https://doi.org/10.17221/177/2021-PPS

Evaluation of the sensitivity of *Leptosphaeria maculans* isolates causing phoma stem canker in oilseed rape in the Czech Republic to boscalid and dimoxystrobin fungicides

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Citation: Fajemisin O., Mazáková J., Ryšánek P. (2022): Evaluation of the sensitivity of *Leptosphaeria maculans* isolates causing phoma stem canker in oilseed rape in the Czech Republic to boscalid and dimoxystrobin fungicides. Plant Protect. Sci., 58: 305–314.

Abstract: Phoma stem canker, caused by *Leptosphaeria* spp. is generally managed with demethylation inhibitor (DMI) fungicides in the Czech Republic. However, there have been reports of resistance to DMIs in *L. maculans* populations. Fungicide resistance management recommends the application of fungicides with different modes of action, either as mixtures or in rotation with other fungicide classes. The objective, therefore, was to evaluate the efficacy of boscalid, dimoxystrobin and a mixture of boscalid and dimoxystrobin, belonging to the succinate dehydrogenase inhibitor and quinone outside inhibitor fungicide groups, respectively, on *L. maculans* isolates. A total of 41 and 285 isolates were tested using the mycelial growth and microtitre plate assays, respectively. The EC_{50} values for the mycelial growth plate method ranged from 0.026–0.984 µg/mL and 0.097–1.653 µg/mL for boscalid and boscalid + dimoxystrobin, respectively. For dimoxystrobin, the EC_{50} range was wide, between 0.053–95.59 µg/mL. The EC_{50} values for the microtitre plate assay ranged from 0.001 496–0.836 3 µg/mL and 7.3×10^{-4}–2.801 µg/mL for boscalid and dimoxystrobin, respectively. The results also showed that the *L. maculans* conidia were more sensitive to boscalid and dimoxystrobin than mycelia.

Keywords: fungicide resistance; blackleg disease; DMI; SDHI; QoI

Leptosphaeria maculans (Desm) Ces & de Not. 1863 [anamorph *Phoma lingam* (Tode ex. Fr.) Desm. 1849] and *Leptosphaeria biglobosa* Shoemaker & H. Brun 2001 are Dothideomycete fungi causing phoma stem canker (also termed blackleg disease) in oilseed rape plants (Shoemaker & Brun 2001; Fitt et al. 2006). This disease is responsible for losses worth more than 900M USD per cropping season (Fitt et al. 2006; Fitt et al. 2008), especially in regions like Canada, Europe, and Australia (West et al. 2001; Howlett 2004). A survey of oilseed rape producing areas in the Czech Republic between 2007 and 2011 showed the presence of both *L. maculans* and *L. biglobosa* species (Mazáková et al. 2017). Mainly, the weather, geographic region, cultivar, and control measures deployed determine the extent of the yield loss and epidemic severity (West et al. 2001; Huang et al. 2007; Ghanbarnia et al. 2009). The management of this disease includes a good crop rotation system, stubble management, use of resistant cultivars, and fungicides (Zhou et al. 1999; Balesdent et al. 2001; West et al. 2002; Gladders et al. 2006; Steed et al. 2007; Huang et al. 2011). These measures help reduce the amount

Supported by the Ministry of Agriculture of the Czech Republic within the framework of The National Agency for Agricultural Research (Projects No. QJ1310227 and No. QK1710397).

https://doi.org/10.17221/177/2021-PPS

of the inoculum, pathogen colonisation, pathogen multiplication, and disease severity. Generally, fungicides are recommended for use in crops where the inoculum level is high and cultivar resistance is low (West et al. 2001). Depending on the region, the chemical control of these pathogens involves the use of a different combination of seed treatments, soil, and foliar fungicides. In Australia, foliar fungicides, in addition to fungicide amended fertilizer, and seed treatments are the main chemical control methods (Marcroft & Potter 2008; Van de Wouw et al. 2021). In Canada, fungicide control measures include seed treatments and foliar fungicides (Fraser et al. 2016). In Europe, however, only foliar fungicides are currently registered for use in the control of phoma stem canker (West et al. 1999; Fitt et al. 2006).

In the Czech Republic, demethylation inhibitor (DMI) fungicides, which include triazoles and imidazoles are one of the most commonly used fungicides in controlling phoma stem canker (Czech Statistical Office, CSO 2021). They are inexpensive and effective against a broad range of plant pathogens (Russell 1995). Fungicides are typically applied in the autumn. They may also be applied in the spring if the oilseed rape is intensively planted. DMI fungicides work by targeting the cytochrome P450 enzyme 14-α-demethylase encoded by the *CYP51* gene. The cytochrome P450 enzyme 14-α-demethylase is important in converting lanosterol to ergosterol. Treatment with these fungicides depletes the amount of ergosterol in the cell, which, in conjunction with an accumulation of 14-α-demethylase sterols, disrupts the membrane structure, preventing active membrane transport resulting in fungistasis (Parker et al. 2014; Price et al. 2015). However, repeated use of single-site fungicides over several years has led to the development of fungicide resistance in many fungal pathogens, leading to a reduced or loss of fungicide efficacy (Brent & Hollomon 2007). This loss of efficacy has been seen in *L. maculans* populations in Australia (Van de Wouw et al. 2017; Yang et al. 2020) and in the Czech Republic (Fajemisin et al. 2022).

In addition to DMI fungicides, boscalid and dimoxystrobin are also registered for use in the Czech Republic against oilseed rape diseases. They belong to the succinate dehydrogenase inhibitor (SDHI) and quinone outside inhibitor (QoI) fungicide classes, respectively (FRAC 2021). SDHI fungicides have protectant, systemic, and translaminar activity depending on the host and pathogen. They inhibit fungal respiration by blocking the binding sites of ubiquinone (UQ binding site) within the mitochondrial complex II (Avenot & Michailides 2010; Sierotzki & Scalliet 2013). Unlike first-generation SDHI fungicides, second generation SDHIs, such as boscalid and fluopyram, have a broad spectrum of activity against plant pathogens (Oliver & Hewitt 2014). QoI fungicides also have protectant and translaminar activity. They inhibit the mitochondrial respiration by binding at the Qo site of cytochrome *b* (CYTB). Cytochrome *b* is a part of the cytochrome *bc* complex located inside the mitochondrial membrane of the fungi and other eukaryotes (Bartlett et al. 2002; Fisher & Meunier 2008). When one of the inhibitors binds, it blocks the electron transfer between cytochrome *b* and cytochrome c_1, which then disrupts the energy cycle within the fungus by halting the production of adenosine triphosphate. SDHI and QoI fungicides both inhibit the fungal respiration. They are also very good at inhibiting spore germination in some fungi, which is an energy-demanding stage in the fungal life cycle (Bartlett et al. 2002).

To delay the chances of resistance developing in fungal populations, it is recommended that the application of fungicides be with different modes of action, either on a rotating schedule or in mixtures (Staub 1991; Brent 1995; Brent & Hollomon 2007). The objectives of this study, therefore, were (1) to evaluate the efficacy of boscalid (SDHI) and dimoxystrobin (QoI) on *L. maculans* isolates collected in the Czech Republic and (2) to compare the inhibition efficacy of boscalid and dimoxystrobin on the *L. maculans* mycelia growth and conidia.

MATERIAL AND METHODS

Sample collection and pathogen isolation. Oilseed rape leaves showing typical phoma leaf spotting symptoms in fields of commercial growers and research stations were collected in the Czech Republic during five growing seasons (2014–2017, and 2019). From each leaf sample, a small section of infected tissue was cut, surface-sterilised in a 20% bleach solution (1% NaClO) for 3 min, rinsed in sterile distilled water three times, and placed in a glass Petri dish with moistened filter paper for two days to grow the pycnidia. The conidia from individual

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pycnidia were harvested with an inoculation needle using a stereomicroscope, then placed onto a Petri dish with potato dextrose agar (PDA) amended with chloramphenicol (100 µg/mL), and incubated in darkness at 20 °C for mycelial growth. Each single pycnidium isolate was sub-cultured onto a new growth medium. In total, 306 *L. maculans* isolates were collected and confirmed as *L. maculans* based on the absence of a yellow pigment on the PDA plates. The isolates that secreted a yellow pigment into the PDA medium were classified as *L. biglobosa* (Williams & Fitt 1999). Polymerase chain reaction using species-specific primers by Liu et al. (2006) and Mahuku et al. (1996), based on the internal transcribed spacer region, was also used to identify both fungal species. Forty-one isolates were collected from seven regions and 24 localities between 2014 and 2017 and tested using the mycelial growth inhibition assay (Table 1). Of these 41 isolates, 29 were collected by the Agrotest Fyto, Ltd. Two hundred and sixty-five *L. maculans* isolates collected from nine regions and 18 localities in 2016, 2017, and 2019 together with 22 isolates from the mycelium growth plate method, were tested using the microtitre plate assay method (Table 2).

Fungicides. Technical grade boscalid and dimoxystrobin (Pestanal®, Sigma Aldrich, USA), were dissolved in dimethyl sulfoxide (DMSO) to produce a 10 mg/mL stock solution. A stock solution of a mixture of both active ingredients (1 000 mg/mL each) was also prepared from the commercial formulations of boscalid and dimoxystrobin (200 + 200 g/L) in sterile distilled water.

Mycelial growth inhibition assay. The stock solutions of the technical grade boscalid and dimoxystrobin, were diluted in DMSO and added to a cooling autoclaved 10% V8 media (100 mL vegetable juice, 2 g $CaCO_3$, and 15 g agar per litre

Table 1. EC_{50} of 41 *Leptosphaeria maculans* isolates from seven regions in the Czech Republic from 2014 to 2017 used in the mycelial growth inhibition assay against boscalid, dimoxystrobin and boscalid + dimoxystrobin

Region	Years	*N*	EC_{50} (µg/mL)		
			boscalid	dimoxystrobin	boscalid + dimoxystrobin
Central Bohemian	2017	6	0.091–0.983	1.362–95.59	0.010–1.341
Hradec Králové	2017	5	0.057–0.451	2.501–62.22	0.837–1.618
Moravian-Silesian	2016	6	0.029–0.419	2.164–22.39	0.098–1.653
Olomouc	2016	6	0.065–0.385	0.053–31.13	0.108–1.39
Praha	2017	2	0.266–0.562	0.145–0.435	0.163–1.02
South Moravian	2015–2016	3	0.064–0.266	0.06–6.187	0.110–0.86
Zlín	2014–2017	13	0.079–0.377	0.184–59.1	0.097–1.057

EC_{50} – effective concentration at which 50% fungal growth was inhibited; *N* – number of isolates collected per region

Table 2. EC_{50} of 285 *Leptosphaeria maculans* isolates from nine regions in the Czech Republic in 2016, 2017 and 2019 used in the microtitre plate assay against boscalid and dimoxystrobin

Region	Years	*N*	EC_{50} (µg/mL)	
			boscalid	dimoxystrobin
Central Bohemian	2016–2017	12	0.009 572–0.082 75	0.005 7–0.12
Hradec Králové	2017, 2019	97	0.001 77–0.254 5	0.000 857–0.33
Karlovy Vary	2019	35	0.003 8–0.079 11	0.002 9–0.10
Moravian-Silesian	2017, 2019	91	0.001 5–0.84	0.001 6–0.97
Olomouc	2017	6	0.004 21–0.085	0.005 816–0.04
Plzeň	2017	10	0.012–0.073	0.000 73–0.35
Praha	2017	7	0.002 6–0.67	0.004 14–2.06
South Moravian	2017	19	0.002 1–0.124 7	0.003–0.17
Zlín	2017	7	0.026–0.065	0.012–2.81

EC_{50} – effective concentration at which 50% fungal growth was inhibited; *N* – number of isolates collected per region

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of distilled water) to produce the following concentrations: 0, 0.001, 0.01, 0.1, 1 and 10 µg/mL. In addition, a stock solution of boscalid + dimoxystrobin was dissolved in sterile distilled water and added to the 10% V8 media to produce the following concentrations: 0, 0.1, 1, 10, 100, 1000 µg/mL. For each isolate, a mycelial plug (5 mm in diameter) was removed from the margin of a two-week-old colony and transferred to the centre of 9 cm Petri dishes containing the amended media. Three plates for each fungicide concentration were used for each isolate. The isolates were incubated in the dark at 20 °C for 14 days. The mean colony diameters were measured at two perpendicular directions and expressed as a percentage of growth inhibition.

Microtitre plate assay. This sensitivity assay was modified from those of Pijls et al. (1994) and Sewell et al. (2017). Briefly, to produce conidial suspensions for this experiment, the mycelia were first cultured on a 20% V8 media (200 mL vegetable juice, 3 g $CaCO_3$, and 16 g agar per litre of distilled water) for ten days under 16 h fluorescent light at 20 °C and 90% relative humidity to enhance the pycnidia production. The conidiospore suspensions were prepared by flooding the Petri dish with 10–15 mL of sterile distilled water and gently scratching the medium surface of the Petri dish with a sterile glass rod. The spore suspensions were filtered using three layers of autoclaved filter cloth with a mesh size of about 0.7 mm. The resulting conidial suspension was adjusted to a concentration of 1×10^7 spores/mL using a light microscope and a Bürker haemocytometer.

The sensitivity of the *L. maculans* isolates to dimoxystrobin and boscalid were tested at twelve concentrations for dimoxystrobin (0, 0.098, 0.195, 0.39, 0.781, 1.562, 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL) and six concentrations for boscalid (0, 0.098, 0.195, 0.39, 0.781, 1.562, 3.125, and 6.25 µg/mL), based on the results from the mycelial growth plate method. Here, aliquots (100 µL) of a fungicide-amended media (2 × potato dextrose broth) were added to wells of flat-bottomed 96 well microtitre plate (GAMEDIUM spol. s r.o., Czech Republic). Aliquots (100 µL) of the individual conidial suspensions containing about 1×10^6 conidia/mL were also added to each well of a single row. There were four replicates for each isolate. The plates were then incubated at 20 °C for four days in the dark. The subsequent fungal growth as indicated by the absorbance was measured with a TECAN sunrise plate reader (Tecan Austria GmbH, Austria) with the program Magellan (version 7.2) at a wavelength of 630 nm in the endpoint mode.

Data analysis. The statistical analyses were carried out using GraphPad Prism (version 8). The effective concentration at which 50% fungal growth was inhibited (EC_{50}) was calculated by non-linear regression (curve-fit). The frequency distributions for the mycelial growth and microtitre plate assay methods were tested for normality using the D'Agostino-Pearson method. The distributions with the microtitre plate method were first assessed for homogeneity of variances and then a *t*-test two-sample assuming unequal variances (Excel) was performed. Spearman's rank correlation test was performed on the logEC_{50} values to test the sensitivity associations between boscalid and dimoxystrobin. The EC_{50} values calculated for each monitoring method were compared using a paired *t*-test. The EC_{50} values calculated for each of these methods were also regressed over each other, and Spearman's correlation was used to assess the level of the correlation significance.

RESULTS

Mycelium growth assay. The effects of boscalid, dimoxystrobin, and boscalid + dimoxystrobin on the mycelial growth of 41 *L. maculans* isolates were assessed. For boscalid, the isolates showed a unimodal frequency curve with a range of 0.026–0.984 µg/mL (resistance factor 1.12–37.85). For dimoxystrobin, the isolates had a wide range of EC_{50} values, from 0.053 to 95.59 µg/mL (resistance factor 1.13–1 803.59). The mean EC_{50} values differed significantly between the isolates treated with boscalid and dimoxystrobin. The mean EC_{50} for dimoxystrobin (3.62 µg/mL) was 21-fold greater than boscalid (0.17 µg/mL) (Table 3). The range of the EC_{50} values for the isolates treated with boscalid + dimoxystrobin was between 0.097–1.653 µg/mL (mean 0.35 µg/mL, resistance factor 1.01–17.04). The mycelium growth of all 41 isolates were inhibited by 100% by boscalid at the highest concentration of 10 µg/mL, while none of the isolates were 100% inhibited by dimoxystrobin at the highest concentration of 10 µg/mL. The minimal inhibitory concentrations (MIC) for all 41 isolates to dimoxystrobin could not, therefore, be calculated (MIC > 10 µg/mL). For boscalid + dimoxystrobin, 22% of the isolates had an MIC between 1 and 10 µg/mL, while

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Table 3. Comparison of the ranges of the sensitivity to boscalid, dimoxystrobin and boscalid + dimoxystrobin of 41 *Leptosphaeria maculans* mycelial isolates and 285 conidial isolates obtained in the Czech Republic

Fungicide	Monitoring method	*N*	Mean EC_{50} (µg/mL)	Range EC_{50} (µg/mL)	MIC (µg/mL)
Boscalid	mycelial growth assay	41	0.17	0.026–0.984	1–10
	microtitre plate assay	285	0.029	0.001 496–0.836 3	–
Dimoxystrobin	mycelial growth assay	41	3.62	0.053–95.59	> 10 (n = 37); 1–10 (n = 4)
	microtitre plate assay	285	0.002	0.000 727–2.801	–
Boscalid + dimoxystrobin	mycelial growth assay	41	0.35	0.097–1.653	0.1–1 (n = 19); 1–10 (n = 22)

EC_{50} – effective concentration at which 50% fungal growth was inhibited; MIC – minimal inhibitory concentrations; *N* – number of isolates

78% had an MIC of 0.1–1 µg/mL. The frequency distribution curves for boscalid, dimoxystrobin, and boscalid + dimoxystrobin were lognormally distributed when tested using the D'Agostino-Pearson normality test (Figure 1). Spearman's correlation analysis indicated there was no correlation (r = 0.09, P = 0.572) of the log-transformed EC_{50} values of boscalid with the log-transformed EC_{50} values of dimoxystrobin (Figure 2).

Microtitre plate assay. The microtitre plate method was carried out on 285 *L. maculans* isolates to test the effects of boscalid and dimoxystrobin on the conidia and assess a larger sample size. Boscalid had an EC_{50} value range of 0.001 496–0.836 3 µg/mL (resistance factor 1.19–559.02), while those of dimoxystrobin had a range of 7.3 × 10^{-4}–2.801 µg/mL (resistance factor 1.17–3 852.82) (Table 3). The mean EC_{50} value of boscalid was 0.029 µg/mL

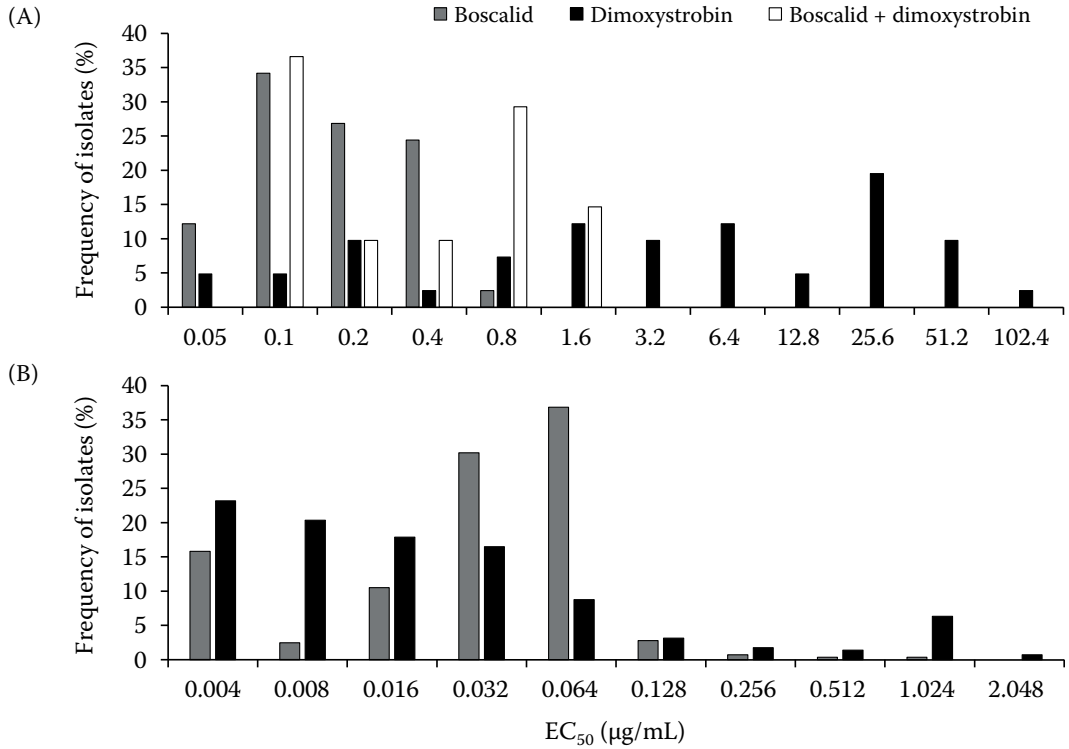

Figure 1. Frequency distribution of the sensitivity of the *Leptosphaeria maculans* isolates collected between 2014 and 2017 from the Czech Republic to the succinate dehydrogenase inhibitor fungicide boscalid, the quinone outside inhibitor dimoxystrobin and boscalid + dimoxystrobin using *in vitro* methods to determine the effective concentration which inhibits (A) the mycelium and (B) conidia by 50% compared to the non-amended control (EC_{50} µg/mL)
Individual isolates are grouped in class intervals when the following interval is two-fold the previous interval. Values on the *x*-axis indicate the midpoint of the interval

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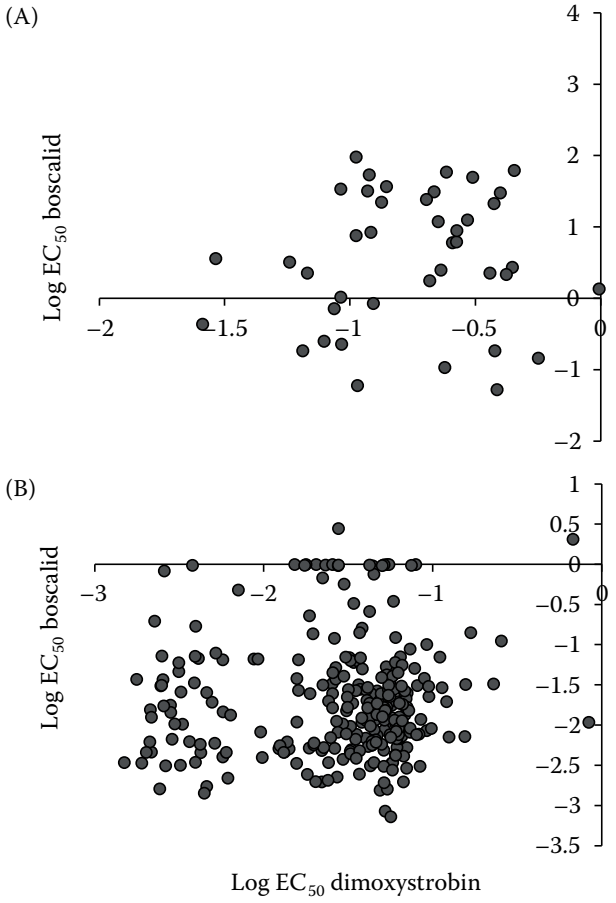

Figure 2. Multiple resistance between boscalid and dimoxystrobin of (A) 41 *Leptosphaeria maculans* mycelial isolates and (B) 285 *L. maculans* conidial isolates from oilseed rape plants obtained in the Czech Republic

and did not statistically differ from the mean EC_{50} value of dimoxystrobin (0.02 µg/mL). Similar to the mycelium growth assay result, the frequency distribution curves showed a lognormal distribution when tested using the D'Agostino-Pearson normality test (Figure 1). Spearman's correlation analysis indicated there was not any multiple sensitivity between the log-transformed EC_{50} values of boscalid and the log-transformed EC_{50} values of dimoxystrobin in any of the *L. maculans* isolates (r = 0.04, P = 0.5) (Figure 2).

Comparison between testing methods. Twenty-two isolates were selected to compare the fungicide testing methods. With the mycelium growth plate method, the mean EC_{50} values of the *L. maculans* isolates were 0.203 µg/mL (resistance factor: 1.5–9.8) for boscalid and 6.014 µg/mL (resistance factor: 2.03–1 804.95) for dimoxystrobin. With the microtitre plate method, the mean EC_{50} values of the *L. maculans* isolates were 0.032 µg/mL (resistance factor: 1.65–33.16) for boscalid and 0.019 µg/mL (resistance factor: 1.02–676.57) for dimoxystrobin. For both boscalid and dimoxystrobin, the mean EC_{50} values from the mycelial growth inhibition assay were significantly higher than the EC_{50} values calculated with the microtitre plate assay method. Spearman's correlation showed that there was not any statistically significant correlation between the fungicide sensitivity testing methods (boscalid: r = 0.225 3, P = 0.313 4; dimoxystrobin: r = 0.217 4, P = 0.331 1) (Figure 3).

DISCUSSION

The management of phoma stem canker in oilseed rape includes the use of fungicides. In the Czech Republic, DMI fungicides are the most widely used fungicide class in controlling phoma

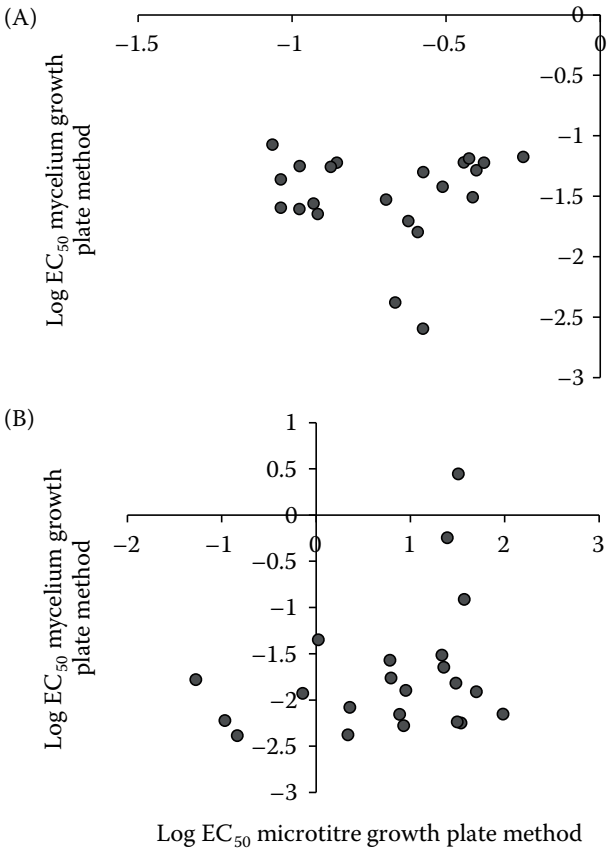

Figure 3. Comparison of the difference between the mycelial growth inhibition and the microtitre plate assay methods for (A) boscalid and (B) dimoxystrobin

No significant correlation was observed between these two methods. Spearman's correlation for boscalid (r = 0.225 3, P = 0.313 4) and for dimoxystrobin (r = 0.217 4, P = 0.331 1)

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stem canker. The continuous use of DMI fungicides has often resulted in resistance development in fungal populations (Brent & Hollomon 2007), including *L. maculans* (Van de Wouw et al. 2017; Yang et al. 2020; Fajemisin et al. 2022). Fungicide resistance management strategies recommend using fungicides with different modes of action rather than a single fungicide class to control plant diseases (Staub 1991; Brent 1995; Brent & Hollomon 2007). This study, therefore, focuses on the efficacy of boscalid and dimoxystrobin, which belong to the SDHI and QoI fungicides classes, respectively, in controlling *L. maculans* isolates collected from oilseed rape plants in the Czech Republic.

Boscalid is an SDHI fungicide with excellent fungicidal activity against plant pathogenic fungi such as *Botrytis*, *Rhizoctonia*, *Sclerotinia*, and *Alternaria*. For example, *Alternaria alternata* isolates never exposed to boscalid in California had an EC_{50} range of 0.089 to 3.435 µg/mL and a mean of 1.515 µg/mL (Avenot & Michailides 2007). *Alternaria alternata* isolates without Pristine® spray in California also had a range of 0.09 to 3.14 and a mean of 1.41 µg/mL (Avenot et al. 2008). Zhang et al. (2009) showed the sensitivity of *Rhizoctonia solani* to boscalid had EC_{50} values from 0.05 to 8.65 µg/mL with a mean of 2.04 µg/mL. Again, the baseline sensitivity of *Sclerotinia sclerotiorum* populations from Germany to boscalid had a range of 0.613 to 2.851 µg/mL with an average of 1.23 µg/mL (Zamani-Noor 2021). *L. maculans* sensitivity to boscalid has not yet been reported. However, fluopyram, another SDHI, has been reported to be effective as a seed dressing (Peng et al. 2020). In this study, boscalid was very effective in inhibiting the mycelial growth and conidia of the *L. maculans* isolates. At the highest concentration of 10 µg/mL, the mycelia of all 41 isolates were completely inhibited. The EC_{50} range was between 0.026–0.984 µg/mL, with a mean EC_{50} of 0.17 µg/mL. Here, the mean was lower than those reported for most pathogens. The low mean and unimodal curve distribution suggests no resistance to boscalid has developed in the *L. maculans* populations in the Czech Republic. Avenot and Michailides (2008) had results showing the mycelial growth of *A. alternata* isolates were more sensitive to boscalid than the conidia. Nevertheless, Zhang et al. (2007), in their study, showed that the conidia of *B. cinerea* isolates were more sensitive to boscalid than the mycelial growth. The EC_{50} values for mycelial growth ranged from 0.09 to 3.69 µg/mL with a mean of 1.07 µg/mL. On the other hand, the EC_{50} values based on conidial growth assay were from 0.02 to 1.68 µg/mL with a mean of 0.42 µg/mL. The *L. maculans* conidia tested for the sensitivity to boscalid in this study also gave an EC_{50} range much lower than those tested for inhibition to the mycelium. This suggests boscalid is more effective at inhibiting the conidial germination in *L. maculans* isolates than the mycelial growth.

QoI fungicides are site-specific fungicides that control many important fungal diseases. They inhibit the mitochondrial respiration in fungal cells at the quinol oxidation site (Qo site) of cytochrome *b* (Bartlett et al. 2002; Fisher & Meunier 2008). Although there have been no published reports of the sensitivity of *L. maculans* to dimoxystrobin, Wang et al. (2020) reported that there was a change in the sensitivity of Canadian *L. maculans* isolates to another QoI, pyraclostrobin from 2011 to 2016. The sensitivity test of the mycelial growth of the *L. maculans* isolates to dimoxystrobin in this study gave a wide range of EC_{50} values, from 0.053–95.59 µg/mL, with a high resistance factor of 1 803.59. This indicates that some *L. maculans* isolates have developed resistance to dimoxystrobin. According to molecular studies, fungicide-resistant strains result if a single point mutation changing glycine to alanine at a position 143 of the CYTB protein (G143A) occurs. The G143A mutation is the most common mutation in plant pathogens resistant to QoI fungicides (Grasso et al. 2006). Another mutation is F129L, which results in the change of phenylalanine to leucine at amino acid position 129 within CYTB (Kim et al. 2003). Further research would be necessary to determine the mechanism of resistance in these isolates.

Out of the 265 *L. maculans* isolates collected in this study, 22 isolates were selected to compare the two sensitivity methods. There was no correlation between the fungicide sensitivity methods. However, the starting material for the mycelium growth plate method was the mycelium, while that of the microtitre plate method was the conidia. Like the SDHIs, QoI fungicides are good inhibitors of spore germination (Bartlett et al. 2002). This was observed in this study as the mean EC_{50} values for the microtitre plate method were much lower than the mean EC_{50} values in the mycelium growth plate method. Nevertheless, the resistance factors varied widely which indicated that the *L. maculans* isolates are more sensitive to boscalid than dimoxystrobin. Therefore,

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both methods would be valid for assessing the fungicide sensitivity in *L. maculans* isolates.

QoI and SDHI fungicides should not be used individually because they have been classified as high risk and medium to high risk by the Fungicide Resistance Action Committee (FRAC 2021). Integrated disease management programmes, therefore, recommend using second-generation SDHIs fungicides, such as boscalid and fluopyram, either as mixing or alternation partners to reduce the possibility of fungicide resistance (Sierotzki & Scalliet 2013). Although fungicide mixtures do not prevent resistant strains from arising, they can slow down the evolution rate of resistance in the pathogen population. A proper mixing partner should provide satisfactory disease control when used alone on the target disease (Staub 1991; Brent 1995; Brent & Hollomon 2007). In this study, the *L. maculans* isolates were sensitive to the boscalid + dimoxystrobin mixture (mean EC_{50} 0.35 µg/mL), probably because one of the mixing agents was boscalid. The absence of multiple resistance between boscalid and dimoxystrobin confirms that although both fungicide classes affect the respiration, they act at different sites in the pathogen (Bartlett et al. 2002).

In summary, the results in this study demonstrate that there is a risk of resistance development among the *L. maculans* populations to the fungicide dimoxystrobin. On the other hand, boscalid was effective in controlling the phoma stem canker in the Czech *L. maculans* isolates. However, to limit the fungicide resistance development of *L. maculans* isolates in the Czech Republic to boscalid, it would be more effective, in the long run, to replace the single site DMI fungicides currently being used with a mixture of boscalid and dimoxystrobin. Future research is needed to test the efficacy of these fungicides in the greenhouse and in the field.

Acknowledgement: We thank Eva Plachká and Pavel Matušinský for collecting and providing samples used in this study, Barbora Slavětinská for her laboratory assistance and Tomáš Hlavsa for statistical analysis.

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Received: December 21, 2021
Accepted: July 13, 2022
Published online: August 31, 2022