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Emergence of Fungicide Sensitivity in *Leptosphaeria maculans* Isolates Collected from the Czech Republic to DMI Fungicides

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Abstract: In the Czech Republic, demethylation inhibitors (DMIs) are used both as fungicides in controlling phoma stem canker and as growth regulators. This heavy use can result in the development of resistant isolates. A total of 45 and 286 *Leptosphaeria maculans* isolates were tested in vitro, using the mycelial growth and microtiter plate assays, respectively. The objective was to determine the sensitivity of *L. maculans* isolates collected in the Czech Republic to the fungicides tetraconazole, metconazole, and prochloraz. The mean EC_{50} values with the mycelial growth plate method were 1.33, 0.78, and 0.40 μg mL^{-1} for tetraconazole, metconazole, and prochloraz, respectively. The mean EC_{50} values for the microtiter plate assay were 3.01, 0.44, and 0.19 μg mL^{-1} for tetraconazole, metconazole, and prochloraz, respectively. All three fungicides also had high variation factors that may be due to inserts in the *ERG11* promoter region. In addition, cross sensitivity among the three fungicides was observed. Overall, the high variation factors and the PCR (polymerase chain reaction) results observed in this study could signify the presence of resistant isolates in *L. maculans* Czech populations, especially in isolates tested for sensitivity to tetraconazole.

Keywords: phoma stem canker; fungicide sensitivity; oilseed rape; DMIs; fungicide

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1. Introduction

Since their introduction into the agricultural fungicide market in the 1970s, demethylation inhibitor (DMI) fungicides (Fungicide Resistance Action Committee (FRAC) group 3) have been a highly effective and inexpensive way of controlling a broad range of fungi [1]. They target the cytochrome P450 enzyme 14-α-demethylase, which is essential for converting lanosterol to ergosterol encoded by the *ERG11* (also known as *CYP51*) gene. By inhibiting the sterol C14-demethylation step during sterol formation in higher fungi, they deplete the amount of ergosterol in the cell and increase the accumulation of 14α-demethylated sterols. Thus, the fungal membrane structure is disrupted, preventing active membrane transport, resulting in fungistasis [2,3].

In the Czech Republic, oilseed rape cultivation accounts for about 15% of the total arable area, making it the second-most frequently grown crop [4]. However, phoma stem canker (also termed blackleg disease) has become a major limiting factor in oilseed rape production [5]. Phoma stem canker is caused by the dothideomycete fungi *Leptosphaeria maculans* (Desm.) Ces. & De Not. (anamorph *Phoma lingam* (Tode) Desm.) [6] and *Leptosphaeria biglobosa* Shoemaker & H. Brun [7]. Yield losses are usually about 10%, but in some seasons can be between 30 and 50% [6]. Control is mainly based on the use of cultural management strategies, resistant cultivars, and fungicides [8]. In the Czech Republic, fungicides registered for control of oilseed rape diseases mainly belong to the DMI fungicide group [9]. Fungicides are generally applied in autumn, but sometimes also in spring if oilseed rape is planted intensively. Many triazoles appear in distinct isomeric forms, which means they can also act as growth regulators [10,11]. In Europe, DMIs, including metconazole and tebuconazole, are currently used in oilseed rape cultivation as both fungicides and

plant growth regulators [11–15]. Because of the site-specific mechanism of action of DMI fungicides, there is the risk that intensive use and overexposure will lead to resistance problems, making it hard to control pathogen populations [16,17].

In fungicide resistance management, it is important to continuously monitor fungicide efficacy, changes in pathogen sensitivity, and effectiveness of fungicide regimes to determine the development of fungicide resistance over time [18]. These would allow for the recommendation and implementation of antiresistance strategies, thus delaying the selection pressure of a fungicide class [18]. There have only been a few studies on the sensitivity of *L. maculans* to fungicides, despite their vast use. Previous sensitivity studies in the UK have reported that although *L. maculans* and *L. biglobosa* differ in their sensitivity to DMI fungicides, they are still effective in controlling phoma stem canker [19,20]. In Australia, however, Van de Wouw et al. [21] and Yang et al. [22] found resistant isolates in *L. maculans* populations. There have been no studies on the sensitivity of *L. maculans* isolates to DMI fungicides in the Czech Republic.

There are three main mechanisms of resistance to DMI fungicides: point mutations within the *ERG11* gene, overexpression of the *ERG11* gene, and overexpression of genes encoding efflux pumps [23]. Huang et al. [24] and Van de Wouw et al. [21] also investigated whether differences in sensitivity among *L. maculans* isolates are a result of changes within the *ERG11*, but no mutations were detected. A recent study of six resistant Australian *L. maculans* isolates, however, showed a 275 bp insertion in two isolates and three long terminal repeat retrotransposons (5263 bp, 5267 bp, and 5248 bp) inserted in the promoter region of three isolates [22]. The objective of this study, therefore, was to determine the sensitivity of *L. maculans* isolates collected in the Czech Republic to three DMI fungicides, tetraconazole, metconazole, and prochloraz, using the mycelium growth plate and microtiter plate assays.

2. Materials and Methods

2.1. Sample Collection and Pathogen Isolation

A total of 310 *L. maculans* isolates were isolated from symptomatic oilseed rape leaves collected from fields of farmers and research stations in 9 regions and 43 localities in the Czech Republic during five growing seasons, 2014–2017, and 2019 (Figure 1). Thirty-one of these isolates were collected by the Agricultural Research Institute Kroměříž between 2014 and 2017 from 4 regions and 25 localities. In 2017, Krukanice and Lužany localities were sprayed with Caramba 0.5 L ha^{-1} (metconazole 60 g L^{-1}) and Staré Smrkovice locality with Horizon 2 × 0.5 L ha^{-1} (tebuconazole 250 g L^{-1}). The spray history of the other localities is unknown. Necrotic leaf discs excised from oilseed rape leaves showing phoma leaf symptoms were immersed in 20% bleach solution (1% NaOCl) for 3 min, rinsed in sterile double-distilled water three times, and placed in a humid chamber. Following isolation, single pycnidium isolates were collected under a stereomicroscope with an inoculation needle and placed onto a Petri dish with PDA (potato dextrose agar) amended with chloramphenicol (100 µg mL^{-1}). After incubation in darkness at 20 °C for growth, the isolates were subcultured onto a new growth medium (PDA or V8 vegetable juice agar).

2.2. Confirmation of L. maculans Isolates

All isolates were confirmed as *L. maculans* using both morphological and genetic characteristics [25]. Morphological characterization of *L. maculans* was based on the presence or absence of yellow pigment on the PDA plates. Isolates with an absence of yellow pigment were categorized as *L. maculans* [25]. Genetic characterization was carried out using species-specific primers by Liu et al. [26] and Mahuku et al. [27]. Fresh mycelia were first ground in liquid nitrogen using a mortar and pestle. Genomic DNA was then extracted using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich®, St. Louis MO, USA), according to the manufacturers' instructions. PCR solutions (25 µL) were made up of sterile double-distilled water, DNA template (1 µL, ~100 ng), 1 × buffer for the *Taq* polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 2.5 mM $MgCl_2$, 0.25 µm each of

dNTP (Thermo Fisher Scientific, Waltham, MA, USA), 0.4 µM each of the relevant primers (Sigma-Aldrich®, St. Louis, MO, USA), and 1 U *Taq* polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Amplification conditions, according to Liu et al. [26], were 95 °C for 2 min, 30 cycles at 95 °C for 15 s, 70 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min (DNA Engine Thermal Cycler Bio-Rad Laboratories, Hertfordshire, UK), while amplification conditions with the pair of primers of Mahuku et al. [27] were 95 °C for 2 min, 35 cycles at 95 °C for 60 s, 55 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 4 min (DNA Engine Thermal Cycler Bio-Rad Laboratories, Hertfordshire, UK). The PCR products were separated on 1% agarose gels containing ethidium bromide at 0.5 µg mL^{-1}, 1 × TBE buffer at 5 V cm^{-1} for 60 min. The gels were visually analyzed under UV light (InGenius LHR and GeneSnap software, Syngene Synoptics Ltd., Cambridge, UK).

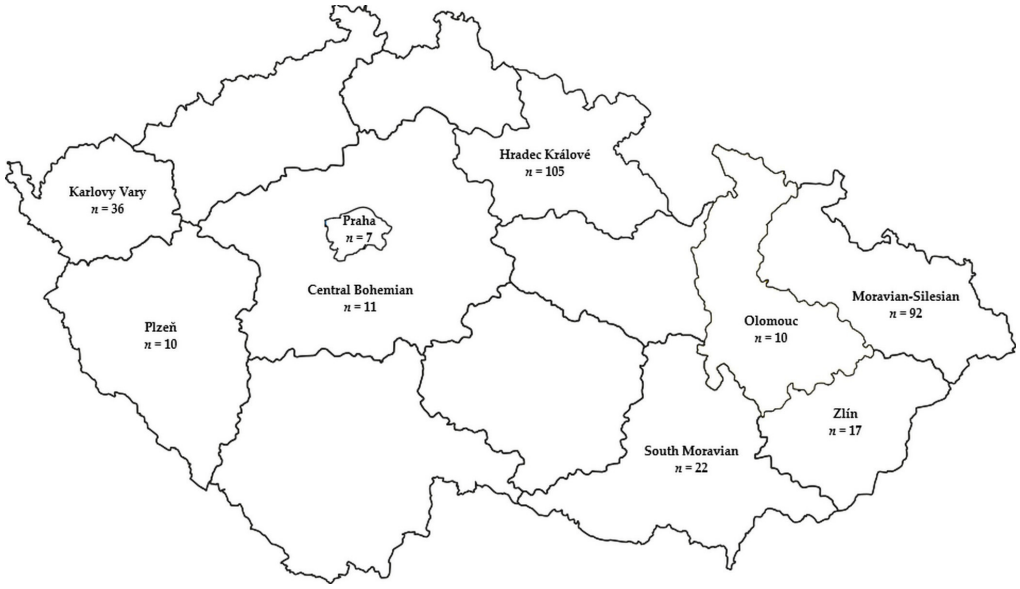

Figure 1. Map of the Czech Republic showing 310 *Leptosphaeria maculans* isolates collected from nine regions between 2014–2017 and 2019. The *n* represents number of isolates collected per region.

2.3. Fungicides

Technical-grade tetraconazole, metconazole, and prochloraz (Pestanal®, Sigma-Aldrich, St. Louis, MO, USA) were dissolved in dimethylsulfoxide (DMSO) to produce a stock solution (10 mg mL^{-1}).

2.4. In Vitro Sensitivity of L. maculans isolates to Tetraconazole, Metconazole, and Prochloraz Using the Mycelium Growth Plate Method

In vitro assessment of inhibition of mycelial growth in the first collected 45 *L. maculans* isolates was determined using the mycelial growth plate method (Table 1). For each isolate, a mycelial plug (5 mm in diameter) was removed from the margin of a two-week-old colony and placed upside down on cooling fungicide-amended V8 juice agar after autoclaving at six concentrations (0, 0.001, 0.01, 0.1, 1.0, and 10 µg mL^{-1}). Isolates were incubated in the darkness (20 °C) for 14 days. The experiment was conducted with three replicates for each isolate. The average fungal growth rate was then measured at two perpendicular directions and expressed as a percentage of growth inhibition. The minimum inhibitory concentration of each fungicide was determined for each isolate as the lowest concentration that inhibited 100% of the growth of a pathogen isolate.

Table 1. EC_{50} range for 45 *Leptosphaeria maculans* isolates from seven regions in the Czech Republic from 2014 to 2017 used in the mycelial growth inhibition assay against DMI fungicides (tetraconazole, metconazole, and prochloraz). N represents the number of isolates collected per region.

Region	Years	N	EC_{50} (μg mL^{-1})		
			Tetraconazole	Metconazole	Prochloraz
Central Bohemian	2016–2017	3	1.277–1.542	0.754–1.5	0.7707–1.463
Hradec Králové	2017	6	0.2411–1.753	0.453–1.717	1.03–1.155
Moravian-Silesian	2016–2017	8	1.143–4.808	0.7088–1.729	0.1596–1.06
Olomouc	2016	7	1.028–1.531	0.3169–1.398	0.035–0.5293
Praha	2017	2	1.3116–1.436	0.281–0.6564	0.1578–0.16
South Moravian	2015–2016	5	1.065–1.332	0.0602–1.451	0.0706–0.7707
Zlín	2014–2017	14	0.7776–1.756	0.06905–1.671	0.0225–1.224

2.5. In Vitro Sensitivity of Conidiospores of L. maculans to Tetraconazole, Metconazole, and Prochloraz Using the Microtiter Plate Method

In vitro assessment of inhibition of conidiospores in 286 *L. maculans* isolates (including 21 isolates from the mycelium growth plate assay method) was carried out using methods modified from Pijls et al. [28] and Sewell et al. [20] (Table 2). First, fresh fungal mycelia of each isolate grown in a 9 cm Petri dish were crushed together with agar using a sterile L-shaped spreader and placed into 20% V8 juice agar. After 10 days under 16 h fluorescent light (Lumilux cool white, Osram GmbH, Munich, Germany) at 20 °C and 90% relative humidity, conidiospores were harvested by flooding the plates with 10–15 mL of sterile double-distilled water and gently scratching the surface of the Petri dish with a sterile glass rod. The suspension was filtered using three layers of autoclaved filter cloth (mesh size of 0.7 mm) to remove pycnidia and mycelia debris. The resulting conidiospore suspension was adjusted to a stock concentration of 1×10^7 spores mL^{-1} using a light microscope and a Bürker hemocytometer.

Table 2. EC_{50} range for 286 *Leptosphaeria maculans* isolates from nine regions in the Czech Republic in 2016, 2017, and 2019 used in the microtiter plate assay against DMI fungicides (tetraconazole, metconazole, and prochloraz). N represents the number of isolates collected per region.

Region	Years	N	EC_{50} (μg mL^{-1})		
			Tetraconazole	Metconazole	Prochloraz
Central Bohemian	2016–2017	11	0.1471–14.74	0.02971–8.105	0.01266–0.9403
Hradec Králové	2017, 2019	100	0.2524–58.62	0.09946–52.58	0.003895–20.7
Karlovy Vary	2019	36	1.172–19.93	0.1278–7.582	0.00613–3.833
Moravian-Silesian	2016, 2017, 2019	88	0.006585–38.3	0.0136–70.69	0.004717–41.95
Olomouc	2016–2017	7	0.1953–14.77	0.05094–9.548	0.034–3.474
Plzeň	2017	9	0.8578–6.38	0.04847–2.959	0.01156–0.1804
Praha	2017	7	0.7815–14.08	0.1198–9.347	0.00459–5.133
South Moravian	2015, 2016, 2017	19	0.3739–59.41	0.07853–59.59	0.03428–56.04
Zlín	2016-2017	9	0.1302–58.84	0.03295–59.28	0.002977–54.6

For the fungicide sensitivity test, 2 × potato dextrose broth (PDB) was amended with technical grade tetraconazole, metconazole, and prochloraz at twelve increasing concentrations (0, 0.098, 0.195, 0.39, 0.781, 1.562, 3.125, 6.25, 12.5, 25, 50, and 100 μg mL^{-1}). Here, fungicide amended media (100 μL) was added to wells of a flat-bottom 96-well microtiter plate (GAMEDIUM spol. s.r.o., Jesenice u Prahy, Czech Republic). In addition, conidiospore suspensions (100 μL) containing about 1×10^6 spores were added to each well of a single row. There were four replicates for each isolate. The plates were then incubated at 20 °C for 4 days in the darkness. Subsequent growth and conidiospore germination, as indicated by absorbance, were measured with the TECAN sunrise plate reader (Tecan

Austria GmbH, Grödig, AustriaCity, Country), software Magellan V7.2 at a wavelength of 630 nm in endpoint mode.

2.6. PCR Amplification of ERG11 Regulatory Region for Molecular Detection of Insertions Conferring Resistance to DMI

A subset of 55 isolates with varying sensitivities to the fungicides based on EC_{50} were selected to investigate, by PCR assay, the absence (sensitive isolates) or presence (resistant isolates) of inserts in the *ERG11* promoter region conferring *ERG11* overexpression. To amplify *ERG11* promoter region from total DNA, PCR reactions were set up in a 25 µL volume as above and carried out using the primer pair EPS1-F/EPS6-R and amplification temperatures by Yang et al. [22].

2.7. Data Analysis

In the fungicide sensitivity experiments, the effective concentration at which 50% fungal growth was inhibited (EC_{50}) for each isolate was calculated [29,30]. The EC_{50} of each fungicide was determined by nonlinear regression (curve-fit) using GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA). The D'Agostino–Pearson method was used to test the normality of frequency distributions (GraphPad Prism version 8.0). Because the data violated the assumption of normality, a nonparametric form of correlation, Spearman's rank correlation test, was performed on logEC_{50} values to test sensitivity associations between tetraconazole and metconazole, prochloraz and metconazole, and prochloraz and tetraconazole.

3. Results

3.1. Mycelium Growth Plate Assay

The mean, minimum, and maximum EC_{50} values of all isolates tested for sensitivity to tetraconazole were 1.33, 0.24, and 4.81 µg mL^{-1}, respectively (variation factor: 20.04). At the highest concentration of 10 µg mL^{-1}, only 11% of the isolates were completely inhibited by tetraconazole, thus making it difficult to determine the minimum inhibitory concentration for the other 89%. Seven percent of the isolates had EC_{50} values below 1 µg mL^{-1}.

L. maculans isolates tested for sensitivity to metconazole had EC_{50} values ranging from 0.06 to 1.73 µg mL^{-1}, representing a 28.83-fold variation factor (EC_{50} mean = 0.78 µg mL^{-1}). Seventy-one percent of the isolates had a minimum inhibitory concentration between 1 and 10 µg mL^{-1}, while the other 29% had minimum inhibitory concentrations greater than 10 µg mL^{-1}. Forty-nine percent of the isolates had EC_{50} values below 1 µg mL^{-1}.

The mean, minimum, and maximum EC_{50} values of all isolates tested for sensitivity to prochloraz were 0.40, 0.02, and 1.46 µg mL^{-1}, respectively (variation factor: 73). Seven percent of the isolates had a minimum inhibitory concentration between 0.1 and 1 µg mL^{-1}; 82% of the isolates had a minimum inhibitory concentration between 1 and 10 µg mL^{-1}; while the remaining 11% had a minimum inhibitory concentration above 10 µg mL^{-1}. Seventy-three percent of the isolates had EC_{50} values below 1 µg mL^{-1}. Data are summarized in Table 1 and Figure 2.

For all 45 *L. maculans* isolates, the frequency distribution curves for the three fungicides were lognormally distributed when tested using the D'Agostino–Pearson normality test.

3.2. Microtiter Plate Assay

For the 286 *L. maculans* isolates carried out with the microtiter plate assay, tetraconazole had EC_{50} values ranging from 0.00659 to 59.51 µg mL^{-1} with a variation factor of 9022.02-fold; metconazole had EC_{50} values ranging from 0.0136 to 70.69 µg mL^{-1} with a variation factor of 5197.80-fold, and prochloraz had EC_{50} values ranging from 0.00298 to 56.04 µg mL^{-1} with a variation factor of 18824.32-fold. The mean EC_{50} values for tetraconazole, metconazole, and prochloraz were 3.01, 0.44, and 0.19 µg mL^{-1}, respectively (Table 2, Figure 2). Frequency distributions of the mean EC_{50} values were lognormally distributed for all three fungicides when tested using the D'Agostino–Pearson normality test.

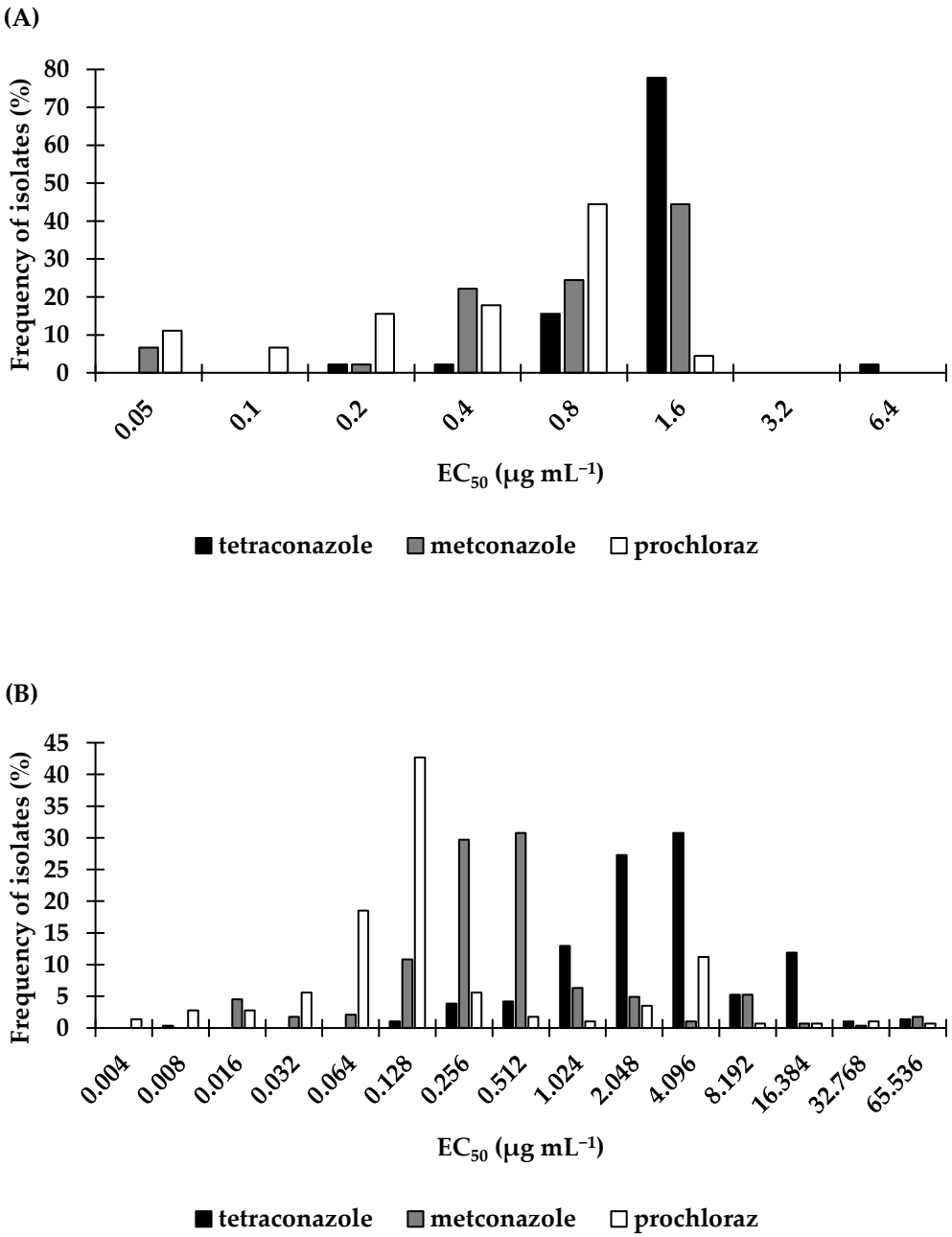

Figure 2. Frequency distribution of sensitivity of *Leptosphaeria maculans* isolates collected between 2014 and 2017 from the Czech Republic to the DMI fungicides; tetraconazole, metconazole, and prochloraz using in vitro methods to determine the effective concentration which inhibits (**A**) mycelium (**B**) conidiospores by 50% compared to the nonamended control (EC_{50} µg mL^{-1}). Individual isolates are grouped in class intervals when following interval is twofold the previous interval. Values on the x-axis indicate the midpoint of the interval.

3.3. Cross Sensitivity

Spearman correlation analysis for mycelium growth plate assay indicated there was no correlation between tetraconazole and metconazole (r = 0.1809, $p = 0.2343$) or prochloraz and tetraconazole (r = 0.1385, $p = 0.3643$). However, there was significant correlation between prochloraz and metconazole (r = 0.6673, $p < 0.0001$) (Figure 3).

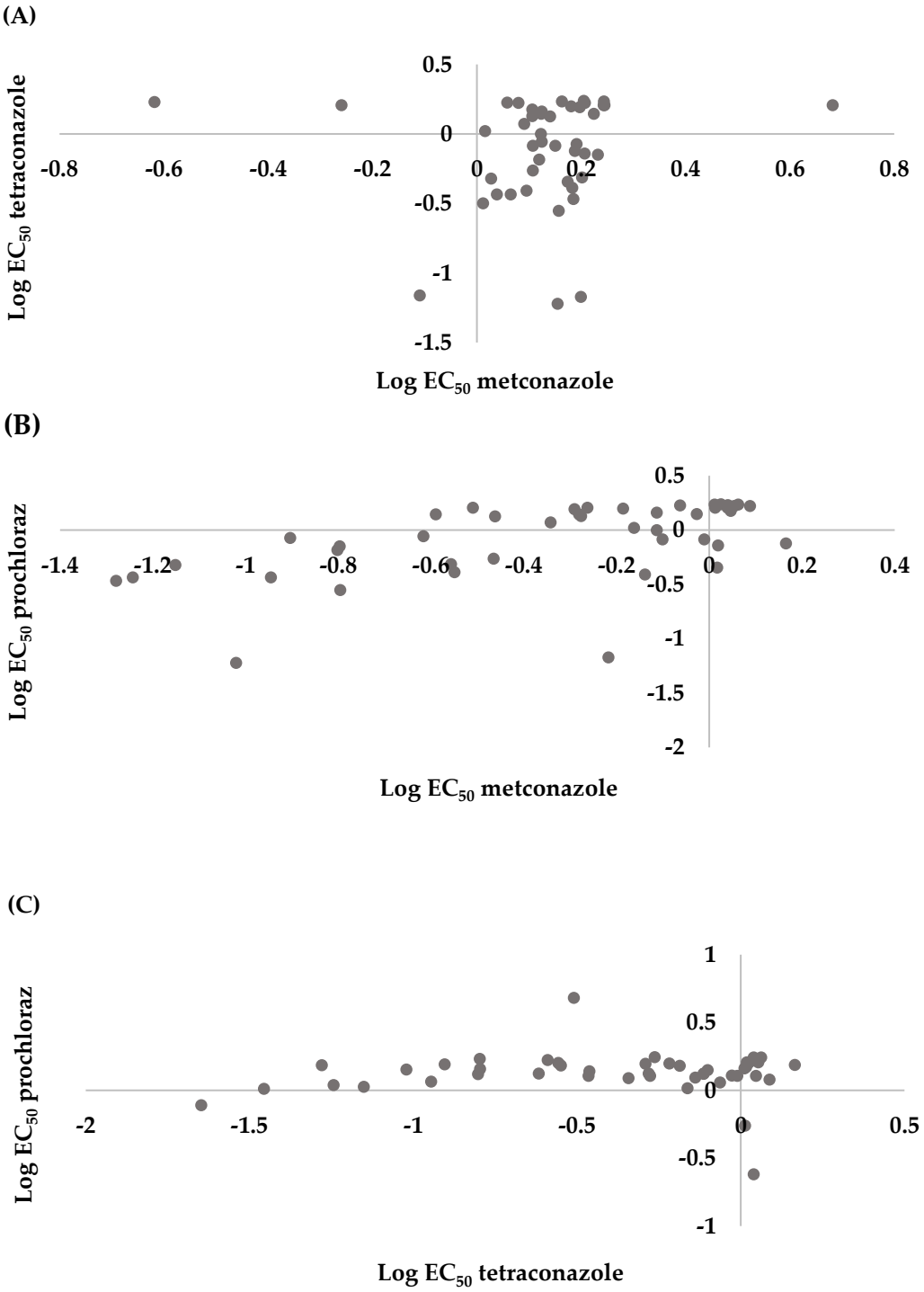

Figure 3. Cross resistance between (**A**) tetraconazole and metconazole (r = 0.1809, p = 0.2343), (**B**) prochloraz and metconazole (r = 0.6673, $p < 0.0001$), and (**C**) prochloraz and tetraconazole (r = 0.1385, p = 0.3643) of 45 mycelial *Leptosphaeria maculans* isolates collected from oilseed rape plants obtained in the Czech Republic.

Spearman correlation analysis for the microtiter plate assay indicated there was a positive correlation between tetraconazole and metconazole (r = 0.3625, $p < 0.0001$), prochloraz and metconazole (r = 0.4623, $p < 0.0001$), and prochloraz and tetraconazole (r = 0.3516, $p < 0.0001$) (Figure 4).

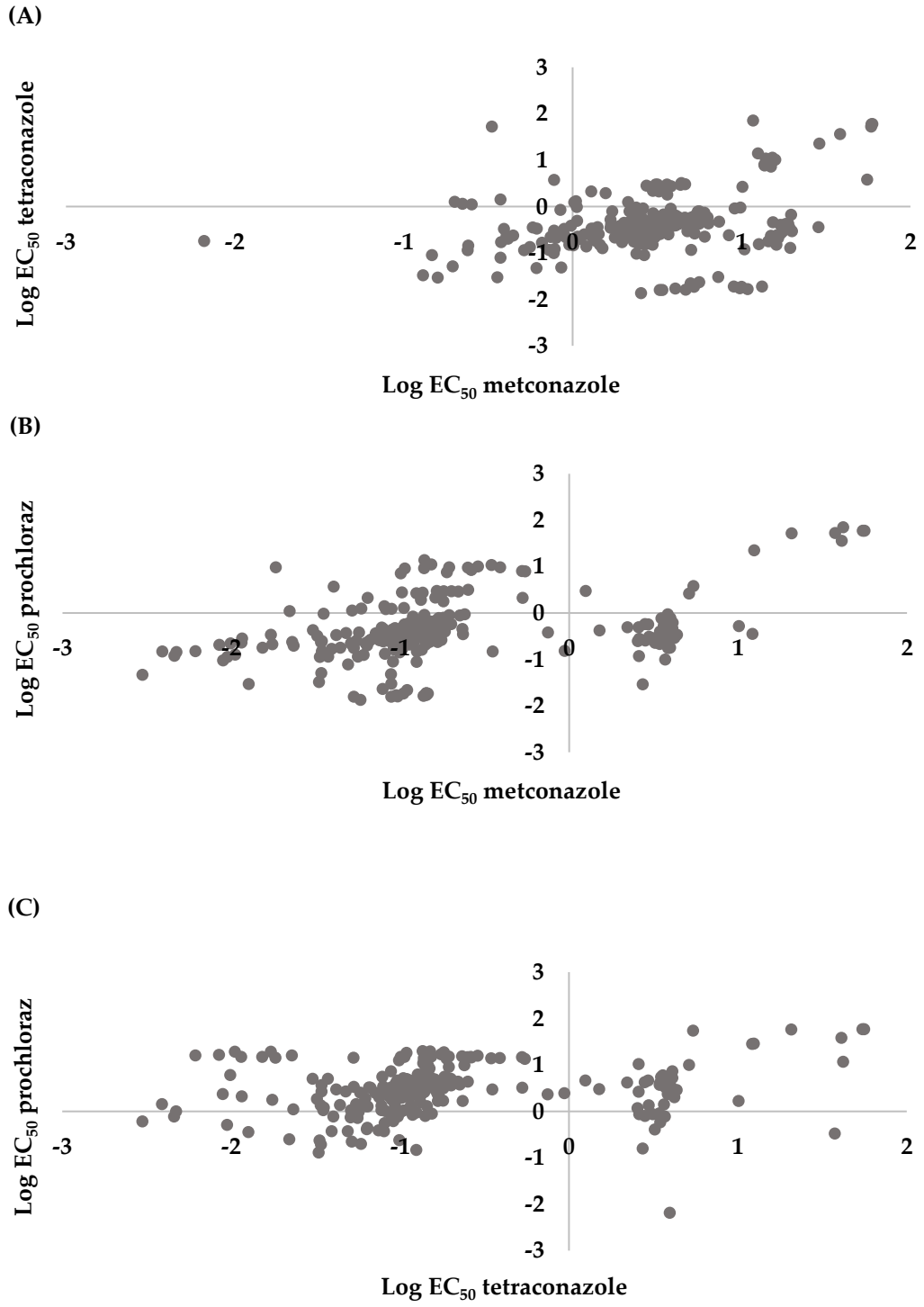

Figure 4. Cross resistance between (**A**) tetraconazole and metconazole (r = 0.3625, $p < 0.0001$), (**B**) prochloraz and metconazole (r = 0.4623, $p < 0.0001$), and (**C**) prochloraz and tetraconazole (r = 0.3516, $p < 0.0001$) of 286 *Leptosphaeria maculans* conidial isolates collected from oilseed rape plants obtained in the Czech Republic.

3.4. Differences between Fungicide Sensitivity Monitoring Methods

The variation factors for all three fungicides in the microtiter plate assay were significantly higher than the variation factors for all three fungicides in the mycelium growth assay.

3.5. Promoter Insertions

Amplification of the *ERG11* promoter region of the 55 isolates with variable sensitivity to all three DMI fungicides showed that 42 isolates (76%) yielded the PCR product size of 1099 bp, representing sensitive isolates, and the other 13 isolates had a PCR product size between 1200 and 1500 bp, representing resistant isolates, indicating an insertion in *ERG11* promoter region [22] (Figure 5). Preliminary sequencing of six sensitive and four resistant isolates revealed that sequences with an insert occur within resistant isolates (data not shown). For tetraconazole, the mean EC_{50} for the sensitive *L. maculans* isolates was 2.4025 μg mL^{-1}, compared with 7.8981 μg mL^{-1} for the resistant isolates. The mean EC_{50} value of the metconazole-sensitive isolates was 0.8464 μg mL^{-1} and of metconazole-resistant isolates was 3.7139 μg mL^{-1}. For prochloraz, the mean EC_{50} values were 0.5637 and 1.6884 μg mL^{-1} for sensitive and resistant isolates, respectively.

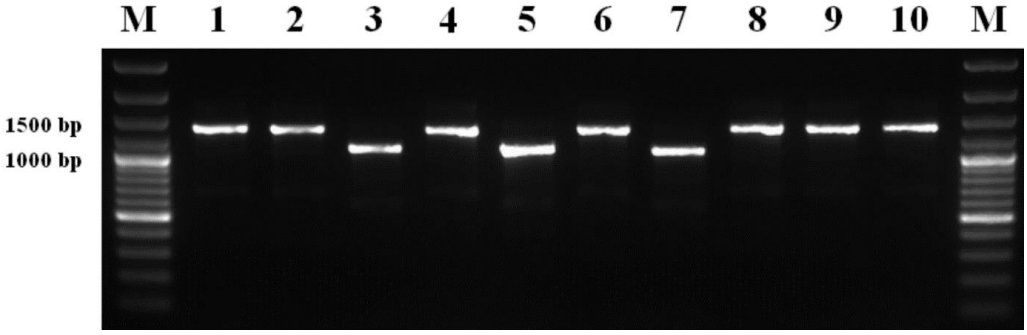

Figure 5. Amplification of the *ERG11* promoter region in 10 selected *Leptosphaeria maculans* isolates using the primers EPS1-F and EPS6-R. Sensitive isolates of *L. maculans* are shown in lanes 3, 5, 7; resistant isolates with an insertion in *ERG11* promoter region are shown in lanes 1–2, 4, 6, 8–10, lane M: GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA).

4. Discussion

DMI fungicides are widely used in agriculture to control plant diseases. They have both preventive and curative properties [31]. These broad-spectrum fungicides have been used successfully for many years in controlling plant pathogens [31], including phoma stem canker [9]. Nevertheless, DMIs are categorized as medium-risk fungicides by FRAC. There have only been a few studies on resistance to fungicides, which have been mostly focused on *L. maculans* sensitivity to strobilurins (quinone outside inhibitor; QoI) [32,33] and other DMI fungicides [20–22,24,34]. However, similar to other phytopathogenic fungi [35–41], resistance to DMI fungicides in *L. maculans* has also been documented [21,22]. This is the first study on the sensitivity of *L. maculans* isolates to DMI fungicides in the Czech Republic. QoI and SDHI fungicides are also used by Czech farmers, but so far only QoI resistance has been reported among *L. maculans* isolates (data not shown).

In vitro fungicide sensitivity studies are used to determine changes in the sensitivity of fungal isolates to a particular active ingredient over time. Testing is typically carried out using mycelial growth assays where the growth of isolates on fungicide amended medium is compared to the growth on nonamended medium [42–44]. This method, though, is usually cumbersome and time-consuming [45]. On the other hand, the microtiter plate assay method allows many isolates to be quickly and simultaneously tested over several fungicide concentrations [28]. In this study, both the mycelium growth assay and the microtiter plate methods were used to test for the sensitivity of *L. maculans* isolates to three DMI fungicides (tetraconazole, metconazole, and prochloraz) registered for use in the Czech Republic for oilseed rape production. In contrast to the mycelium growth plate method, the microtiter plate method was able to test a large sample size (286 isolates).

Both the mycelium growth plate method and the microtiter plate assay showed that, of the three fungicides, tetraconazole had the highest mean EC_{50}. Here, the mean EC_{50} for *L. maculans* isolates to the three fungicides were similar to the mean EC_{50} of resistant

isolates in some studies, and in other studies similar to the mean EC_{50} of sensitive isolates. This made it difficult to determine the sensitivity of the isolates to these fungicides based on mean EC_{50} alone. However, this is not surprising because none of the other studies were based on the sensitivity of *L. maculans* isolates to tetraconazole, metconazole, and prochloraz, but rather to flusilazole, fluquinconazole, prothioconazole, and tebuconazole [20–22,24,34]. Resistance to DMI fungicides is quantitative, meaning it develops in a stepwise gradual progression [18]. It results from the modification of many interacting genes [18]. Therefore, depending on the number of gene changes, isolates exhibit a range of sensitivity to the fungicide [18]. Our current study showed that when tested for mycelial growth inhibition, *L. maculans* isolates had variation factors of 20.04, 28.83, and 73 for tetraconazole, metconazole, and prochloraz isolates, respectively. With the microtiter plate method and a larger number of isolates, this variation factor was much higher, at 9022.02 (tetraconazole), 5197.80 (metconazole), and 18,824.32 (prochloraz). Because metconazole and tetraconazole are triazole fungicides commonly used in the Czech Republic as both fungicides and growth regulators, the high mean EC_{50} values and variation factors suggest high-intensity use of these fungicides may have led to the selection of resistance in some isolates.

Prochloraz is an imidazole fungicide registered in the Czech Republic against both oilseed rape and cereal diseases [9]. Nevertheless, in oilseed rape plants, this fungicide is more commonly used in managing sclerotinia stem rot [4]. Even though *Sclerotinia sclerotiorum* is the main target organism for fungicide application, both pathogens usually occur on the same oilseed rape plant and field and are affected by a fungicide containing prochloraz at the same time [9]. *L. maculans* tested for sensitivity to prochloraz in the current study had a high mean EC_{50} and variation factor, which also suggests a selection pressure with prochloraz. One reason for this could be through the inadvertent exposure of *L. maculans* populations to prochloraz residues used in controlling cereal diseases, as oilseed rape plants are usually grown in rotation with cereals [9]. Another reason for the high mean EC_{50} values and variation factor could be the presence of cross resistance between prochloraz, metconazole, and tetraconazole shown in this study. Cross resistance usually occurs between fungicides with similar modes of action [46]. Thus, fungal isolates which are resistant to one fungicide will often be resistant to another closely related fungicide, even if they have not been exposed to the other closely related fungicide [46], as has been seen between DMI fungicides in *Cercospora beticola* isolates [47], and between tebuconazole and difenoconazole in *Didymella bryoniae* [48]. In addition, Ishii et al. [49] demonstrated cross resistance between mefentrifluconazole and other DMI fungicides: propiconazole, difenoconazole, and tebuconazole. However, cross resistance was only observed with all three fungicides in the isolates tested with the microtiter plate method and between metconazole and prochloraz in the isolates tested with the mycelium growth plate method. The lack of cross resistance between tetraconazole and metconazole and between prochloraz and tetraconazole could be because of the small sample size (45 isolates) tested with this method. Czech farmers have reported a decrease in the efficacy of triazole fungicides (personal communication). However, it is unknown whether this is as a result of the emergence of *L. maculans* resistance to this fungicide group or because of improper fungicide use. Results based on EC_{50} values show that reduced sensitivity of *L. maculans* to triazole fungicides used to control of phoma stem canker could be related to the decreased efficacy observed under field conditions.

Molecular mechanisms of DMI resistance have been studied where overexpression of the *ERG11* gene is sometimes associated with inserts upstream of the *ERG11* gene [50]. The use of molecular markers for rapid PCR-based detection have been recently developed [50], where similar PCR tests have been used to find inserts in isolates of *Venturia inaequalis* and *Blumeriella jaapii* resistant to myclobutanil [51,52]. Yang et al. [22] designed primers to test for insertions in the coding and promoter regions of *ERG11*. Using these primers, 76% of the isolates tested were shown to have PCR products between 1200 and 1500 bp, suggesting

resistance in these isolates. However, these results are not conclusive, as further testing on other isolates and more sequencing data would be needed to confirm the size of the insert.

5. Conclusions

This study has shown that tetraconazole, metconazole, and prochloraz have not effectively inhibited in vitro *L. maculans* populations in the Czech Republic. This insensitivity could be a result of an insertion in the promoter region of the *ERG11* gene. In addition, cross resistance occurring between DMI fungicides was observed in this study. Therefore, it is recommended that DMI fungicides be used either in rotation with other fungicide classes or as tank mixes to reduce the chances of fungicide resistance developing, as sole reliance on one fungicide class is also not advisable in modern agriculture. However, because in vitro assays only provide insight into possible resistant development, they are not entirely accurate predictors of field efficacy, and future studies would be needed to confirm the field performance of commercially formulated products.

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