 83.30 ppm (representing 0.01; 0.1; 1; and 10% of the recommended doses). The control media was amended by distilled water. Each treatment was tested in 3 replications. The rate of the mycelium growth inhibition was calculated according to the formula of Pandey *et al.* (1982).

In the fully inhibited isolates, the fungicidal or fungistatic activity of the active ingredients were evaluated. The fungicidal effect was verified by the transfer of the fungus from the poisoned PDA plates to the plates without the fungicidal component.

The resistance factor was expressed as the EC_{50} of the least sensitive/most sensitive isolates. Isolates with a resistance factor of less than 2 were classified as sensitive (Leroux *et al*. 2010).

Statistical analyses. The EC_{50} value was determined by the linear regression method with logarithmically transformed concentration data. All the obtained data were subjected to the ANOVA and the means were compared using Fisher's least significant difference (LSD) procedure ($P \leq 0.05$). The mean EC_{50} values between the two active ingredients – azoxystrobin and picoxystrobin, were compared using a *t*-test, the medians were compared by a Mann-Whitney *W*-test.

RESULTS

The highest final concentration (83.30 ppm) of both tested active ingredients completely suppressed the growth of 23 out of 30 *S. sclerotiorum* isolates. The effect of both active ingredients were mostly fungistatic, a fungicidal effect was determined in 5 isolates (3 isolates per active ingredient). The less sensitive isolate exhibited 61.94 and 61.70% inhibition of the mycelial growth on the media amended by azoxystrobin and picoxystrobin, respectively (Table 1).

Table 1. The mean inhibition of the mycelial growth (%) of the *Sclerotinia sclerotiorum* rapeseed isolates from the stems and flower petals on the PDA medium amended with azoxystrobin and picoxystrobin at concentrations of 83.30; 8.33; 0.83 and 0.08 ppm under *in vitro* conditions

Isolate from	Concentration (ppm)							
	83.30		8.33		0.83		0.08	
	inhibition (%)	SD	inhibition (%)	SD	inhibition (%)	SD	inhibition (%)	SD
Azoxystrobin								
Petals (n = 15) (min.–max.)	97.67[a] (85.16–100.00)	5.16	81.24[a] (46.62–100.00)	16.03	27.78[a] (4.94–74.21)	18.38	10.70[a] (2.43–37.35)	10.52
Stems (n = 15) (min.–max.)	95.73[a] (61.94–100.00)	10.51	79.94[a] (51.40–100.00)	17.68	30.98[a] (7.37–91.40)	25.67	9.68[a] (1.00–28.23)	8.64
Total (n = 30)	96.70	8.29	80.59	16.79	29.38	22.26	10.19	9.59
Picoxystrobin								
Petals (n = 15) (min.–max.)	96.09[a] (72.00–100.00)	10.08	74.95[a] (18.69–100.00)	25.75	51.29[a] (13.06–73.63)	18.54	11.50[a] (1.15–44.55)	12.21
Stems (n = 15) (min.–max.)	95.72[a] (61.70–100.00)	11.21	78.32[a] (35.56–100.00)	22.34	54.35[a] (28.05–90.74)	23.06	7.75[a] (0.51–31.84)	8.78
Total (n = 30)	95.90	10.60	76.63	24.03	52.82	20.86	9.63	10.74

Inhibition – the different letters indicate significant differences according to Fisher's LSD test ($P \leq 0.05$); SD – the standard deviation

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Table 2. The azoxystrobin and picoxystrobin concentrations for 50% inhibition (EC_{50}) of the mycelial growth of *Sclerotinia sclerotiorum* isolated from the flower petals and stems of the rapeseed grown in the Nitra Region, Slovakia

Isolate from	Azoxystrobin			Picoxystrobin		
	EC_{50} (ppm)	SD	RF	EC_{50} (ppm)	SD	RF
Petals (n = 15) (min.–max.)	2.29^{a*} (0.19–6.03)	1.47	1.27–31.74	3.24^{a*} (0.10–22.43)	5.75	4.90–224.30
Stems (n = 15) (min.–max.)	3.18^{a*} (0.15–15.56)	3.81	4.95–103.73	3.01^{a*} (0.11–18.60)	6.55	2.18–169.09
Total mean (n = 30)	2.73^{a**}	2.90	1.27–103.73	3.12^{a**}	6.13	1.10–224.3
Total median (n = 30)	1.99^{a***}	2.79		1.23^{a***}	5.15	

The different letters indicate significant differences according to –* Fisher's LSD test ($P \leq 0.05$) in the columns; ** t-test in the row; *** Mann-Whitney W-test in the row; SD – the standard deviation; RF – the resistance factor

At the concentration of 8.33 ppm, the growth of 2 isolates cultivated on the PDA medium amended with azoxystrobin was completely inhibited, while the mycelium growth on the PDA medium amended by picoxystrobin was fully inhibited in 6 isolates. The less sensitive isolate exhibited 46.62 and 18.69% inhibition of the mycelial growth on the media amended by azoxystrobin and picoxystrobin, respectively (Table 1).

The sensitivity of the individual isolates was highly variable at the active ingredient concentration of 0.83 ppm. The inhibitory effect ranged between 4.94 and 91.40% among the isolates tested for azoxystrobin (Table 1); and from 13.06 to 90.74% between the isolates tested for picoxystrobin (Table 1).

At the lowest concentration (0.08 ppm), the mycelial growth was comparable, azoxystrobin supressed the growth of the *S. sclerotiorum* isolates by 1.00–37.35% (Table 1) and the suppression by picoxystrobin ranged between 0.51–44.55% (Table 1).

The total average inhibition rates of azoxystrobin and picoxystrobin were comparable in almost all tested concentrations, except that of 0.83 ppm. At this concentration, the inhibition rate of picoxystrobin (52.82%) was much higher than that of azoxystrobin (29.38%). By comparing the inhibition rates between the isolates from the flower petals and stems, significant differences were not observed (Table 1).

The EC_{50} values for azoxystrobin varied from 0.15–15.56 ppm with an overall mean at 2.73 ppm. For picoxystrobin, the EC_{50} values varied from 0.11–22.43 ppm with an overall mean at 3.12 ppm. The EC_{50} values of the isolates from the flower petals and stems were not significantly different for both azoxystrobin and picoxystrobin. The median was higher for azoxystrobin (1.99 ppm) than for picoxystrobin (1.23 ppm). The differences between the azoxystrobin and picoxystrobin EC_{50} means and medians were not significant. The resistance factors (RF) ranging from 1.27–103.73 for azoxystrobin and 1.10–224.30 for picoxystrobin suggest a shift in the *S. sclerotiorum* population sensitivity towards the resistance (Table 2). Both histograms were unimodal, the EC_{50} of the majority of the isolates were up to 3.00 ppm for azoxystrobin and 2.00 ppm for picoxystrobin (Figure 1) with a RF up to 20.00.

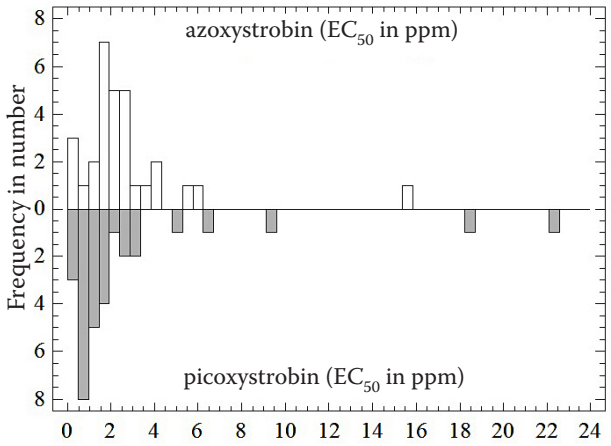

Figure 1. The frequency distribution (in numbers) of the *Sclerotinia sclerotiorum* isolates from the rapeseed grown in the Nitra Region, Slovakia according to their mycelial sensitivity to azoxystrobin and picoxystrobin. The sensitivity is expressed as the EC_{50} in ppm

DISCUSSION

The concentration of 83.30 ppm completely suppressed the growth of most isolates. Some isolates were found to be fully inhibited even at a 10-fold

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lower concentration. Although the mycelial growth of most isolates was fully suppressed under the *in vitro* conditions, the fungicidal effect was recorded in only 5 isolates exposed to azoxystrobin and picoxystrobin. Considering that azoxystrobin and picoxystrobin have been used since 1998 and 2004, respectively (Commission Implementing Regulation (EU) No. 540:2011), we assume that the tested isolates were previously exposed to strobilurins and, thus, several less-sensitive strains of the *S. sclerotiorum* population emerged.

We found considerable diversity in the obtained data concerning the activity of each active ingredient to the mycelial growth. Some possible reasons for these discrepancies could include genotypic and phenotypic variability among the different isolates within a distance of 30 km, since the *S. sclerotiorum* epidemics are initiated by both intrinsic and extrinsic sources of ascospores. Most of the primary ascospores infect flower petals in a 100-meter radius from the apothecia (Adams & Ayres 1979). However, ascospores can be carried on an air current over much longer distances (Williams & Stelfox 1980).

The overall mean EC_{50} value for azoxystrobin was 2.73 ppm (0.15 to 15.56 ppm). Several isolates were found to be very sensitive, their EC_{50} values were comparable to the EC_{50} determined by the *in vitro* inhibition of the mycelial growth in China (0.11–0.62 ppm) (Duan *et al.* 2012) or in Australia (0.006–0.30 ppm) (Hailstones 2011). In north central US, the overall mean azoxystrobin EC_{50} of the isolates was 1.01 ppm for the mycelium (Munoz 2016). Very sensitive isolates of *S. sclerotiorum* have been found in the main European rapeseeds producing countries (UK, Germany, France, and Poland), with an overall mean at 0.16 ppm (Matysiak *et al.* 2011).

For picoxystrobin, the range of the EC_{50} values of the isolates tested in the current study varied from 0.11 to 22.43 ppm with an overall mean at 3.12 ppm. None of the isolates were found to be as sensitive as the *S. sclerotiorum* isolates collected during 2006–2007 in Europe from the main rapeseeds producing countries with an overall mean EC_{50} at 0.02 ppm. Each EC_{50} for picoxystrobin was statistically lower than those obtained for the same isolate with azoxystrobin (Matysiak *et al.* 2011). In our study, we found that the EC_{50} was lower for azoxystrobin than that for picoxystrobin in 6 isolates.

Our study showed that the overall mean EC_{50} of all 30 tested isolates was higher for picoxystrobin (3.12 pm) than for azoxystrobin (2.73 ppm). Because the mean values are more sensitive to extreme values, the median could describe each sample group better than their arithmetic mean. In contrary to the means, the median EC_{50} value of all the tested isolates was higher for azoxystrobin (1.99 ppm) than for picoxystrobin (1.23 ppm). In north central US, the overall azoxystrobin EC_{50} median of the isolates collected between 2000 and 2008 was at 0.57 ppm for the mycelium without any significant change since its introduction to the market (Munoz 2016). The median of the EC_{50} of isolates collected in 2015 in the Nitra Region was 3.49 times higher. We assume that geographically isolated populations may display genetic and phenotypic differentiations due to differential selection resulting from environmental variations and differences in crop rotation and control practices (Attanayake *et al.* 2013).

The EC_{50} values between the isolates from the flower petals and stems were not significant for both azoxystrobin and picoxystrobin. Since the stem infections of the rapeseed originate from the flower petals, we did not expect any differences in the values of the EC_{50} or the mycelial growth inhibition.

Since there is no information on the baseline sensitivity of *S. sclerotiorum* in Slovakia, the EC_{50} values of the most sensitive isolates could be considered akin to the baseline sensitivity. The ratios between the EC_{50} values of the least and most sensitive isolates varied for azoxystrobin by 103.73-fold and for picoxystrobin by 224.30-fold. The majority of our isolates showed an RF of up to 20.00. Leroux *et al.* (2010) considered isolates with RF values between 10 and 100 as moderately resistant. Isolates with a resistance factor of less than 2 were classified as sensitive (Delp & Deckker 1985; Leroux *et al.* 2010). In this study, an RF of less than 2 was observed in 3 isolates tested to azoxystrobin, and 2 isolates tested to picoxystrobin. Currently 29 QoI fungicides (azoxystrobin, dimoxystrobin) are authorised for the control of *sclerotinia* stem rot in rapeseeds in Slovakia (ÚKSÚP 2019). Of the 27 azoxystrobin-based fungicides, 12 are single components with a high risk of resistance development (according the FRAC 2018). Two picoxystrobin-based fungicides were authorised until October 2017. In the literature, there is only a little information on the field efficacy of the single component fungicides used in this study. The result of the field efficacy of azoxystrobin (Bradley *et al.* 2006; Matysiak *et al.* 2011) and picoxystrobin (Matysiak *et al.* 2011) suggests the effective reduction of *sclerotinia* stem rot incidences. At present, there is no evidence of reduced field performance of the QoIs used alone, but their

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use in a co-formulation with a mixture partner with a different mode of action, should help to prevent the future erosion of their activity.

Further research on the factors affecting the *S. sclerotiorum* sensitivity and the distribution of the isolates with declined sensitivity throughout Slovakia could prove beneficial towards the fundamental understanding of these results.

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