Fungicide sensitivity, avirulence genes and genetic variability of *Leptosphaeria maculans* isolates in the Czech Republic

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Doctoral Thesis

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Statement of authorships

I hereby declare that I have written my dissertation thesis entitled “Fungicide sensitivity, avirulence genes and genetic variability of *Leptosphaeria maculans* isolates in the Czech Republic” independently and by my own. All literature sources used in this thesis are properly cited according to requirements of the Faculty of Agrobiology, Food and Natural Resources, CULS Prague, and are listed in the chapter References and *vice versa*.

Moreover, it is also to be declared that the research work presented here is original and has not been submitted to other institutions for any degree or diploma.

Fear not, for I am with you;

Be not dismayed, for I am your God;

I will strengthen you, I will help you,

I will uphold you with my righteous right hand.

Isaiah 43:10 (ESV)

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Abstract

After the double zero breakthrough, which lowered the erucic acid and glucosinolates, oilseed rape is now one of the most important oilseeds in the world. It is useful for food, feed for animals, and as a biofuel. Unfortunately, the presence of a variety of pests and diseases has led to severe yield losses. One of the most important diseases of oilseed rape is phoma stem canker or blackleg disease. It is caused by a *Leptosphaeria maculans/ Leptosphaeria biglobosa* complex. The use of fungicides and genetic resistance have over the years mitigated these yield losses. However, these two control methods are usually not durable and after a few years lead to fungicide resistance and breakdown of genetic resistance. This study, therefore, focuses on improving our understanding of fungicide sensitivity, race structure as well as gene flow of *L. maculans* populations in the Czech Republic.

During this study, *L. maculans* isolates were collected from 10 regions across the Czech Republic between 2014 and 2020. First, the fungicide sensitivity of *L. maculans* isolates to nine active ingredients (tebuconazole, tetraconazole, metconazole, prochloraz, propiconazole, prothioconazole, boscalid, dimoxystrobin, and trifloxystrobin), and six commercial fungicides (Caramba, Tilmor, Horizon 250 EW, Bumper Super, Pictor, and Efilor) were evaluated using two established methods, the mycelium growth plate, and microtitre plate method. These fungicidal substances represent three fungicide classes commonly used in the Czech Republic to control oilseed rape pathogens: (Demethylation inhibitors [DMI], Quinone outside inhibitors [QoI], and Succinate dehydrogenase inhibitors [SDHI]). In addition, the frequency of avirulence alleles and race structure of *L. maculans* isolates was determined. Also tested in this study were the mating type and genetic variability because understanding the composition of *L. maculans* populations is one of the first steps in maintaining the durability of control methods.

The results of the fungicide sensitivity tests showed that the DMI fungicides were less effective at controlling *L. maculans* isolates than the other fungicides tested, suggesting that resistance to this group of fungicides may have begun. However, boscalid which belongs to the SDHI fungicide group is currently the most effective fungicide for controlling this pathogen. Cross and multiple sensitivity studies also showed that these fungicides are more effective when two fungicides belonging to different fungicide classes are used as mixtures. For the study on the frequency of avirulence alleles and race structure of *L. maculans* isolates, it was determined

that the major resistance gene *Rlm7* is the most effective resistance gene present in oilseed rape cultivars in the Czech Republic. However, there is still the danger that after several years, *L. maculans* isolates will become virulent to this resistance gene. Therefore, rotating the oilseed rape cultivars with different resistance genes or introgressing the *Rlm7* gene into a new breeding line might be the best way of preserving this major resistance gene. Interestingly, the third study on mating types and genetic variability showed that in the Czech Republic, there is currently only one population structure, which provided considerable insight into how genes flow among the *L. maculans* populations.

Keywords: Phoma stem canker, fungicide sensitivity, avirulence genes, mating types, population structure

Author's publications

Peer reviewed articles

Fajemisin, O., Mazáková, J., Ryšánek, P. (2022). Emergence of fungicide sensitivity in *Leptosphaeria maculans* isolates collected from the Czech Republic to DMI fungicides. Agriculture 12, no. 2: 237. https://doi.org/10.3390/agriculture12020237.

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. Accepted for publication Plant Protection Science.

Conference

Fajemisin, O.; Mazakova, J.; Rysanek, P. (2018): Effects of three fungicides on *Leptosphaeria maculans* isolates collected in the Czech Republic. 17th meeting of the IOBC-WPRS Working Group "Integrated Control in Oilseed Crops", ICOC, University of Zagreb, Faculty of Agriculture, Zagreb, Croatia, 18-20 September 2018, Book of Abstracts p. 170-172.

Burketová, L.; Jindřichová, B.; Stehlík, D.; Trdá, L.; Mazáková, J.; Fajemisin, O.; Ryšánek P. (2018): Leptosphaeria species causing blackleg disease in oilseed rape and *L. maculans* race incidence in the Czech Republic. 17th meeting of the IOBC-WPRS Working Group "Integrated Control in Oilseed Crops", ICOC, University of Zagreb, Faculty of Agriculture, Zagreb, Croatia, 18-20 September 2018, Book of Abstracts p. 189-190.

Poster presentation: Effects of three fungicides on *Leptosphaeria maculans* isolates collected in the Czech Republic: Czech and Slovak Conference of Plant protection, Mendel University Brno, 5-7 September, 2018.

Poster presentation: Effects of fungicides on *Leptosphaeria maculans* isolates collected in the Czech Republic: British Society for Plant Pathology conference. Arms race: Evolution of plant pathogens and their hosts Bristol, 2-3 September, 2019.

Certified works

Burketová, L., Jindřichová, B., Pospíchalová, R., Stehlík, D., Fajemsin, A. O., Maňasová, M., Mazáková, J., Ryšánek, P., Zouhar, M., Plachká, E., Rychlá, A., & Vrbovský, V., 2021. Detekce genů avirulence v izolátech *Leptosphaeria maculans*. Certifikovaná metodika.

Burketová, L., Jindřichová, B., Pospíchalová, R., Stehlík, D., Fajemsin, A. O., Maňasová, M., Mazáková, J., Ryšánek, P., Zouhar, M., Plachká, E., Rychlá, A., & Vrbovský, V., 2021. Identifikace specifických genů rezistence k *Leptosphaeria maculans* v odrůdách řepky a šlechtitelských materiálech. Certifikovaná metodika.

Burketová, L., Jindřichová, B., Pospíchalová, R., Stehlík, D., Fajemsin, A. O., Maňasová, M., Mazáková, J., Ryšánek, P., Zouhar, M., Plachká, E., Rychlá, A., & Vrbovský, V., 2021. Mapa výskytů patogenů *Leptosphaeria maculans* a *L. biglobosa* a jejich ras v ČR. Specializovaná mapa s odborným obsahem.

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Abbreviations

°C	Degree Celsius
µg	Microgram
µl	Microlitre
µmol	Micromole
µM	Micromolar
λ	Simpson's index
%	Percent
χ^2	Chi square
ABC	ATP-binding cassette
AFLP	Amplified fragment length polymorphism
Avr	Avirulence gene
bp	Base pairs
CANOLA	Canadian oilseed rape
cm	Centimetres
DMg	Margalef index
DMI	Demethylation inhibitors
DNA	Deoxyribonucleic acid
E.5	Genotypic evenness
EC50	Effective concentration which inhibits 50%
EDTA	Ethylene-diamine-tetra-acetic acid
EW	Emulsion in water
eMLG	Expected multilocus genotypes
FAO	Food and agricultural organisation
FRAC	Fungicide resistance action committee
g	Gram
Hexp	Nei's unbiased gene diversity
h	Hours
ha	hectares
ITS	Internal transcribed spacer
K	Number of clusters
km	Kilometre

L	Litre
m	Metre
MFS	Major facilitator superfamily
mg	Milligram
MIC	Minimum inhibitory concentration
mM	Millimolar
ml	Milliliter
mm	Millimeter
min	minute
MLG	Multilocus genotypes
N	Total number of isolates per site in each region
NaOCl	Sodium hypochlorite
P	Probability
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PCoA	Principal coordinates analysis
PG	Pathogenicity group
PGR	Plant growth regulator
R	Resistance gene
RAPD	Random amplified polymorphic DNA
rbarD	Standardized index of association
QoI	Quinone outside inhibitor
r	Correlation coefficient
s	Seconds
S	Number of races
SDHI	Succinate dehydrogenase inhibitors
TBE	Tris-EDTA
TE	Tris-borate-EDTA
v	version
V	Volts
V8	Vegetable juice

1. General Introduction

Phoma stem canker is a devastating disease of oilseed rape plants; caused by a *Leptosphaeria maculans/Leptosphaeria biglobosa* complex. This disease has been responsible for losses worth more than £100 million per cropping season in North America, Australia, and Europe. The control of this disease can be through chemical, cultural, biological, or genetic means. Unfortunately, maintaining the durability of these control methods has in recent years become very difficult, which has resulted in reports of resistance of *L. maculans* isolates to different fungicide classes. In addition, changes in virulence of *L. maculans* populations often lead to the breakdown of major gene resistance in oilseed rape cultivars.

Fungicide resistance usually occurs with the continuous use of single site fungicides, especially those of the same fungicide class. To prevent resistance from occurring, it is important to continuously monitor fungicide use and fungicide sensitivity in pathogen populations. *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 non-amended medium. This method, though, is usually cumbersome and time-consuming. Another method available that can be used to test for fungicide resistance is the microtiter plate assay method. This allows many isolates to be quickly and simultaneously tested over several fungicide concentrations. In fungicide resistance management, baseline sensitivity studies are first carried out to assess the response of fungal populations previously unexposed to the fungicide. Prior to doing this work, baseline sensitivity was not carried out. Therefore, the sensitivity of the isolates could not be compared to the sensitivity of isolates collected before the use of each fungicide and shifts in the *L. maculans* population's sensitivity could not be determined. Nevertheless, the results of this study provide valuable information on the current state of fungicide sensitivity in *L. maculans* populations in the Czech Republic, which is important for future monitoring of changes in sensitivity.

Genetic resistance is the most effective and cost-effective method of controlling phoma stem canker. There are two types of resistance described in *Brassica napus*: quantitative resistance and qualitative resistance. Adult resistance, also known as quantitative resistance, is controlled by multiple minor genes and is extremely durable. Qualitative resistance on the other hand is expressed at the seedling stage and is usually controlled by single dominant major resistance

(*R*) genes. It is based on Flor's gene for gene interaction theory, which states that for every resistance gene (*R*), an avirulence gene exists (*Avr*). However, because of their proximity to transposable elements and placement in gene-deficient AC isochores, *Avr* genes are under mutational pressure. As a result, qualitative resistance is not as long-lasting as quantitative resistance. Thus, the *Avr* gene profile can be understood using a differential set of oilseed rape genotypes, which is necessary for selecting *R* genes effective against the pathogen population in the Czech Republic. Oilseed rape breeders can then use the information gained to select effective *R* genes to introgress into new cultivars.

L. maculans reproduces both sexually and asexually. This can give rise to evolutionary processes such as can have an impact such as mutations, migration, genetic drift, selection, and recombination. It is therefore also important to understand the genetic variability in *L. maculans* populations, which can be instrumental in knowing how genes flow within *L. maculans* populations, thereby aiding in predicting potential problems with management strategies.

Therefore, this work focuses on improving our understanding of fungicide sensitivity, genetic resistance as well as gene flow of *L. maculans* populations in the Czech Republic.

2. Literature review

2.1 Oilseed rape

Oilseed rape also called rapeseed or rape (*Brassica napus* subsp. *napus*) belongs to the *Brassicaceae* (cruciferous or mustard) family (Figure 1). This family comprises about 330 genera and 3709 species, including crops (vegetables, oil plants, and spices), weeds (e.g. *Raphanus raphanistrum* L., *Sinapis arvensis* L., and *Thlaspi arvense* L.), and *Arabidopsis thaliana* (a model plant that has been very useful in scientific research). In addition to the already mentioned oilseed rape, the most widely cultivated crops of the genus *Brassica* are varieties and convarieties of wild cabbage (*Brassica oleracea* L.), e.g. cabbage, kale, brussels sprout, broccoli, and kohlrabi, subspecies and varieties of *Brassica rapa* L., e.g. Chinese or Napa cabbage, white turnip and turnip rape, black mustard (*B. nigra*), and swede (*B. napus* subsp. *rapifera*) (Al-Shehbaz et al., 2006; Warwick et al., 2010).

Figure 1. Field of flowering oilseed rape (*Brassica napus* subsp. *napus*) plants.

Cytological studies in 1935 by Nagaharu U revealed the taxonomic relationships between the genus, showing *Brassica napus* L. is derived from the spontaneous interspecific hybridization of *Brassica rapa* (genome AA, 2n = 20) and *Brassica oleracea* (genome CC, 2n = 18), resulting in an amphidiploid genome (Koh et al., 2017) (Figure 2).

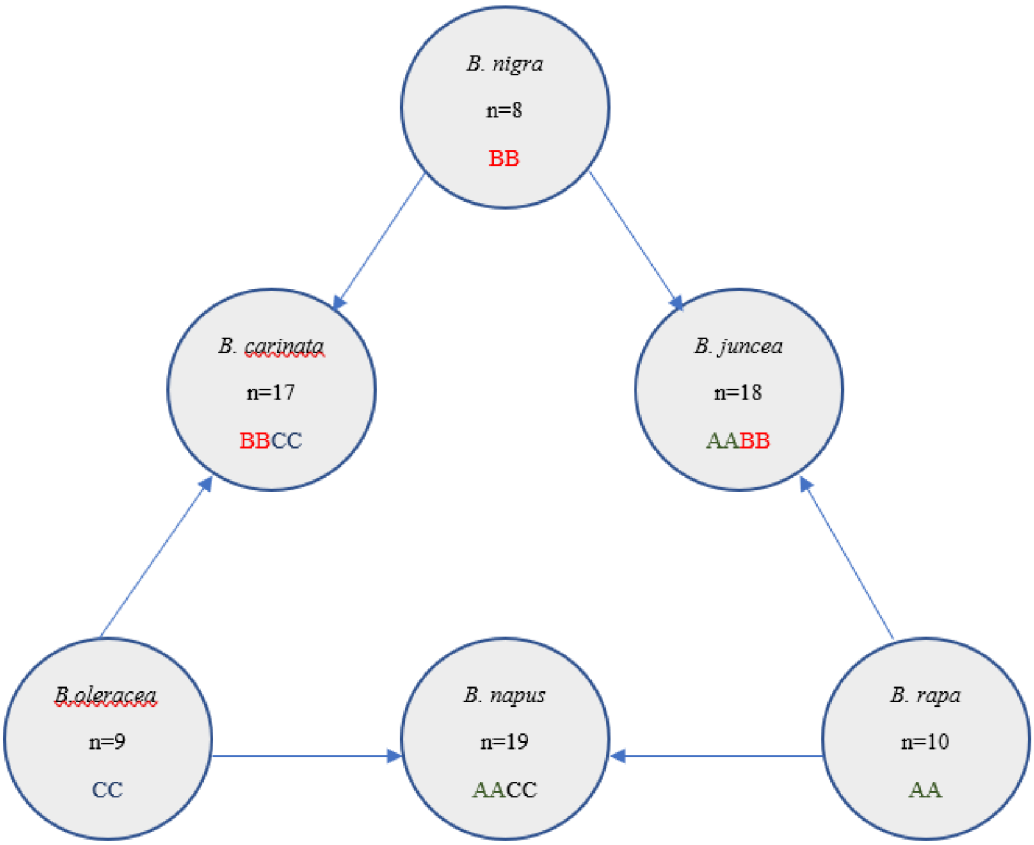

Figure 2. The triangle of U representing the genomic relationships among *Brassica* species according to U (1935).

2.1.1 History of the oilseed rape plant

Oilseed rape plants were first grown in India 3000 years ago, and between 500 and 200 BC were introduced to China and Japan (Downey & Rakow, 1987). In Asia and the Mediterranean regions, the oil was used as a lighting source (Gupta & Pratap, 2007). However, because of the high cost of vegetable oil, the poorer population was forced to also use it as a source of nutrition. At this time, the use of oilseed rape as a source of nutrition was not ideal because of the high content of insalubrious erucic acid and glucosinolates (Gupta & Pratap, 2007). It is unclear when oilseed rape was first cultivated in Europe and used in place of animal fat. This practice appears to have originated in Western Europe, from where the plant migrated to northern and later southern Germany in the sixteenth century. By the eighteenth and nineteenth centuries, oilseed rape became widely used in central Europe, where rapeseed oil (from *B. napus* or *B. rapa*) was primarily used as lamp oil or for technical purposes (Krzymanski, 1998).

In 1978, the Canadian company Western Canadian Oilseed Crushers Association registered a product called canola, which is an abbreviation for CANadian Oil Low Acid. It was used to describe an edible oil crop with low erucic acid (less than 2 %) and low levels of glucosinolates. This was known as the double zero breakthrough. As a result, today, canola or oilseed rape is now a spring annual or winter plant grown worldwide. It is one of the few edible oilseeds worldwide adapted to regions of cool, moist temperate zones or subtropical zones with high altitude areas (Downey & Rakow, 1987). Since 1945, production has immensely increased, especially in Canada and northern Europe. Annual world production has been estimated to have increased to 73.16 million tonnes as at 2021; Canada (19.49 million tonnes) and the European Union (16.29 million tonnes) are the largest producers of oilseed rape in the world today (FAO, 2021) (Figure 3).

In Europe and Asia, winter oilseed rape is mainly cultivated, whereas, in Canada and Australia, only spring forms are cultivated. Unlike winter oilseed rape, the spring form does not require vernalization and is not winter hardy. Therefore, spring oilseed rape is sown in the spring, and stem development begins immediately after germination (Snowdon et al., 2007). It is grown for a variety of purposes, including vegetable oil for humans, as an invaluable biodiesel plant, to produce animal feed, and as a break crop. These uses make it the second leading oil crop that contributes to the economies and health of people around the world today (Abbadi & Leckband, 2011).

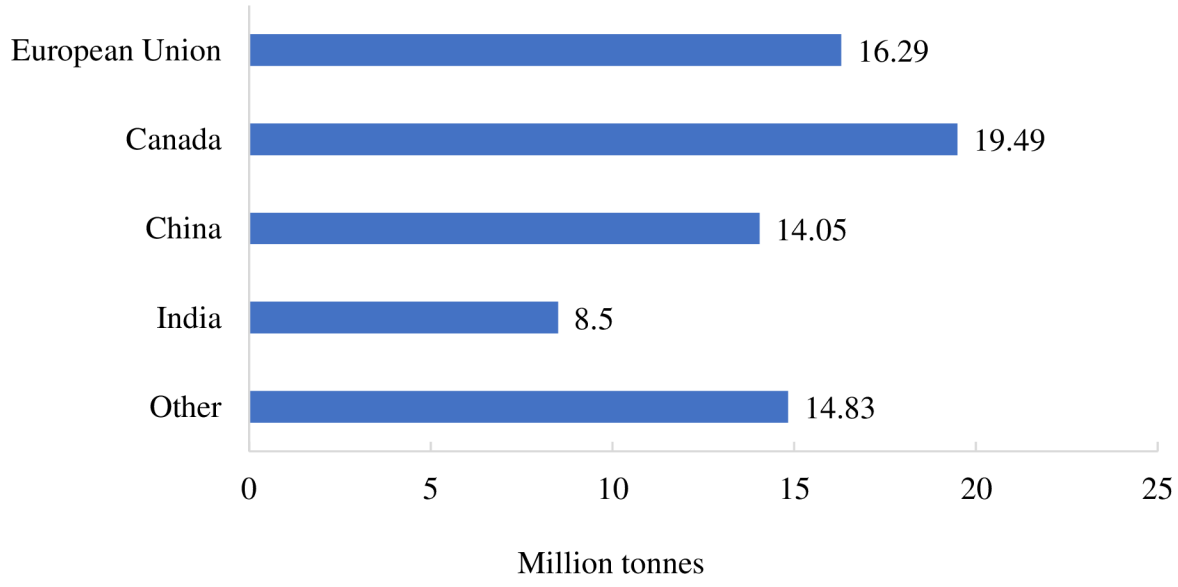

Figure 3. Leading producers of oilseed rape in 2020/2021
Source: FAO US Department of Agriculture
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2.1.2 Oilseed rape production in the Czech Republic

In the Czech Republic, oilseed rape cultivation accounts for about 15 % of the total arable area, making it the second most frequently grown crop (Czech statistical office, 2022). The Land Fund Act of 1992 led to the restitution of ownership rights to former owners and the privatization of state land. Therefore, the Czech Republic became one of Europe's largest producers of oilseed rape. Between 1991 and 2018, the cultivation area increased to 411 802 ha. However, in the last three years, there has been a significant decrease in sowing area, which as of 2021 was 342 315 ha (Figure 4). Furthermore, the harvest yield in 2021 was the lowest in the last decade, at 1 024 928 tonnes (Czech statistical office, 2022) (Figure 4). The decrease in average yields in the Czech Republic may be as a result of a reduction in sowing area, warm temperatures, or the incidence of pests and diseases.

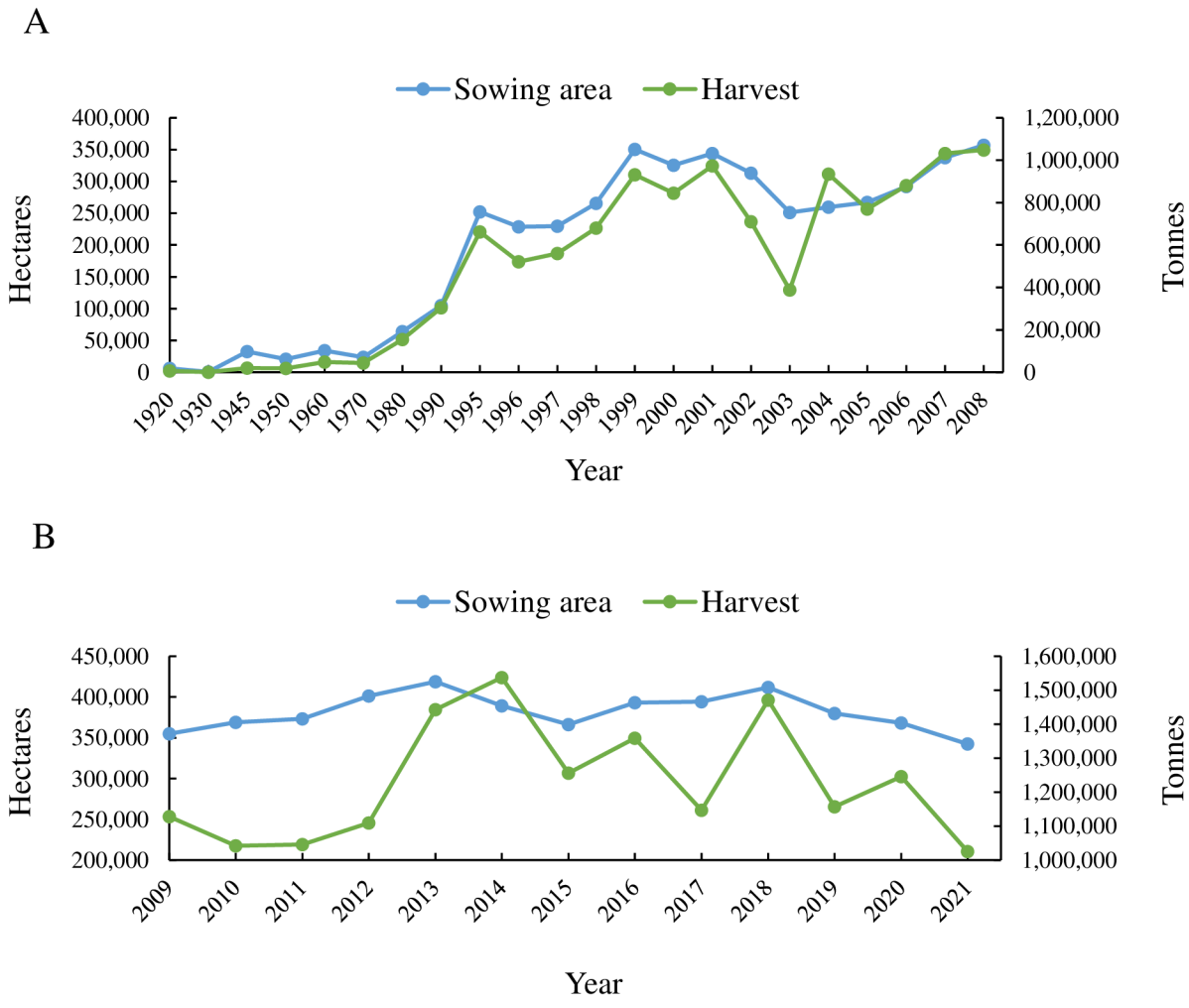


Figure 4. Sowing area and harvest of oilseed rape plants between (A) 1920 and 2008 (B) 2009 and 2021 in the Czech Republic.

2.1.3 Pests and diseases of oilseed rape plants

The most important pests in oilseed rape producing regions are pollen beetle (*Brassicogethes aeneus* [Fabricius, 1775]), the flea beetles of the genus *Phyllotreta crucifera,* cabbage stem flea beetle (*Psylliodes chrysocephalus* [Linnaeus, 1758]), rape stem weevil (*Ceutorhynchus pallidactylus* [Marsham, 1802]), turnip gall weevil (*Ceutorhynchus assimilis* [Paykull, 1792]), cabbage seedpod weevil (*Ceutorhynchus obstrictus* [Marsham, 1802]), cabbage aphid (*Brevicoryne brassicae* [Linnaeus, 1758]), diamondback moth (*Plutella xylostella* [Linnaeus, 1758]), cabbage root fly (*Delia radicum* [Linnaeus, 1758]), and brassica pod midge (*Dasineura brassicae* [Winnertz, 1853]) (Walker & Booth, 2001). Phoma stem canker caused by *Leptosphaeria maculans* [Ces. & De Not., 1863]/ *Leptosphaeria biglobosa* [Shoemaker & H. Brun, 2001]), Sclerotinia stem rot (caused by *Sclerotinia sclerotiorum* [(Lib.) de Bary, 1984]), and clubroot (caused by *Plasmodiophora brassicae* [Woronin, 1877]) are the most common diseases found in all oilseed rape producing regions worldwide. Diseases specific to Europe are Verticillium stem striping (caused by *Verticillium longisporum* [(C. Stark]) Karapapa, Bainbr & Heale, 1997]), light leaf spot (caused by *Pyrenopeziza brassicae* [B. Sutton & Rawl., 1979]) and Mycosphaerella ring spot (caused by *Mycosphaerella brassicicola* [(Duby) Lindau, 1897]) (Zheng et al., 2020) (Figure 5).

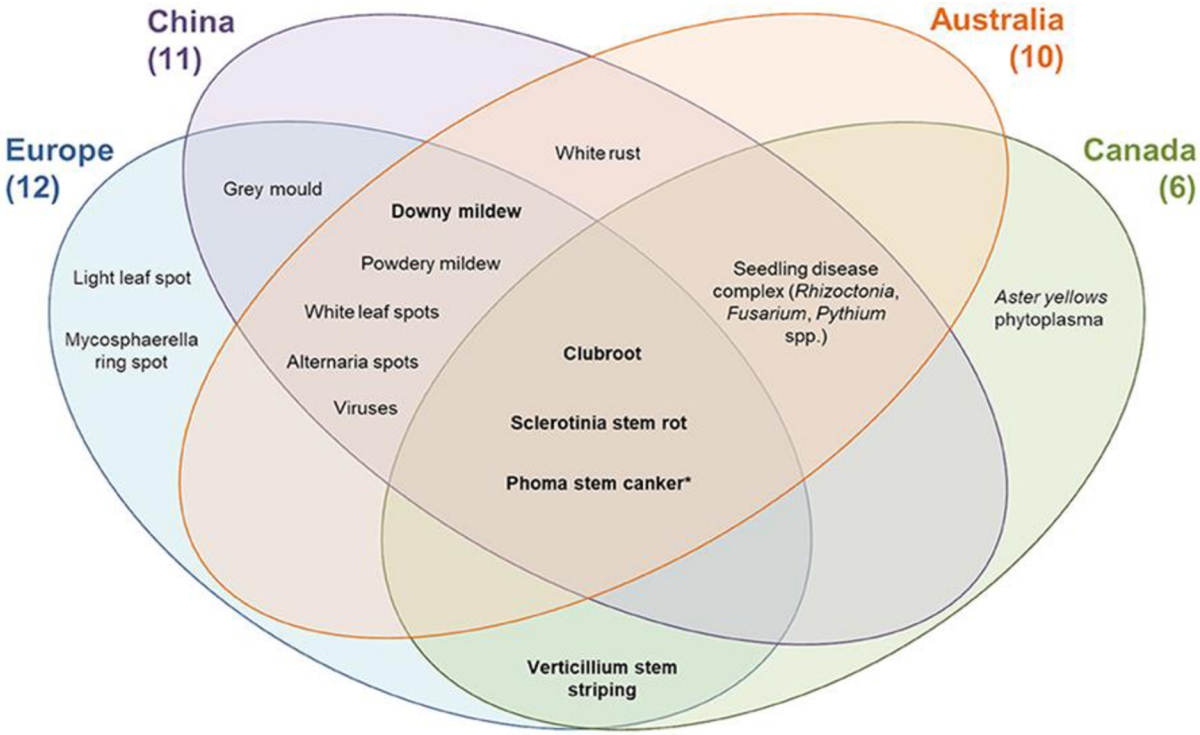


Figure 5. Fungal diseases of oilseed rape plants in China, Europe, Canada and Australia (Zheng et al., 2020).

2.2 The pathogens: *Leptosphaeria maculans/Leptosphaeria biglobosa*

2.2.1 Taxonomy and classification

Leptosphaeria maculans (Desm.) Ces. & de Not., 1863 (anamorph = *Phoma lingam* [Tode ex Fr.] Desm., 1849) and *Leptosphaeria biglobosa* Shoemaker & Brun, 2001 are the causal agents of phoma stem canker (Fitt et al., 2006; West et al., 2001). They are hemibiotrophic and heterothallic fungi, previously classified as belonging to the *Loculoascomycetes* class, which includes over 6000 fungal species (Silva-Hanlin & Hanlin, 1999). Now, they belong to the *Dothideomycetes* class (even though the class *Loculoascomycete* is still used by some authors) (Howlett et al., 2001).

Until 2001, isolates were considered as one species with two major groups or pathotypes. The reason was that there were marked differences based on plant assays (isolate pathogenicity and virulence), culture characteristics (morphology, growth rate, pigment production), biochemical characteristics (sirodesmin production, serology) and molecular characteristics (isozyme and DNA polymorphism) (Williams & Fitt 1999). Isolates that were slow growing but aggressive in plants, and could produce the toxin sirodesmin PL were designated as A, highly virulent or Tox^{+} (*L. maculans*), while isolates that were fast growing, non-aggressive in plants, and unable to produce the toxin sirodesmin PL were designated as B, weakly virulent or Tox^{0} (now *L. biglobosa*) (Johnson and Lewis, 1994; Williams & Fitt, 1999; Balesdent et al., 1992; Koch et al., 1989; Taylor et al., 1991; Voigt et al., 2001). These isolates were also seen to have yellow pigmentation on potato dextrose agar and red pigmentation in liquid media, whereas *L. maculans* isolates produce no pigmentation. However, based on the differences in the morphology of their fruiting bodies (pseudothecia), Shoemaker & Brun (2001) described the previously considered B group as a new species, *L. biglobosa*.

Phylogenetic analysis carried out using ITS rDNA, actin, and tubulin sequences by Mendes-Pereira et al. (2003) and Voigt et al. (2005) separated both species into subclades based on geographical origin and host plant. Thus, *L. maculans* was classified into two subclades: '*brassicae*' and '*lepidii*', while *L. biglobosa* isolates were classified into seven subclades: '*americensis*', '*australensis*', '*brassicae*', '*canadensis*', '*erysimii*', '*occiaustralensis*' and '*thlaspii*' (Figure 6). Further phylogenetic analysis of nrDNA sequence data has led to the reclassification

of *L. maculans* and *L. biglobosa* into *Plenodomus lingam* and *P. biglobosus*, respectively (de Gruyter et al., 2009, 2013). However, *L. maculans* and *L. biglobosa* are the scientific names currently still being used in literature.

According to the latest taxonomy (Schoch et al., 2020; Index Fungorum Partnership, 2022), *L. maculans* and *L. biglobosa* belong to:

Kingdom: Fungi

Subkingdom: Dikarya

Phylum: Ascomycota

Subphylum: Pezizomycotina

Class: Dothideomycetes

Subclass: Pleosporomycetidae

Order: Pleosporales

Suborder: Pleosporineae

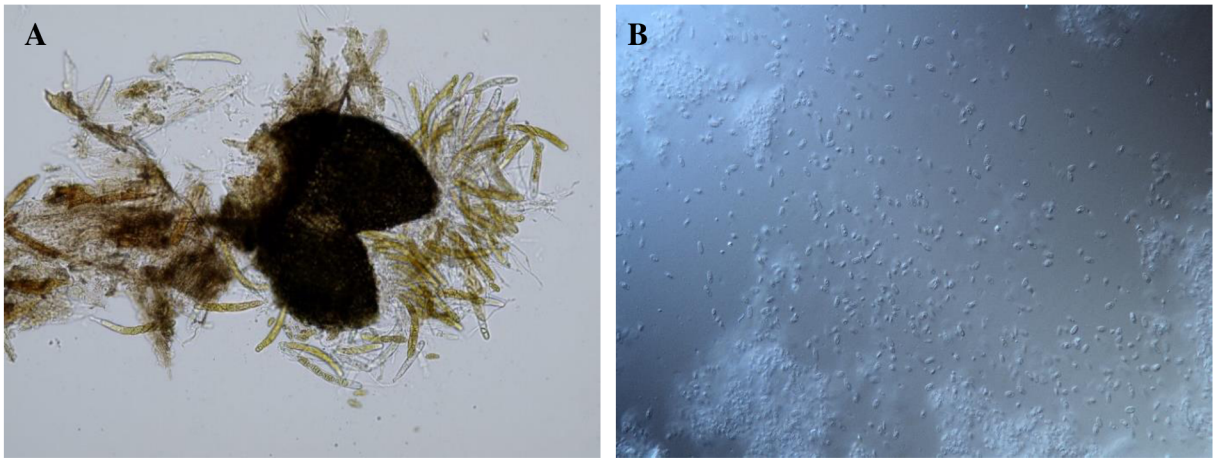
Family: Leptosphaeriaceae

Genus: *Leptosphaeria* (*Plenodomus*)

2.2.2 Morphology

L. maculans and *L. biglobosa* have similar morphologies. The asexual fruiting bodies are known as pycnidia. Two types of pycnidia are produced, one on living host tissue and the other on infected residue. Pycnidia on the living host are 200–600 µm in diameter, with a wall up to 18 µm thick, globose and black, and a papilla or neck. Pycnidia on residue are sclerotiod of variable shape, 200–500 µm in diameter, composed of several layers of thick-walled (pseudosclerenchymatous) cells, black, and with a narrow ostiole. Pycnidiospores are 1.2–2 × 3–5 µm in size, hyaline, mostly straight, gluttulate, and unicellular. Ascospores range in colour from hyaline to yellow-brown. They are produced in groups of eight, have five septa, are biseriate, cylindrical to ellipsoidal, with rounded ends, and measure 35–70 × 5–8 µm. Pseudothecia (ascomata) have a diameter of 300–500 µm (Shoemaker & Brun, 2001; Vakili

Zarj et al., 2017; Williams, 1992) (Figure 6). Differences between *L. maculans* and *L. biglobosa* can be found in the ascomata. The ascomata of *L. biglobosa* differ from those of *L. maculans* in that they have a long beak that is typically inflated through the proliferation of the beak wall cells at the upper part. The ascomata of *L. maculans* are globoid with a barely discernible papilla. The ascomata of *L. biglobosa* resemble miniature dumbbells (Shoemaker



& Brun, 2001).

Figure 6. Morphology of *Leptosphaeria maculans*: (A) pseudothecium with protruding asci and ascospores (B) pycnidiospores.

2.3 The disease: Phoma stem canker

2.3.1 Economic importance and worldwide distribution

Phoma stem canker (or blackleg disease) is a disease of many plants in the *Brassicaceae* family, including spring and winter oilseed rape varieties, brassica vegetables, *Crambe* spp., *Eruca* spp., *Erysimum* spp., *Lepidium* spp., *Raphanus* spp., *Sisymbrium* spp., and *Thlaspi* spp. (Kaczmarek & Jedryczka, 2011). It is found in all oilseed rape-producing countries (Fitt et al., 2006; West et al., 2001). *L. maculans* is thought to be far more dangerous than *L. biglobosa*. It is the more aggressive pathogen and usually causes severe epidemics and substantial yield losses (Fitt et al., 2008). *L. maculans* has been spreading in areas such as Canada and Poland where only *L. biglobosa* had previously been present (Liu et al., 2007; Fitt et al., 2008). This occurs because the climatic and agronomic conditions may be favourable for *L. maculans*. After

all, these two related *Leptosphaeria* species occupy similar ecological niches (West et al., 2002; Fitt et al., 2006).

A linear relationship between Phoma stem canker disease severity and yield loss; and between disease severity and seed yield has been reported. *L. maculans* has also been estimated to cause losses worth more than £1000 million per cropping season at £400 per tonne (Barnes et al., 2010), making it now one of the most serious and economically important diseases of oilseed rape in the world (Fitt et al., 2006). On average, there is an estimated yield loss of <10 % with a maximum loss of 30–50 %. In France, a yield loss of 5–20 % was reported, and in the UK between 1987 and 1995, 8–29 % yield loss was recorded. Epidemics causing significant levels of disease occurred in regions of France in 2000 and the lower Eyre Peninsular in Australia in 2003. In Australia, losses resulting in up to 90 % have been recorded (Sprague et al., 2006; Fitt et al., 2006; Zhou et al., 1999). In China, however, *L. biglobosa* '*brassicae*' is the only subspecies identified to date, and even though it causes 10–30 % yield loss, it is still not as damaging as *L. maculans* (Liu et al., 2014; Cai et al., 2018). Nevertheless, some Chinese oilseed rape cultivars have been very susceptible to *L. maculans* when grown as crops in countries such as the UK, Poland, and Australia, where *L. maculans* is widespread while others have shown some resistance in field experiments in Canada (Zhang et al., 2014). In the Czech Republic, phoma stem canker has become a major limiting factor in oilseed rape production (Mazáková et al., 2017).

2.3.2 Life cycle

Hemibiotrophic pathogens *L. maculans* and *L. biglobosa* have a similar life cycle (Howlett et al., 2001; West et al., 2001). They survive non-host periods as mycelium and pseudothecia in crop residues (saprophytic stage) (Gladders & Musa, 1980; Hall, 1992; West et al., 2001). Survival times differ between regions, less than two years in Europe and up to 4 years in Australia (West et al., 2001; Fitt et al., 2006). An epidemic is usually initiated by airborne ascospores which are released from the pseudothecia (Gladders &Musa, 1980). Ascospore release does not differ significantly across a temperature range of 5°C to 20°C but is significantly increased by rainfall (Huang et al., 2005). Rain-splash has been estimated to have a limited spread, primarily within 14 cm (Travadon et al., 2007), but spores can be spread by wind over distances of up to 10 km (Piliponyte-Dzikiene et al., 2014). Ascospores adhere to the

cotyledons and true leaves of new crops and germinate in humid or wet conditions to produce primary infection. Infection of cotyledons is usually through the stomata or via wounds (Chen & Howlett, 1996). The fungus initially colonizes the tissue as a biotroph, but later becomes necrotrophic. During this stage of the disease cycle, the fungus produces asexual spores (pycnidia), and phoma leaf spots develop (Gladders & Musa, 1980; West et al., 2001). In Australia, pycnidiospores produced in the centre of phoma leaf spot lesions are disseminated by rain splash and may initiate further infections. In this case, the life cycle is polycyclic (Li et al., 2006). Outside Australia, this rarely occurs, and the lifecycle is therefore considered to be monocyclic. A symptomless and biotrophic phase follows when the fungus then colonizes the intercellular spaces between mesophyll cells and grows down the petiole mainly in xylem vessels or between cells of the xylem parenchyma and cortex (Hammond et al., 1985; Johnson & Lewis, 1994). The fungus then invades and kills the cells of the stem cortex, resulting in either a blackened canker at the base of the stem for *L. maculans* or upper or lower stem lesions for *L. biglobosa* (necrotrophic stage). Sometimes the fungus penetrates the root, causing the cortex to blacken and rot (West and Fitt, 2005). Lodging or restricted water uptake causes premature pod shattering, resulting in yield losses (Davies, 1986) (Figure 8).

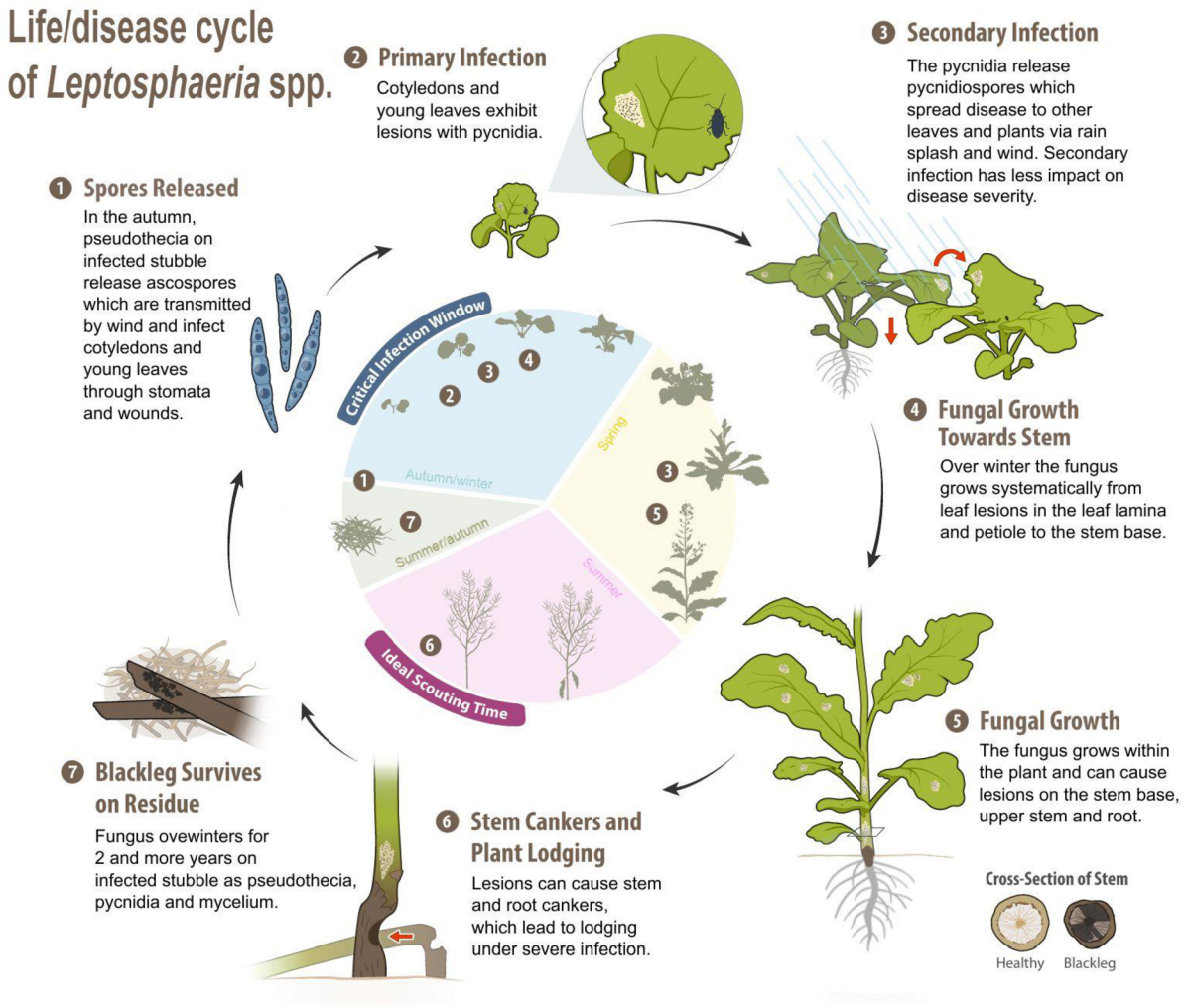


Figure 7. Life cycle of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* on oilseed rape (Adapted by Canola Council of Canada, https://www.canolacouncil.org/canola-encyclopedia/diseases/blackleg/).

2.3.3 Symptoms

Both causal agents of Phoma stem canker can infect any part of the plant, including the cotyledons, true leaves, stems, pods, roots, and seeds (West et al., 2001). *L. maculans* and *L. biglobosa* are genetically different and exhibit distinct disease symptoms on the oilseed rape plant. Symptoms may be found on cotyledons and the first true leaves, and a severe infection may cause damping-off at the seedling stage. *L. biglobosa* lesions on the leaves are smaller and darker, containing only a few pycnidia. On the other hand, *L. maculans* lesions on the leaves

are round or irregular shaped, confined by leaf veins. As the leaf lesions mature, they become pale often with a darker margin, necrotic and greyish, typically >15-mm with numerous pycnidia. In oilseed rape crops, typical phoma stem canker symptoms develop after flowering.

The pathogen enters the stem via the leaf petiole causing the disease's main symptom, a blackened stem base canker, which gave rise to the disease's name, 'blackleg'. The basal stem canker causes crop lodging and premature ripening. It is generally considered that *L. biglobosa* is typically restricted to the upper stem (Brun et al., 1997; Ansan-Melayah et al., 1997; West et al., 2001). Recent research has nevertheless revealed the presence of *L. biglobosa* on lower stems in Lithuania, Poland, UK, and the Czech Republic (Brazauskiene et al. 2011; Stonard et al. 2010; Jedryczka & Lewartowska 2006; Mazakova et al., 2017). The fungus growing on the pods may infect the seeds. Seeds that have been infected can become shrivelled and discoloured but may have no external symptoms (Figure 7).

Figure 8. Disease symptoms of phoma stem canker as seen on oilseed rape plants: (A) stems (B) leaves (C) cotyledons.

2.4 Control of blackleg disease

2.4.1 Cultural control

Ascospores are a good source of primary inoculum and ascospore development often coincides with seedling development. A good crop rotation system and stubble management reduce the rate of infection by ascospores from colonized tissues. The crop rotation should be at least four years apart, as closer rotations increase the amount of stubble residues in subsequent oilseed rape crops (Gladders et al., 2006). Stubble management is carried out by destruction of infected residues by burning, raking and burying (Huang et al., 2003). In areas such as Western Australia where there is a greater use of minimum tillage, there has been an increase in the amount of infected residue remaining on the soil surface especially as the dry climate promotes its persistence. Here, to reduce inoculum, deep ploughing is recommended but this is difficult on heavy soils which is why ploughing is delayed by farmers to allow seed split at harvest to germinate and produce. This leaves a very short period of about three weeks in which fields that have previously grown oilseed rape can be ploughed before the new crop of oilseed rape seedlings emerge. In Europe however, since infected residues break down more quickly, the problem is not as acute as in Australia. Because the number of ascospores in the air increases greatly for a period and then declines, delaying the sowing date so the seedlings would escape this period can allow the crop to have produced enough leaves by the time ascospores are released to evade infection at the most sensitive stage (West et al., 1999; Aubertot et al., 2004). In China and India, labour intensive cultural practices such as removal of the whole plant at harvest and subsequently flooding of the field for the following rice crop helps to destroy the inoculum. This may be why phoma stem canker is not considered to be a problem in these countries (Zhang et al., 2014; Liu et al., 2014).

2.4.2 Biological control

Biological control involves the use of antagonistic microorganisms to suppress pathogens. This method is becoming popular as it is considered environmentally friendly and harmless to birds, fish, bees and other animals. It is especially useful in crops where fungicide resistance has been reported to occur. The main mechanisms employed by biological control agents (BCA) against

fungal pathogens are competition, antibiosis, and mycoparasitism. In the control of *L. maculans*, *Penicillum verrucosum* has been found to produce a metabolite toxic to the pathogen (Kharbanda & Dahiya, 1990). *Paenibacillus polymyxa* syn. *Bacillus polymyxa*, strain PBKI has also been found to inhibit the growth of isolates of *L. maculans* infecting oilseed rape (Kharbanda et al., 1999). In the Czech Republic, *Pythium oligandrum* and *Bacillus subtilis* are registered as fungicidal biopreparations (Central institute for supervising and testing in agriculture, 2021).

2.4.3 Chemical control

Chemical control involves the use of fungicides to reduce the impact of plant pathogens in cropping systems. Currently, there are over 200 fungicides recommended for use in food production. About 57 different modes of action are available but only a few modes of action dominate the market. The three most popular classes of chemicals are the Demethylation inhibitors (DMI), Quinone outside inhibitors (QoI), and Succinate dehydrogenase inhibitors (SDHI). These classes have a single site mode of action that targets different pathways in fungal pathogens (FRAC, 2021).

In the control of phoma stem canker, fungicides are recommended for use in oilseed rape crops where the inoculum is high, cultivar resistance is low and there is a high yield potential (Steed et al., 2007; West et al., 1999). Depending on the region, 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 methods of chemical control (Marcroft & Potter, 2008; Van de Wouw et al., 2017). 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 (Fitt et al., 2006; West et al., 1999).

2.4.3.1 Chemical control of oilseed rape pathogens in the Czech Republic

All three fungicide groups (DMI, SDHI and QoI) are registered for control of oilseed rape diseases in the Czech Republic (Central institute for supervising and testing in agriculture, 2021). Fungicides are generally applied in autumn, but sometimes also in spring if oilseed rape

is planted intensively. DMI fungicides include triazoles (tebuconazole, prothioconazole, difenoconazole, metconazole, mefentrifluconazole, cyproconazole) and imidazoles (prochloraz). QoI fungicides such as methoxy-acrylates (azoxystrobin), methoxy-carbamates (pyraclostrobin), oximino-acetates (trifloxystrobin), oximino-acetamides (dimoxystrobin), and dihydro-dioxazines (fluoxastrobin) are some fungicide groups used in the Czech Republic. Also used are the SDHI e.g. pyridinyl-ethyl benzamides (boscalid) and pyridinyl-ethyl-benzamides (fluopyram). Another group is Benzamides such as pyridinylmethyl-benzamides (fluopicolide) (Central institute for supervising and testing in agriculture, 2021).

Demethylation inhibitor (DMI) fungicides: These are the preferred fungicides because of their low cost and effectiveness against a broad range of plant pathogens (Russell, 1995). In agriculture, the first triazole active ingredients introduced for use were imazalil and triadimefon. They have been very successful in controlling plant pathogenic fungi such as *Zymoseptoria tritici*, *Blumeria graminis*, *Mycosphaerella fijiensis* and *Venturia nashicola*. 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 (Parker et al., 2014; Marichal et al., 1999).

Quinone outside inhibitor (QoI) fungicides: These are an important class of fungicides also called the strobilurins. They were developed from natural derivatives such as strobilurin A, oudemasin A and myxothiazol. The first strobilurin active ingredient to be sold was azoxystrobin in 1996. Others are kresoxim-methyl, metominostrobin, trifloxystrobin, picoxystrobin, famoxadone and fenamidone (Bartlett et al., 2002). They have protectant and translaminar activity and are very good at inhibiting spore germination in some fungi, which is an energy-demanding stage in the fungal life cycle (Bartlett et al. 2002). Their mode of action is the inhibition of mitochondrial respiration by binding at the Qo site of cytochrome b. Cytochrome b is part of the cytochrome bc complex, located in the inner mitochondrial membrane of fungi and other eukaryotes. When one of the inhibitors binds, it blocks electron transfer between cytochrome b and cytochrome c, which in turn disrupts the energy cycle within the fungus by halting the production of ATP. Since the natural products and their synthetic analogues can displace each other from the binding site, they are reversibly bound. QoIs are

used in a wide variety of crops including cereals, fruits, tree nuts, vegetables, turf grasses and ornamental plants to control fungal diseases.

Succinate dehydrogenase inhibitors (SDHI) fungicides: They have been used internationally since the late 1960s. These are fungicides with protectant, systemic and translaminar activity depending on the host and pathogen. They are used for crops such as vegetables, cereals, grape, fruit, beans, onions, garlic and brassicas. SDHIs inhibit fungal respiration by blocking the ubiquinone binding sites in the mitochondrial complex II. Carboxin and oxycarboxin are known as the Generation I SDHIs. They are highly effective against basidiomycete pathogens such as rusts or *Rhizoctonia* spp. The newer generation or Generation II have a broad spectrum of activity against plant pathogens. They work 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, generation II SDHIs such as boscalid and fluopyram have a broad spectrum of activity against plant pathogens (Oliver & Hewitt, 2014). This group includes boscalid, fluxapyroxal, penthiopad, isopyrazum and fluopyram. They are intended for use in integrated disease management programmes or as mixing or alteration partners to prevent fungicide resistance (Avenot & Michailides, 2010).

2.4.3.2 Fungicides as growth regulators

Plant growth regulators (PGRs) are chemicals that are used to alter plant growth by increasing branching, suppressing shoot growth, increasing return bloom, removing excess fruit, or changing fruit maturity. These include auxins, gibberellins and inhibitors of gibberellin biosynthesis, cytokinins, and abscisic acid. Many fungicides appear in distinct isomeric forms, which means they can also act as growth regulators (Berry & Spink, 2009; Davis et al., 1988). In Europe, DMIs, including metconazole and tebuconazole, are currently used in oilseed rape cultivation as both fungicides and plant growth regulators (Davis et al., 1988; Fletcher et al., 1986; Luster & Miller, 1992; Coules et al., 2002; Zamani-Noor & Knüfer, 2018). Triazoles reduce the rate of photosynthesis by reducing stomatal conductance. Strobilurins on the other hand reduce ethylene concentration thus resulting in delayed senescence (Ijaz & Honermeier, 2012). Fungicides are used in oilseed rape as growth regulators usually at lower (ca ½)

concentration than as fungicides, which represents high risk of the resistance selection in fungal pathogens population.

2.4.3.3 Fungicide resistance

The continuous use of fungicides, especially those that are single-site specific, results in resistant strains or populations of pathogens, thereby reducing their efficacy and lifespan (Hollomon, 2015; Ma & Michailides, 2005). This has been reported in many plant-pathogen interactions with QoI, SDHI, and DMI fungicides (Cools & Fraaije, 2013; Dooley et al., 2016; Wierczorek et al., 2015; Dubos et al., 2013; Sierotzski & Scalliet, 2013)

SDHI fungicides are of medium to high risk (FRAC code 7). As a result, since their registration against many plant pathogenic fungi, resistance has been documented. Over 40 SdhB, SdhC and SdhD alterations of the SDHI binding site have been linked to reduced sensitivity to SDHIs. Some of these are C-T79N and C-N865 which confer moderate resistance and C-H152R which confers complete resistance to SDHIs (Avenot & Michailides, 2010; Sierotzki & Scalliet, 2013).

QoI fungicides (FRAC code 11) are classified as highly risky fungicides. According to molecular studies report that QoI fungicide-resistant strains result if a single point mutation changing glycine (G) to alanine (A) at a position 143 of the CYTB protein (G143A) occurs. G143A mutation is the most common mutation in plant pathogens resistant to QoI fungicides. It has been described as governing the expression of high resistance. Another mutation is F129L, which results in the change of phenylalanine (F) to leucine (L) at amino acid position 129 within CYTB (Bartlett et al., 2002; Fisher & Meunier, 2008). As a result, fungicide resistance to QoI fungicides has been seen in over 30 pathogen species including *Alternaria solani*, *Blumeria graminis*, *Plasmopara viticola*, *Magnaporthe oryzae*, *Pyricularia grisea*, *Venturia inaequalis*, and *Mycosphaerella graminicola* (Fraaije et al., 2002; Fraaije et al., 2005; Ishii et al., 2001; Kim et al., 2003; Ma & Michailides, 2003; Pasche et al., 2005; Siertzski et al., 2000; Steinfeld et al., 2001; Vincelli & Dixon, 2002; Wong & Wilcox, 2000).

DMI fungicides (FRAC code 3) are classified as medium risk fungicides. There are three main mechanisms of resistance to DMI fungicides: point mutations within the *CYP51* gene, overexpression of *the CYP51* gene, and overexpression of genes encoding efflux pumps. Point mutations in the *CYP51* enzyme are the most reported mechanism of resistance in field isolates.

These mutations can alter the fungicide's affinity for the enzyme, resulting in fungicide tolerance. An example of this mutation is in *Zymoseptoria tritici,* where over 30 different substitutions and deletions have been reported in the field. Overexpression of the *CYP51* gene in fungal pathogens is caused by mutations in the promoter region upstream of the *CYP51* gene by the insertion of tandem repeats or transposable elements. Overexpression of the target enzyme causes a reduction in the fungicide sensitivity exhibited by the fungus (Parker et al., 2014; Price et al., 2015). Therefore, higher doses or more active compounds are necessary to inhibit fungal growth. It has been observed in several fungi such as *Venturia inaequalis, Monilinia fructicola*, *Zymoseptoria tritici*, and *Penicillium digitatum*. In eukaryotic cells, efflux transporters are important in removing toxins and fungicides. Overexpression of the genes that encode these transporters can result in fungicide resistance. ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters are the most common genes linked to fungicide efflux. Examples of this can be seen in *Penicillium digitatum* (Sun et al., 2013)*, M. fructicola* (Luo & Schnabel, 2008) *Venturia inaequalis* (Schnabel & Jones, 2001), and *Zymoseptoria tritici* (Cools & Fraaije, 2013).

There have only been a few studies on the sensitivity of *L. maculans* to fungicides despite their vast use. There has been no report of resistance to any SDHI fungicide to date. In Canada nevertheless, results showed that *L. maculans* populations lost sensitivity to the QoI fungicide pyraclostrobin from 2011 to 2016. 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 (Huang et al., 2011; Eckert et al., 2010; Sewell et al., 2017). In Australia however, Van de Wouw et al. (2017) and Yang et al. (2020) found resistant isolates in *L. maculans* populations. There have been no studies on the sensitivity of *L. maculans* isolates to QoI or DMI fungicides in the Czech Republic.

Many molecular methods including the use of polymerase chain reaction (PCR) have been developed to detect resistance in various plant pathogenic fungi to fungicides. Huang et al. (2011) and Van de Wouw et al. (2017) also investigated whether differences in sensitivity among *L. maculans* isolates are as a result of changes within the *ERG11*, but no mutations were detected. However, 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 (Yang et al., 2020).

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, and gain insight into potential resistance problems. These would allow for the recommendation and implementation of anti-resistance strategies, thus delaying the selection pressure of a fungicide class (Thomas et al., 2012; Bauske et al., 2018).

2.5 Genetic Control

Genetic resistance is the most important and sustainable means of controlling phoma stem canker (Balesdent et al., 2001). It is also the most cost effective and environmentally sound method. Field resistance of oilseed rape to *L. maculans* is known to combine both quantitative and qualitative resistance. Quantitative resistance is said to be race non-specific and mediated by multiple minor genes of partial effect which reduces symptom severity and epidemic progression over time. It is very durable and can sometimes result in high levels of protection. This type of resistance is expressed at the adult stage of *Brassica* species (Delourme et al., 2006). Qualitative or R-gene mediated resistance on the other hand is controlled by one or a few major genes which are usually dominant and expressed at the seedling plant stage in cotyledons and first true leaves (Brun et al., 2010). This type of resistance obeys the gene-for-gene interaction model that states that for each resistance (*R*) gene in a host, there is a corresponding avirulence (*Avr*) gene in the pathogen (Flor, 1955).

Differential interactions between *Brassica* species and *Leptosphaeria maculans* pathosystems have been studied at the seedling stage using cotyledons. Using the differential interactions, the *L. maculans* isolates were classified into pathogenicity groups (PG) based on their virulence on the *Brassica* genotypes Westar, Quinta and Glacier. These pathogenicity groups were PG 2 (avirulent on Quinta and Glacier), PG 3 (avirulent on Quinta but virulent on Glacier) and PG 4 (virulent on all three cultivars) (Koch et al., 1991, Mengistu et al., 1991). Another classification system was by Badawy et al. (1991), who classified six pathogenic groups termed A1–A6 using four different genotypes Quinta, Glacier, Westar and Jet Neuf. These classifications however are limited as it can only result in either 4 or 6 pathogenicity groups based on whether the reaction is resistant or susceptible. After 18 more resistance genes were identified meaning that the number of possible pathotypes or races could theoretically be 262144 (Balesdent et al., 2005), a new classification system to accommodate all possible physiological races and overcome all the limitations of the PG system was proposed. Using this classification system, at least 18 major genes have been identified. These include *Rlm1, Rlm2, Rlm3, Rlm4, Rlm5, Rlm6, Rlm7, Rlm8, Rlm9, Rlm10, Rlm11, RlmS, LepR1, LepR2, LepR3, LepR4, BLMR1* and *BLMR2* (Marcroft et al., 2012) (Table 1).

About 16 avirulence (*Avr*) genes have been identified with the *Avr* genes of *L. maculans* named *AvrLm* or *AvrLep* to show to which resistance genes they correspond (Table 1). Some of these genes are located in genetic clusters like *AvrLm1-2-6* cluster (Balesdent et al., 2002) and the

AvrLm 3-4-7-9-AvrLepR1 cluster (Balesdent et al., 2005; Ghanbarnia et al., 2015) and of these, *AvrLm1* (Gout et al., 2007), *AvrLm4-7* (Parlange et al., 2009), *AvrLm6* (Fudal et al., 2007), AvrLm*11* (Balesdant et al., 2013) and *AvrLmJ1* (Van de Wouw et al., 2014) have been cloned. When pathogen avirulence genes are recognized directly or indirectly by plant resistance genes, a set of defence responses termed the effector triggered immunity which eventually leads to a hypersensitive response (Jones & Dangl, 2006).

Table 1 An overview of the *R* genes identified so far in *Brassica* spp. and their corresponding *Avr* genes in *Leptosphaeria maculans*

R gene	*Avr* gene
Rlm1	*AvrLm1–3*[d]
Rlm2	*AvrLm2*
Rlm3	*AvrLm3*
Rlm4	*AvrLm4–7*[de]
Rlm5	*AvrLm5–9*[d]
Rlm6	*AvrLm6*
Rlm7	*AvrLm4–7*[de]
Rlm8	*AvrLm8*
Rlm9	*AvrLm5–9*[d]
Rlm10	*AvrLm10A/AvrLm10B*[i]
Rlm11	*AvrLm11*
Rlms	*AvrLms*
LepR1	*AvrLepR1*
LepR2	*AvrLepR2*
LepR3	*AvrLm1–L3*[d]
LepR4	*AvrLepR4*
BLMR1	?
BLMR2	?

[d] dual recognition specificity genes

[e] gene of *AvrLm7* is epistatic to the gene of *AvrLm3* and the gene of *AvrLm9*

[i] interaction of two contiguous genes

? not yet identified

L. maculans has a high evolutionary potential which makes it able to adapt to novel resistance genes over time (McDonald & Linde, 2002). This resistance breakdown is as a result of sexual recombination during its life cycle, mutation, a high gene flow through large scale dissemination of ascospores and its large population size (Rouxel et al., 2003). Also, the intensive cultivation of oilseed rape genotypes with the same major gene has been reported to affect the durability of resistance genes over time. Examples of this include the rapid breakdown of Surpass resistance in Australia that led to the complete disappearance of the corresponding avirulent gene as well as the breakdown of the *Rlm1* resistance gene in France after only three years (Li et al., 2003; Sprague et al., 2006). One of the newest major genes, *Rlm7* has the fewest number of corresponding virulent isolates, making it one of the major genes still effective today. The combination of the high evolutionary potential of *L. maculans* and the effect intensive cultivation of genotypes with major genes makes *Rlm7* particularly susceptible to loss of durability (Daverdin et al., 2012).

2.6 Genetic variation in *Leptosphaeria maculans* populations

L. maculans has both asexual and sexual reproduction systems in its life cycle. The presence of sexual reproduction and airborne ascospores facilitates pathogen recombination, and long-distance dispersal, whereas asexual reproduction allows for mutations resulting in a rapid increase of virulent races (Hayward et al., 2012). Sexual reproduction is critical for generating genetic variation in *L. maculans* populations. *L. maculans* has only one mating type and two idiomorphs (MAT1.1 and MAT 1.2) (Cozijnsen & Howlett, 2003). In order to mate successfully, isolates must have different idiomorphs. As a result, rapid shifts in the field population can result from evolutionary processes such as mutation, migration, genetic drift, selection, and/or recombination. Knowledge of the amount and distribution of genetic variation within and among populations of a plant pathogen can assist in predicting the evolutionary potential of a pathogen to overcome major gene and quantitative resistance (McDonald & Linde, 2002).

A wide range of genetic markers can be used to study the genetic diversity of *L. maculans* populations in oilseed rape fields around the world. AFLP (amplified fragment length polymorphism), RFLP (restriction fragment length polymorphism), and RAPD (random amplified polymorphic DNA) fingerprinting studies have revealed that the populations of

L. maculans in Australia and Canada are made up of different alleles (Barrins et al., 2002; Goodwin & Annis, 1991; Purwantara et al., 2000). Following on from these techniques, newer methods based on variable number tandem repeat loci, such as minisatellites and microsatellites, have gained popularity due to their ability to produce size-specific alleles based on the repeat number. Minisatellites are made up of 10–60 bp short repeats, whereas microsatellites are made up of 1–6 bp repeated motifs. These are frequently used as molecular markers in genetic distance and population diversity studies. Several minisatellites from *L. maculans* have been identified and characterized over the last two decades (Attard et al., 2001; Eckert et al., 2005; Jedryczka et al., 2010). In the Czech Republic, no studies on the genetic structure of *L. maculans* field populations have been conducted.

3. Hypotheses

- The continuous use of fungicides both as plant protection agents and as growth regulators by oilseed rape farmers in the Czech Republic has increased the risk of resistance to fungicides in *L. maculans* populations.
- Avirulence genes are subject to mutational pressure which provides *L. maculans* the ability to overcome major gene resistance in oilseed rape. Knowledge of avirulence genes (*AvrLm*) in *L. maculans* populations and resistance genes (*Rlm*) in oilseed rape crops within a location would help with the deployment of oilseed rape cultivars.
- The genetic diversity in *L. maculans* populations represent a pool of possible variants that can result in an increase in virulent and fungicide resistant isolates.

4. Objectives

- To determine the efficacy of selected fungicides and level of sensitivity of individual *L. maculans* isolates against different fungicides (active ingredients and commercial fungicides).
- To survey the incidence of avirulence alleles and race structure of *L. maculans* isolates in all main cultivation areas of oilseed rape in the Czech Republic.
- To determine the genetic diversity and population structure of *L. maculans* isolates in the Czech Republic using mating types and 10 minisatellite markers.

5 Materials and methods

5.1 General materials and methods

5.1.1 Sample collection and spray history

Oilseed rape leaves showing typical phoma leaf spotting symptoms were collected from the fields of commercial growers and research stations in the Czech Republic during seven growing seasons (2014−2020) (Figure 10). Twenty-nine of these isolates were collected by the Agrotest Fyto Ltd between 2014 and 2017. In 2017, Krukanice, Lužany locality was sprayed with Caramba 0.5 l/ha (metconazole 60 g/l) and Stare Smrkovice (Chomutice) locality with Horizon 250 EW 2 × 0.5 l/ha (tebuconazole 250 g/l). The spray history of the other localities is unknown. From each leaf sample, a small section of infected tissue was excised, surface-sterilized in 20% bleach solution (1% NaOCl) for 3 min, rinsed in sterile distilled water three times, and placed in Petri dishes with three layers of moistened filter paper for two days to encourage the formation of pycnidia.

5.1.2 Pathogen isolation

Single pycnidium isolates:

To obtain *Leptosphaeria* spp. single pycnidium isolates, an individual pycnidium or ooze (cirrhus) containing pycnidiospores was harvested with a sterile inoculating needle under a stereomicroscope and placed into a Petri dish with PDA (potato dextrose agar; HIMEDIA LABORATORIES, Einhausen Germany) amended with chloramphenicol (100 µg/ml). After incubation at 20 °C in darkness for growth, single pycnidium isolates were subcultured by transferring a mycelial plug from the colony edge onto new growth medium.

Single pycnidiospore isolates:

To obtain single pycnidiospore isolates, an individual pycnidium was transferred to a sterile 0.5-ml microcentrifuge tube containing 50-µl sterile double-distilled water and crushed with a sterile pipette tip. Pycnidiospores released from the pycnidium were properly mixed and the

resulting pycnidiospore suspension was adjusted to a concentration of approximately 1000 pycnidiospores/ml using a light microscope and a Bürker hemocytometer slide. An aliquot of 100-µl containing about 100 pycnidiospores was transferred onto surface of PDA amended with chloramphenicol (100 µg/ml) in a Petri dish using an inoculating L-shaped spreader and incubated at 20 °C in darkness. A small agar plug with an individual hypha was picked up with a sterile inoculating needle under a stereomicroscope from a germinating pycnidiospore and placed into a new Petri dish and again incubated at the same conditions.

5.1.3 Maintenance and storage of *Leptosphaeria* species isolates

Petri dishes with *Leptosphaeria* isolates were sealed with thermoplastic film Parafilm M and maintained in darkness at 20 °C on PDA or 10% V8 agar (consisting of 100 ml V8 vegetable juice, 2 g $CaCO_3$, and 15 g agar per litre of double-distilled water) plates at 20 °C. To subculture *Leptosphaeria* isolates a small piece of mycelia was removed from the edge of the actively growing culture and transferring onto a new media plate. For long term storage, isolates were stored as pycnidiospore stocks in 25% glycerol at -80 °C.

5.1.4 Production of pycnidiospores

Fresh fungal mycelia of *L. maculans* isolates were crushed together with agar using a sterile spatula (or L-shaped spreader or a glass microscope slide) and grown on new 2% V8 juice agar media (consisting of 200 ml V8 vegetable juice, 3 g $CaCO_3$, and 16 g agar per litre of double-distilled water). Petri dishes were fixed with 3M Micropore^{TM} tape and incubated under 16 h fluorescent light at 20 °C and 90% relative humidity to enhance pycnidia production. About 10–14 days when pycnidia were mature, inoculum was prepared by flooding the Petri plates with 10–15 ml of sterile double-distilled water and gently scratching the surface of plates with a sterile glass rod. The pycnidia was then filtered using an autoclaved filter cloth with a mesh size of about 0.7 mm. Using a light microscope and a Bürker haemocytometer slide, the resulting pycnidiospore suspension was adjusted to a concentration of 1×10^7 spores/ml. They were then stored in 0.5-ml or 2-ml microcentrifuge tube at -20 °C or as pycnidiospore stocks in 25 % glycerol at -80 °C until needed.

5.1.5 DNA extraction

Fresh mycelia of *Leptosphaeria* isolates were ground in liquid nitrogen using a mortar and pestle. Genomic DNA was extracted using the GenElute Plant Genomic DNA Miniprep Kit (Sigma Aldrich®, St. Louis MO, USA) according to the manufacturers' instructions. DNA was resuspended in 100 µl of elution buffer and stored at -20 °C.

5.1.6 Differentiation between *L. maculans* and *L. biglobosa* isolates

All isolates were first characterised to species using both cultural and genetic characteristics (Fitt et al., 2006; Williams & Fitt, 1999; Liu et al., 2006, Mahuku et al., 1996). The isolates were confirmed as either *L. maculans* or *L. biglobosa* according to the presence or absence of yellow pigment on the PDA plates. Those isolates that did not produce yellow pigment in solid medium were classified as *L. maculans*. The isolates that secreted yellow pigment into PDA medium were classified as *L. biglobosa* (Williams & Fitt, 1999).

Genetic characterization was done using species-specific primers by Liu et al. (2006) and Mahuku et al. (1996) (Table 2). The solution for multiplex PCR of 25 µl for Liu et al. (2006) primers contained sterile double-distilled water, 1 µl (~100 ng) DNA template, 1× buffer for the *Taq* polymerase, 2.5 mM $MgCl_2$, 0.25 mM each of dNTP, 0.4 µM each of the relevant primers, and 1 U *Taq* polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Amplification conditions 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). Reactions with Mahuku et al. (1997) primers, were made up of 1 µl (~100 ng) DNA template, 24 µl master reaction mixture consisting of sterile double-distilled water, 1× buffer for the *Taq* polymerase, 2.5 mM $MgCl_2$, 0.25 mM each of dNTP, 0.4 µM each of the relevant primers, and 1 U *Taq* DNA polymerase. The total reaction volume was 25 µl. Amplification conditions were 95 °C for 2 min; 35 cycles at 94 °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× TBE (89 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.3) at 100 V for 60 min. MassRuler DNA Ladder Low Range (Thermo Fisher Scientific) was included in each gel as a molecular size standard. Visualisation was performed

with the gel documentation system InGenius LHR and GeneSnap software (Syngene, Synoptics Ltd, Cambridge, UK).

Table 2. Forward and reverse primers used in the differentiation of *Leptosphaeria maculans* and *L. biglobosa* isolates.

LmacR was used as a reverse primer for both *L. maculans* and *L. biglobosa* isolates

Pathogen	Primer	ITS sequence (5´–3´)	Reference
L. maculans	LmacF	CTTGCCCACCAATTGGATCCCCTA	Liu et al., 2006
	LmacR	GCAAAATGTGCTGCGCTCCAGG	
	HV17S	CCCATTTTCAAAGCACTGCC	Mahuku et al., 1996
	HV26C	GAGTCCCAAGTGGAACAAACA	
L. biglobosa	LbigF	ATCAGGGGATTGGTGTCAGCAGTTGA	Liu et al., 2006
	LmacR	GCAAAATGTGCTGCGCTCCAGG	
	WV17S	CCCTTCTATCAGAGGATTGG	Mahuku et al., 1996
	WV58C	GCTGCGTTCTTCATCGATGC	

5.2 Experiment 1: Fungicide Sensitivity

5.2.1 Fungicides used

Nine fungicide active ingredients (Pestanal®, Sigma Aldrich®, St. Louis MO, USA) belonging to the DMI (Demethylation inhibitors), SDHI (Succinate dehydrogenase inhibitors) and QoI fungicides (Quinone outside inhibitors) chemical groups (Table 3) were used. In addition, commercial fungicides mixtures commonly used in the Czech Republic were tested. These were Caramba, Tilmor, Efilor, Horizon 250 EW, Bumper Super and Pictor (Table 4).

Technical grade fungicides were dissolved in dimethyl sulphoxide (DMSO) to produce a 10 mg/ml stock solution. Commercial fungicides were dissolved in sterile double-distilled water to produce a 1000 mg/ml stock solution. The stock solutions were stored at room temperature before dilution for the *in vitro* experiments.

Table 3. Active ingredients used in testing efficacy on individual *L. maculans* isolates collected in the Czech Republic.

Active ingredient	Chemical group	Group name	Mode of action
Tetraconazole	Triazole	DMI (Demethylation inhibitors)	Inhibits the biosynthetic pathway of ergosterol required for fungal cell membranes
Tebuconazole			
Metconazole			
Prothioconazole			
Propiconazole			
Prochloraz	Imidazole		
Boscalid	Pyridine-Carboxamides	SDHI (Succinate dehydrogenase inhibitors)	Inhibit fungal respiration by blocking the ubiquinone binding sites in the mitochondrial complex II
Trifloxystrobin	Oximino-acetates	QoI fungicides (Quinone outside inhibitors)	Inhibits mitochondrial respiration by disrupting the cytochrome bc1 in complex III
Dimoxystrobin	Oximino-acetamides		

Table 4. Commercial fungicides used in this study, their active ingredients and the concentration of active ingredient used in this study.

Fungicide	Active Ingredient	Concentration (µg/ml)								
Horizon 250 EW	Tebuconazole (250 g/l)	0	0.0001	0.001	0.01	0.1	1	10	100	1000
Bumper Super	Prochloraz (400 g/l)	0	0.0004	0.0044	0.044	0.44	4.4	44	444	4444
	Propiconazole (90 g/l)	0	0.0001	0.001	0.01	0.1	1	10	100	1000
Tilmor	Prothioconazole (80 g/l)	0	0.0001	0.001	0.01	0.1	1	10	100	1000
	Tebuconazole (160 g/l)	0	0.0002	0.002	0.02	0.2	2	20	200	2000
Pictor	Dimoxystrobin (200 g/l)	0	0.001	0.01	0.1	1	10	100	1000	
	Boscalid (200 g/l)	0	0.001	0.01	0.1	1	10	100	1000	
Efilor	Boscalid (133 g/l)	0	0.0022	0.022	0.22	2.2	22	222	222	
	Metconazole (60 g/l)	0	0.001	0.01	0.1	1	10	100	1000	
Caramba	Metconazole (60 g/l)	0	0.001	0.01	0.1	1	10	100	1000	

5.2.2 *In vitro* sensitivity of *L. maculans* isolates to fungicides using the mycelium growth plate method

Stock solutions of technical grade fungicides were diluted in dimethyl sulphoxide (DMSO) to produce the following concentrations: 0, 0.001, 0.01, 0.1, 1 and 10 µg/ml. Petri plates amended only with DMSO (1 µl/ml) were used as controls. Similarly, for the commercial fungicides, stock solutions were dissolved in sterile double-distilled water to produce final concentrations (Table 4). 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 9 cm Petri dishes containing the cooling amended media (10% V8 juice agar) after autoclaving. There were three replicates for each isolate. Isolates were incubated in the dark at 20 °C for 14 days. 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.

5.2.3 *In vitro* sensitivity of *L. maculans* isolates to fungicides using the microtitre plate assay

In vitro assessment of inhibition of pycnidiospores was carried out using methods modified from those of Pijls et al. (1994) and Sewell et al. (2017).

For the fungicide sensitivity test, 2× Potato dextrose broth (PDB) was amended with technical grade tetraconazole, metconazole, and prochloraz at twelve increasing concentrations of 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 mycelium growth plate method. Here, fungicide amended media (100 µl) was added to wells of flat bottom 96-well microtitre plate (GAMEDIUM spol. s.r.o., Jesenice u Prahy, Czech Republic). Also, pycnidiospore suspensions (100 µl) containing about 1×10^6 spores was 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 pycnidiospore germination as indicated by absorbance were measured with the TECAN sunrise plate reader (Tecan Austria GmbH, Grödig, Austria), software Magellan V7.2 at a wavelength of 630 nm in endpoint mode.

5.2.4 PCR amplifications 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 (5´-AGCACCCATGGACCACGG-3´) and EPS6-R (5´-CAGGATAAAGGAGGCGAAG-3´) and amplification temperatures (95 °C for 2 min; 35 cycles of 94 °C for 40 s, 58 °C for 30 s, and 72 °C for 1 min; and 72 °C for 5 min) by Yang et al. (2020).

5.2.5 Data analysis

In the fungicide sensitivity experiments, the effective concentration at which 50% fungal growth was inhibited (EC_{50}) for each isolate was calculated (Wong & Midland, 2007; Wong & Wilcox, 2002). The EC_{50} of each fungicide was determined by non-linear regression (curve-fit) using GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, United States). 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 non-parametric form of correlation, Spearman's rank correlation test was performed on $logEC_{50}$ values to test sensitivity associations.

5.3 Experiment 2: Determination of avirulence alleles and race structure of *L. maculans* isolates in the Czech Republic

5.3.1 Plant materials and experimental design

Seven host differential genotypes of oilseed rape with previously characterized major resistance genes (*Rlm*, Table 5) were requested from abroad (INRA, Institut de Génétique Environnement et Protection des Plantes, France) and multiplied. These genotypes were Columbus, Bristol, Jet Neuf, MT29, 02.22.2.1, 01.23.2.1, and Goeland. The genotype Westar (harbouring no known major genes) served as a control for successful infections. Seeds of genotypes were sown in plastic trays (6 cm × 6 cm) filled with the potting soil or perlite. Each column in the plastic tray represented one cultivar. One seed per cultivar was sown in each cell. Plants were grown under controlled conditions in a 14/10 h day/night cycle, at 22/20 °C, and light intensity 150 μmol m^{-2} s^{-1}.

Table 5 Differential set of *B. napus* genotypes used for detection of avirulence genes (*AvrLm*) of *Leptosphaeria maculans* isolates

Genotype	Major resistance genes	References
Columbus	*Rlm1*, *Rlm3*	Balesdent et al., 2006
Bristol	*Rlm2*, *Rlm9*	Balesdent et al., 2006
Jet Neuf	*Rlm4*	Balesdent et al., 2006
MT29	*Rlm1,9*	Delourme et al., 2008
02.22.2.1	*Rlm3*	Delourme et al., 2006
01.23.2.1	*Rlm7*	Delourme et al., 2004
Goeland	*Rlm9*	Balesdent et al., 2006
Westar	No resistance gene	Balesdent et al., 2002

5.3.2 Cotyledon inoculation and disease assessment

Pycnidiospores isolated from oilseed rape cultivars in the field were used as inoculum. Inoculation was carried out according to a method developed by Williams & Delwiche (1979). Cotyledons of the differential genotypes were inoculated with the *L. maculans* isolates 10 days after transplanting. A small puncture was made with a needle on the four lobes of each cotyledon. Ten microliters of inoculum (10^7 pycnidiospores/ml) were then pipetted on each of the four small punctures. This was done to find which isolates of *L. maculans* were virulent and which isolates were avirulent on the genotypes. There were 10 replications for each genotype. To maintain high humidity, trays containing the inoculated plants were covered with a black polyethylene cover film for 48 hours and placed into the growth chamber. After 4 days, the true leaves were removed from all plants to delay the senescence of cotyledons.

5.3.3 Scoring

After 14 days, cotyledons of each plant were evaluated for interaction phenotype (IP) and visually scored using a rating scale between 0 (no symptoms) and 9 (severe symptoms) based on lesion size and necrosis or chlorosis (Koch et al., 1991) (Figure 9). Plants that had a rating scale of 0–4 when inoculated with the *L. maculans* isolates would be considered resistant and the *L. maculans* isolates would be considered as having the avirulence allele of the resistance gene, while those having a rating of 5–9 when inoculated would be considered susceptible with the *L. maculans* isolates lacking that avirulence allele (Figure 9). Races of each *L. maculans* isolate were marked according identified avirulence genes (*AvrLm*).

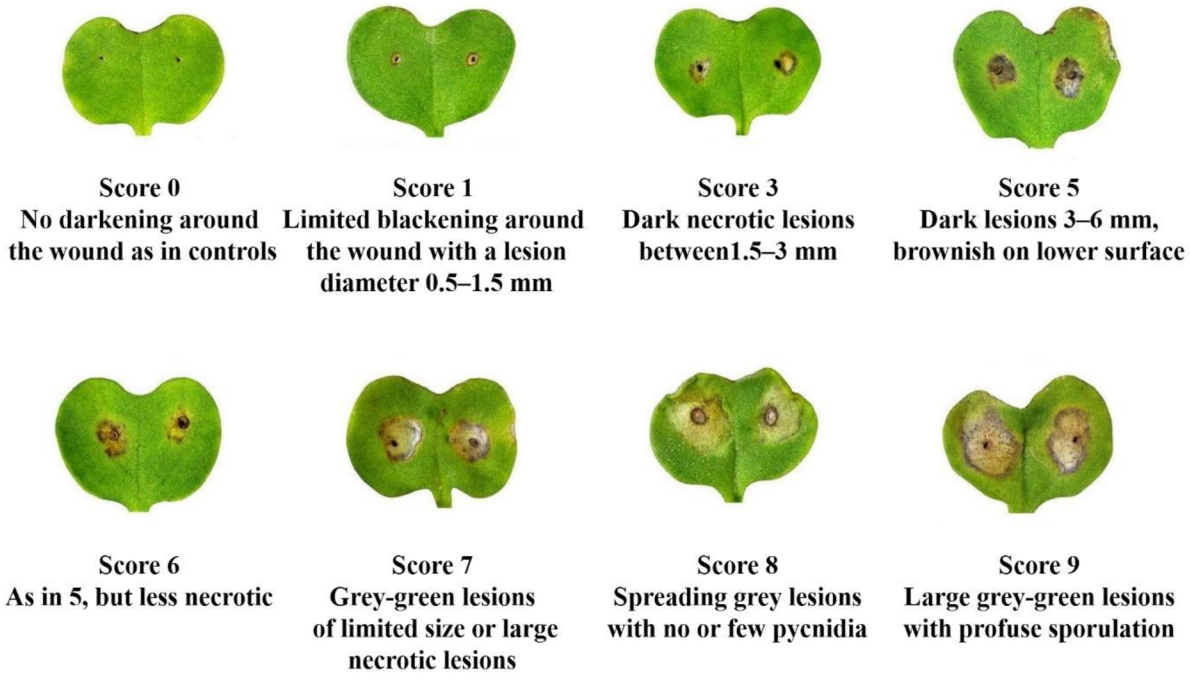


Figure 9. Rating scale by Koch et al. (1991), showing symptom characteristics which was used to score disease severity of *Leptosphaeria maculans* on cotyledons of *Brassica napus* to aid in the visual assessment of the cotyledons.

5.3.4 Data analysis

The number of compatible (susceptible) reactions observed in the cotyledon tests was represented relative to the number of isolates collected per region for phenotypic characterization of the *L. maculans* populations. To compare race diversity across sampling regions, the Margalef index (DMg) was calculated using the formula: DMg = (S-1) / [LN (N)], where S = number of races and N = total number of isolates per site in each region.

5.4 Experiment 3: Mating Type and genetic variability

5.4.1 Multiplex PCR for determination of mating types

A multiplex PCR assay previously designed by Cozijnsen & Howlett (2003) was used to determine the distribution of MAT 1.1 and MAT 1.2 *L. maculans* idiomorphs collected (Table 6). PCR reactions were set up in a 25 µl volume containing 1 µl DNA template, 1× buffer for the *Taq* polymerase, 2.5 mM $MgCl_2$, 0.25 mM each of dNTP, 0.4 µM each of the relevant primers, and 1 U *Taq* DNA polymerase (Thermo Fisher Scientific). The reactions were run according to the following protocol: 95 °C for 2 min; 35 cycles at 94 °C for 60 s, 58 °C for 30 s; 72 °C for 60 s; and a final extension at 72 °C for 4 min. All PCR reactions were performed using the DNA Engine Thermal Cycler (Bio-Rad Laboratories, Hertfordshire, UK). The samples were loaded on 1% agarose gel and run for 1 hour at 100 V.

Table 6. Forward and reverse primers used in the differentiation of *Leptosphaeria maculans* isolates into MAT 1.1 and MAT 1.2

MAT-R was used as a reverse primer for both *L. maculans* MAT 1.1 and MAT 1.2 idiomorphs.

Primer	Sequence (5´–3´)	Reference
MAT1.1F	CTCGATGCAATGTACTTGG	Cozijnsen & Howlett, 2003
MAT1.2F	AGCCGGCGGTGAAGTTGAAGCCG	
MAT-R	TGGCGAATTAAGGGATTGCTG	

5.4.2 Minisatellite analyses

Ten minisatellite markers (MinLm1, MinLm585, MinLm555, MinLm2452, MinLm5, MinLm6, MinLm935-2, MinLm2451, MinLm4, and MinLm8) were used to analyse genetic diversity (Table 7) of Czech *L. maculans* populations. All DNA samples were adjusted to a final concentration of 50 ng/µl in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Loci were amplified via PCR in 25 µl reactions containing 20.65 µl sterile double-distilled water, 1× buffer for the *DreamTaq* polymerase, 0.25 mM each of dNTP, 0.4 µM each of the relevant primers, and 1 U *DreamTaq* DNA polymerase (Thermo Fisher Scientific) and 1.0 µl DNA. PCR

amplifications were carried out as above. PCR products were separated by electrophoresis on 2% agarose gels containing ethidium bromide with a concentration of 0.5 µg/ml and electrophoresed in 1× TBE at 100 V for 60 min. Visualisation was performed with the gel documentation system InGenius LHR and GeneSnap software (Syngene, Synoptics Ltd, Cambridge, UK). A GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific) was used included in each gel as a molecular size standard.

Table 7. Primer pairs of 10 minisatellite markers used in this study to study the genetic diversity and population structure.

Primer	Sequence (5´–3´)	Motif size (bp)	Annealing temperature (°C)	Reference
MinLm1F	CACCCACATCTCCCTCTTAC	23	59	Attard
MinLm1R	GGGAGGGGGAGGTGTGTATG			et al., 2001
MinLm585F	GTCCAAGAGGGGTCTAATG	26	60	Jedryczka
MinLm585R	TGCAATACCTATCAACTATGCTA			et al., 2010
MinLm555F	CACTGTCATTCCTCCTCCTGGTT	63	60	Jedryczka
MinLm555R	AATTGAATGATTTGCGACACA			et al., 2010
MinLm2452F	GTACATGGGCGGACAGGC	21	60	Jedryczka
MinLm2452R	CATTTACACTGCACACCTGCTCA			et al., 2010
MinLm5F	TACGCTTCTCATTCGGTCCT	16	60	Eckert
MinLm5R	CGGGTGGCAGCATTCAC			et al., 2005
MinLm6F	GGAAGGAACACACGGTGAC	21	60	Eckert
MinLm6R	AATTGAATGATTTGCGACACA			et al., 2005
MinLm935-2F	AGTAGGCAACACAACAGCACACA	39	58	Jedryczka
MinLm935-2R	CCCTCTCTGCCATTTTCCATTAG			et al., 2010
MinLm2451F	GGGGCGAATGGTATGTTTATAGT	24	58	Jedryczka
MinLm2451R	CGGACACAATACTCACCACCTC			et al., 2010
MinLm4F	ACCAGGTGGAGTTGATAACAT	51	55	Eckert
MinLm4R	TCCTGCGAATCCCATTAG			et al., 2005
MinLm8F	ATTTGCTGGCGGTGTAGGTA	27	53	Travadon
MinLm8R	TGTTTGTACATGTGGTAAGTAAAGCA			et al., 2011

5.4.3 Data analysis

Mating type distribution was analysed with a χ^2 test. Polymorphic DNA bands were scored manually to determine the number of haplotypes and create input file formats required for other analysis. Genetic analysis data were generated using minisatellite markers. The total number of alleles at each locus and in each population was determined. The genotypic richness, represented by the number of observed multilocus genotypes (MLG) and expected MLG (eMLG); genotypic evenness (E.5) and genotypic diversity in the form of Simpson's index (λ) and Nei's unbiased gene diversity (Hexp) were calculated using the raw data set in the *poppr* package of the R statistical language, v2.9.3 (Kamvar et al., 2014; Kamvar et al., 2015; Jombart, 2008; Thioulouse et al., 2018; R Core Team, 2022). The R statistical language was also used to test linkage disequilibrium with a standardized index of association (rbarD) using 999 permutations. The population structure was determined using STRUCTURE HARVESTER (Earl & VonHoldt, 2012; STRUCTURE v2.3.4; Hubisz et al., 2009; Evanno et al., 2005).

6. Results

6.1 Isolate collection and identification

More *L. maculans* (n = 530) than *L. biglobosa* isolates (n = 13) were collected during the seven growing seasons 2014–2020 from 10 regions in the Czech Republic (Figure 10). *L. biglobosa* isolates were only found in the Pilsen region; Lužany (n = 4) and Pernarec (n = 9). A large-scale sampling of oilseed rape genotypes was collected from research stations where "trap plants" (known to harbour no major resistance genes), genotypes that harbour major resistance genes, and new breeding lines were planted.

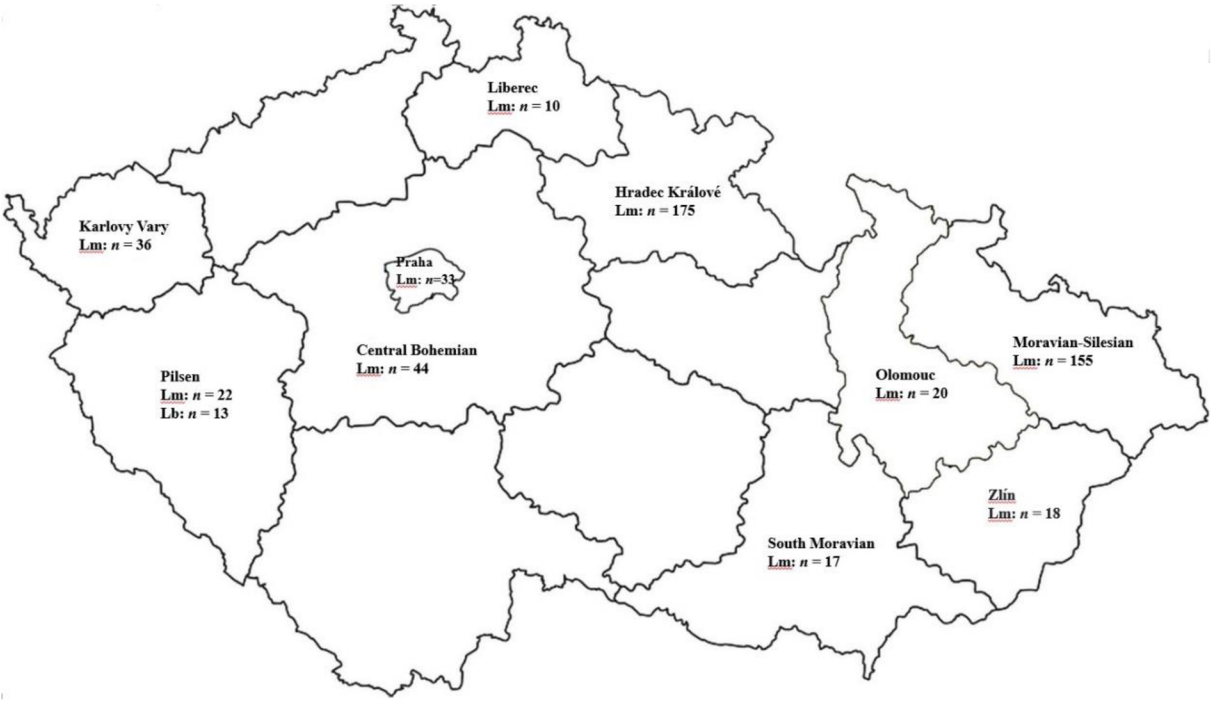


Figure 10. Map of the Czech Republic showing 474 *Leptosphaeria maculans* (Lm) isolates and 13 *Leptosphaeria biglobosa* (Lb) isolates collected from 10 regions in 2014, 2015, 2016, 2017, 2019 and 2020. n represents number of isolates collected per region.

Based on cultural characteristics on PDA (Figure 11) and PCR assay using species specific primers (Figure 12), isolates were classified as either *L. maculans* or *L. biglobosa*. PCR reaction with LmacF, LbigF/LmacR primers (Liu et al., 2006) yielded either 331 or 444 bp DNA fragments, indicating their affiliation to *L. maculans* or *L. biglobosa* species, respectively

(Figure 12 A B). Primer sets HV17S/HV26C and WV17S/WV58C (Mahuku et al., 1996) amplified all *L.* maculans and *L.* biglobosa samples, respectively, with PCR products of approximately 370 and 230 bp (Figure 12 B), respectively. There were no discrepancies between the cultural characteristics and results of PCR assay used to identify isolates to species level.

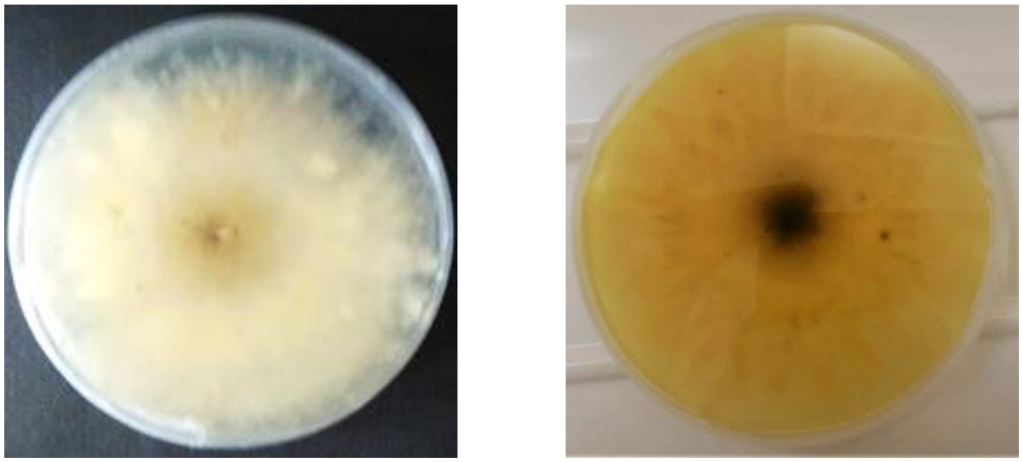


Figure 11. Differentiation between *Leptosphaeria maculans* (left) and *L. biglobosa* (right) based on cultural characteristics on PDA. *L. biglobosa* isolates give a yellow pigmentation which is absent in *L. maculans* isolates.

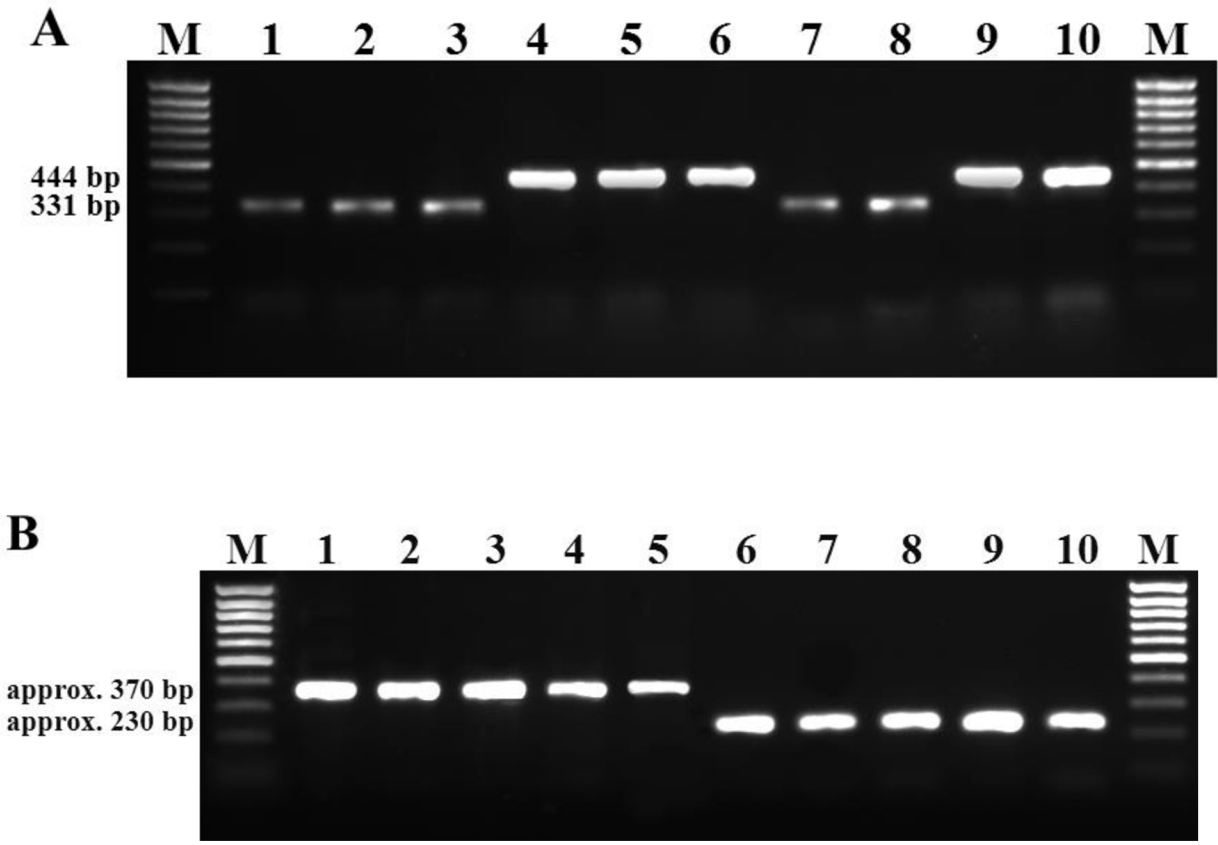


Figure 12 Differentiation between *Leptosphaeria maculans* and *L. biglobosa* based on using LmacF, LbigF/LmacR primers (A) and HV17S/HV26C and WV17S/WV58C primers (B)
In figure A: isolates of *L. maculans* are shown in lanes 1–3 and 7–8; isolates of *L. biglobosa* were shown in lanes 4–5 and 9–10. In figure B: isolates of *L. maculans* are shown in lanes 1–5; isolates of *L. biglobosa* were shown in lanes 6–10. Lane M: MassRuler DNA Ladder Low Range.

6.2 Experiment 1: Fungicide Sensitivity

6.2.1 Mycelium growth plate assay – active ingredients

6.2.1.1 *In vitro* sensitivity of *Leptosphaeria maculans* isolates to the SDHI fungicide boscalid

The EC_{50} values of the 118 isolates tested for sensitivity to boscalid ranged from 0.014–0.989 µg/ml (mean: 0.26 µg/ml; variation factor: 70.64). All isolates were 100% inhibited by boscalid at the highest concentration of 10 µg/ml (MIC = 1–10 µg/ml). The frequency distribution for the isolates collected between 2014 and 2019 showed a unimodal curve skewed to the right (Figure 13).

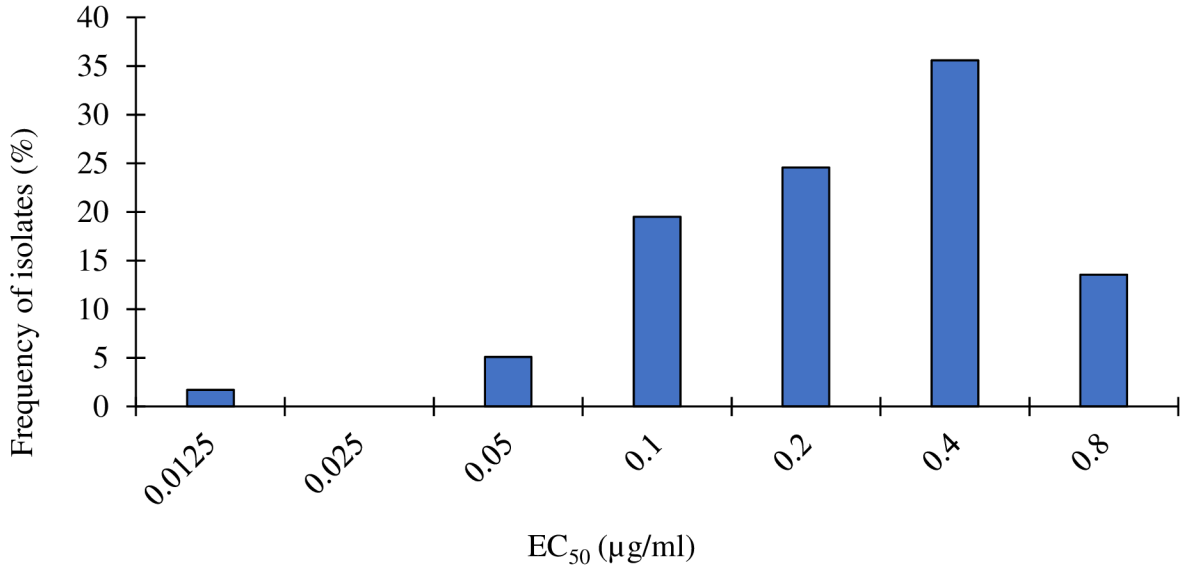


Figure 13. Frequency distribution of the sensitivity of 118 *Leptosphaeria maculans* isolates collected from oilseed rape fields between 2014 and 2017 to boscalid.
Individual isolates are grouped in class intervals when following interval is twofold the previous interval. Values on the x-axis indicate the midpoint of the interval.

6.2.1.2 *In vitro* sensitivity of *Leptosphaeria maculans* isolates to the QoI fungicides dimoxystrobin and trifloxystrobin

The mean, minimum and maximum EC_{50} values of 109 isolates tested for sensitivity to trifloxystrobin were 0.03, 0.004, and 0.34 µg/ml, respectively (variation factor: 77.40). At the highest concentration of 10 µg/ml, none of the isolates were completely inhibited by trifloxystrobin. All 109 isolates however had EC_{50} values below 1 µg/ml (Figure 14).

The mean, minimum and maximum EC_{50} values of 50 isolates tested for sensitivity to dimoxystrobin were 1.87, 0.02, and 95.59 µg/ml, respectively (variation factor: 4713.5). At the highest concentration of 10 µg/ml, only 62 % of the isolates were completely inhibited by dimoxystrobin, thus making it difficult to determine the MIC for the other 38 %. Forty-six percent of the isolates had EC_{50} values below 1 µg/ml (Figure 14).

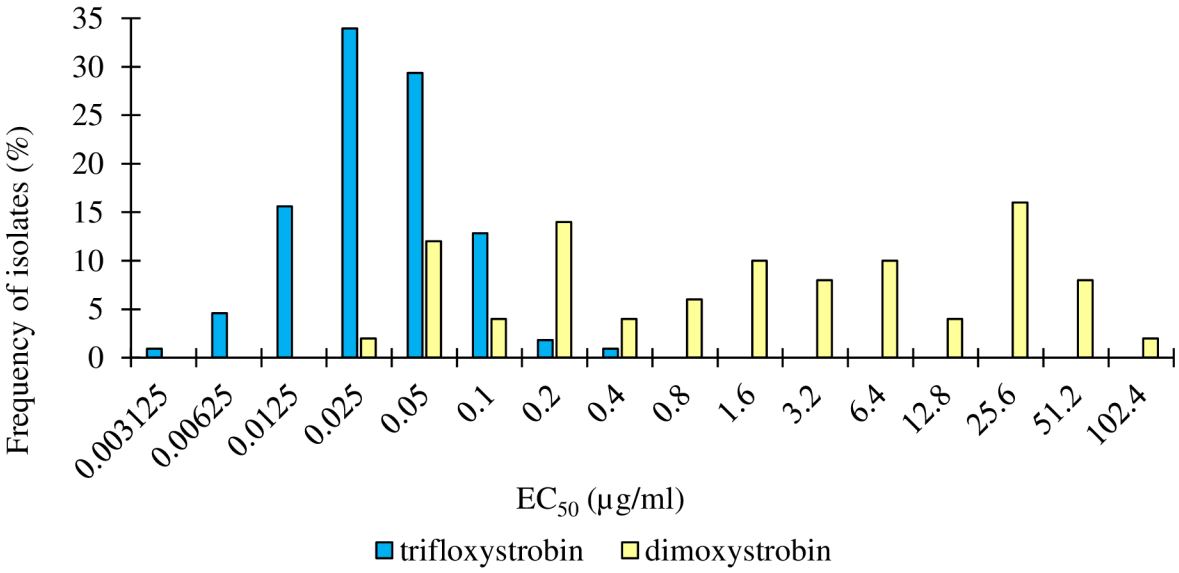


Figure 14. Frequency distribution of the sensitivity of *Leptosphaeria maculans* isolates collected from oilseed rape fields between 2014 and 2017 to trifloxystrobin and dimoxystrobin. Individual isolates are grouped in class intervals when following interval is twofold the previous interval. Values on the x-axis indicate the midpoint of the interval.

6.2.1.3 *In vitro* sensitivity of *Leptosphaeria maculans* isolates to the DMI fungicides tetraconazole, metconazole, prochloraz, prothioconazole, tebuconazole, and propiconazole

The mean, minimum and maximum EC_{50} values of 112 isolates tested for sensitivity to tetraconazole were 1.49, 0.28, and 6.47 µg/ml, respectively (variation factor: 23.26). At the highest concentration of 10 µg/ml, only 11 % of the isolates were completely inhibited by tetraconazole, thus making it difficult to determine the MIC for the other 89 %. Three percent of the isolates had EC_{50} values below 1 µg/ml (Figure 15).

Fifty-three *L. maculans* isolates tested for sensitivity to metconazole had EC_{50} values ranging from 0.02 to 1.73 µg/ml, representing an 84.88-fold variation factor (mean EC_{50} = 0.77 µg/ml). Seventy-one percent of the isolates had an MIC between 1 and 10 µg/ml, while the other 29 % had minimum inhibitory concentrations greater than 10 µg/ml. Forty-seven percent of the isolates had EC_{50} values above 1 µg/ml (Figure 15).

The mean, minimum, and maximum EC_{50} values of 55 isolates tested for sensitivity to prochloraz were 0.40, 0.02, and 1.77 µg/ml, respectively (variation factor: 78.88). Seven percent of the isolates had an MIC between 0.1 and 1 µg/ml; 82 % of the isolates had an MIC between 1 and 10 µg/ml; while the remaining 11 % had an MIC above 10 µg/ml. Seventy-five percent of the isolates had EC_{50} values above 1 µg/ml (Figure 15).

The mean, minimum and maximum EC_{50} values of 52 isolates tested for sensitivity to tebuconazole were 1.52, 0.11, and 2.42 µg/ml, respectively (variation factor: 21.74). At the highest concentration of 10 µg/ml, only 10 % of the isolates were completely inhibited by tetraconazole, thus making it difficult to determine the minimum inhibitory concentration (MIC) for the other 90 %. Six percent of the isolates had EC_{50} values below 1 µg/ml (Figure 15).

The mean, minimum and maximum EC_{50} values of 59 isolates tested for sensitivity to propiconazole were 1.06, 0.13, and 2.42 µg/ml, respectively (variation factor: 17.96). At the highest concentration of 10 µg/ml, only 37 % of the isolates were completely inhibited by tetraconazole, thus making it difficult to determine the MIC for the other 63 %. Thirty-two percent of the isolates had EC_{50} values below 1 µg/ml (Figure 15).

The mean, minimum and maximum EC_{50} values of 56 isolates tested for sensitivity to prothioconazole were 0.63, 0.0015, and 1.82 µg/ml, respectively (variation factor: 1176.888).

At the highest concentration of 10 µg/ml, only 63 % of the isolates were completely inhibited by prothioconazole, thus making it difficult to determine the MIC for the other 37 %. Forty-six percent of the isolates had EC_{50} values below 1 µg/ml (Figure 15).

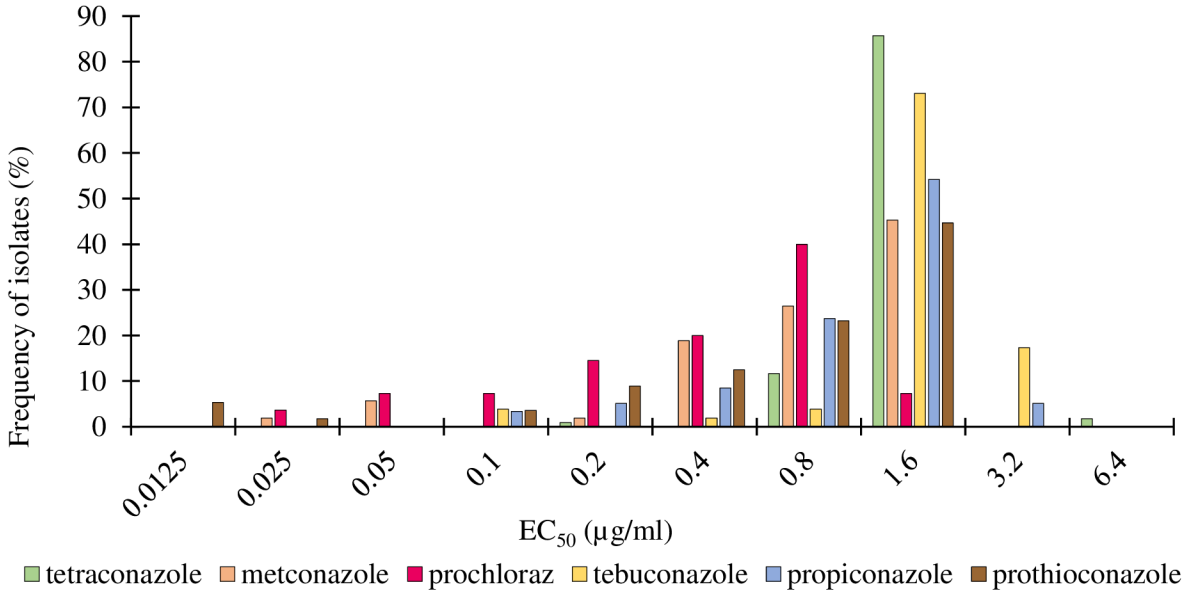
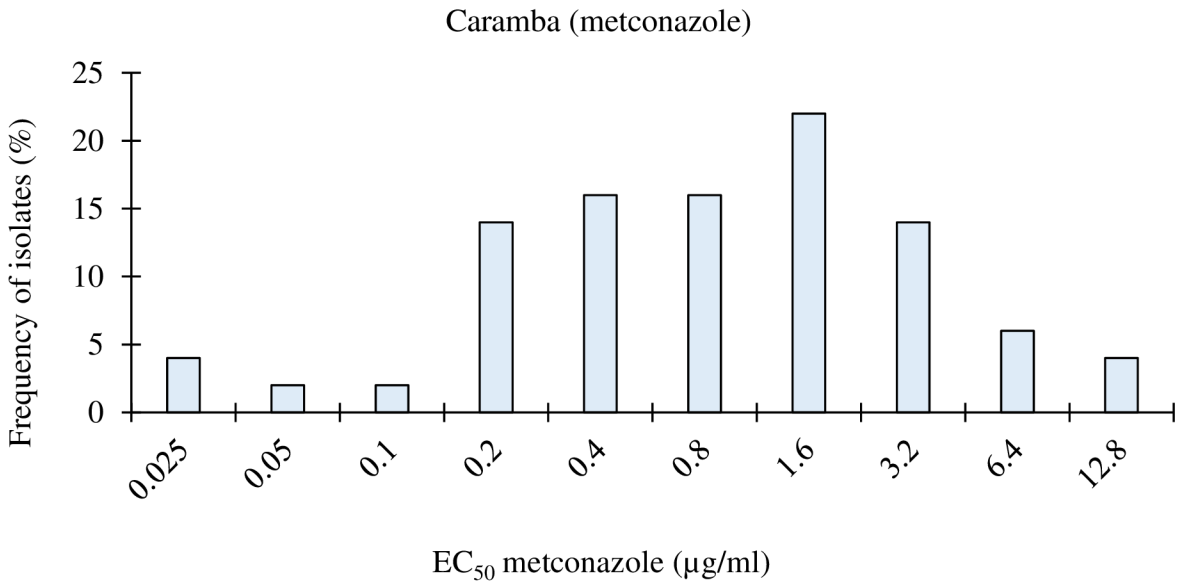


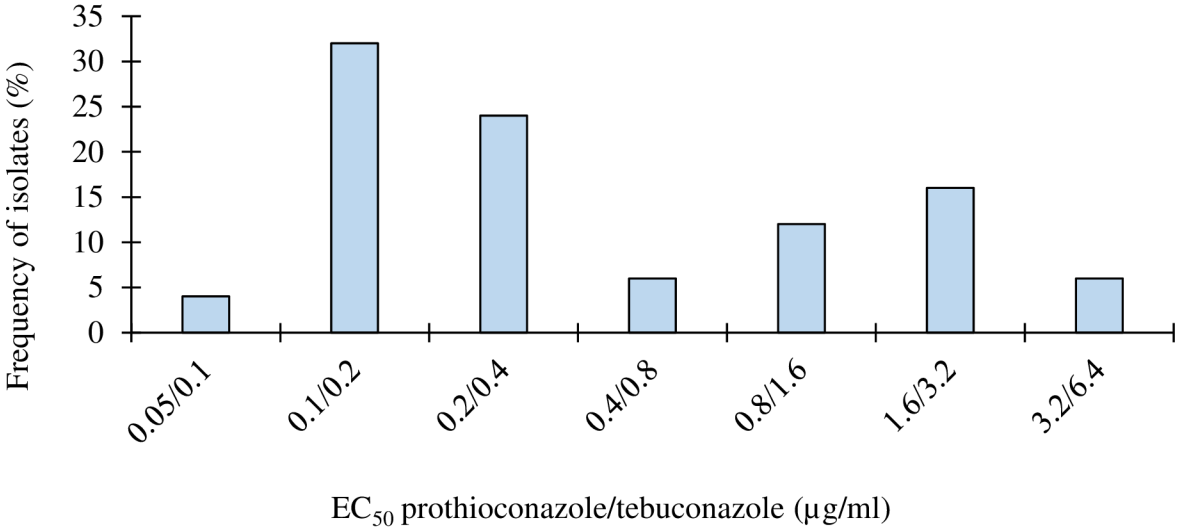
Figure 15. Frequency distribution of sensitivity of *Leptosphaeria maculans* isolates collected between 2014 and 2017 from the Czech Republic to the DMI fungicides tetraconazole, metconazole, prochloraz, tebuconazole, propiconazole, and prothioconazole
Individual isolates are grouped in class intervals when following interval is twofold the previous interval. Values on the x-axis indicate the midpoint of the interval.

6.2.1.4 *In vitro* sensitivity of *Leptosphaeria maculans* isolates to commercial mixtures

Fifty *L. maculans* isolates were tested for their sensitivity to six commercial mixtures: Caramba, Tilmor, Pictor, Horizon 250 EW, Bumper and Efilor. The mean, minimum and maximum EC_{50} values of isolates tested for sensitivity to Caramba (metconazole) were 0.91, 0.014, and 15.9 µg/ml. For Tilmor, which is composed of tebuconazole and prothioconazole, the mean EC_{50} values were 0.63 (range: 0.001–9.169) and 0.32 (range; 0.00053–4.58) µg/ml, respectively. Pictor which is composed of an equal amount of dimoxystrobin and boscalid had mean, minimum and maximum EC_{50} values of 0.36, 0.086 and 1.65 µg/ml, respectively. Horizon is made up of tebuconazole. Tebuconazole had a mean EC_{50} of 2.88 and range of 0.01–16.5 µg/ml, while prochloraz had a mean of 12.66 µg/ml and range of 0.44–72.87 µg/ml. Bumper is made up of prochloraz and propiconazole. Prochloraz had a mean EC_{50} of 1.39 and range of 0.27–10.62 µg/ml, while propiconazole had a mean EC_{50} of 0.32 and range of 0.062–2.42 µg/ml. Efilor is made up of boscalid and metconazole. Boscalid had a mean EC_{50} of 0.47 and range of 0.034–6.14 µg/ml, while metconazole had a mean EC_{50} of 0.22 and range of 0.015–2.78 µg/ml (Figure 16).

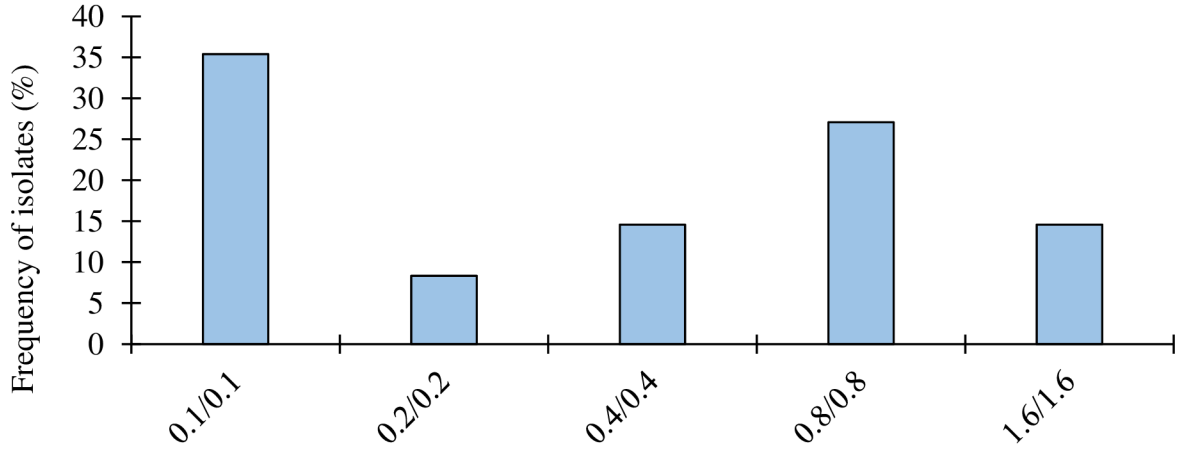


Tilmor (prothioconazole + tebuconazole)



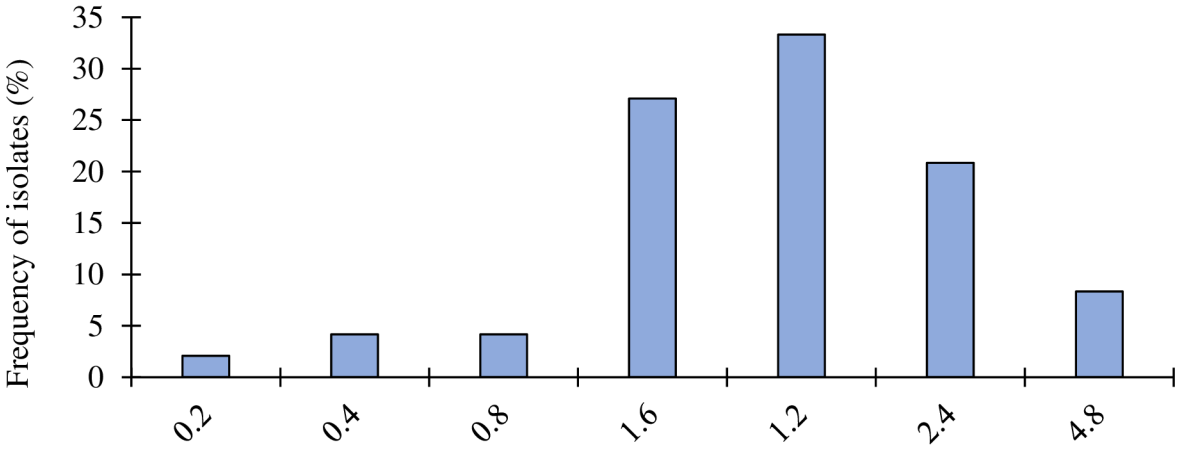
EC_{50} prothioconazole/tebuconazole (µg/ml)

Pictor (boscalid + dimoxystrobin)



EC_{50} boscalid/dimoxystrobin (µg/ml)

Horizon 250 EW (tebuconazole)



EC_{50} tebuconazole (µg/ml)

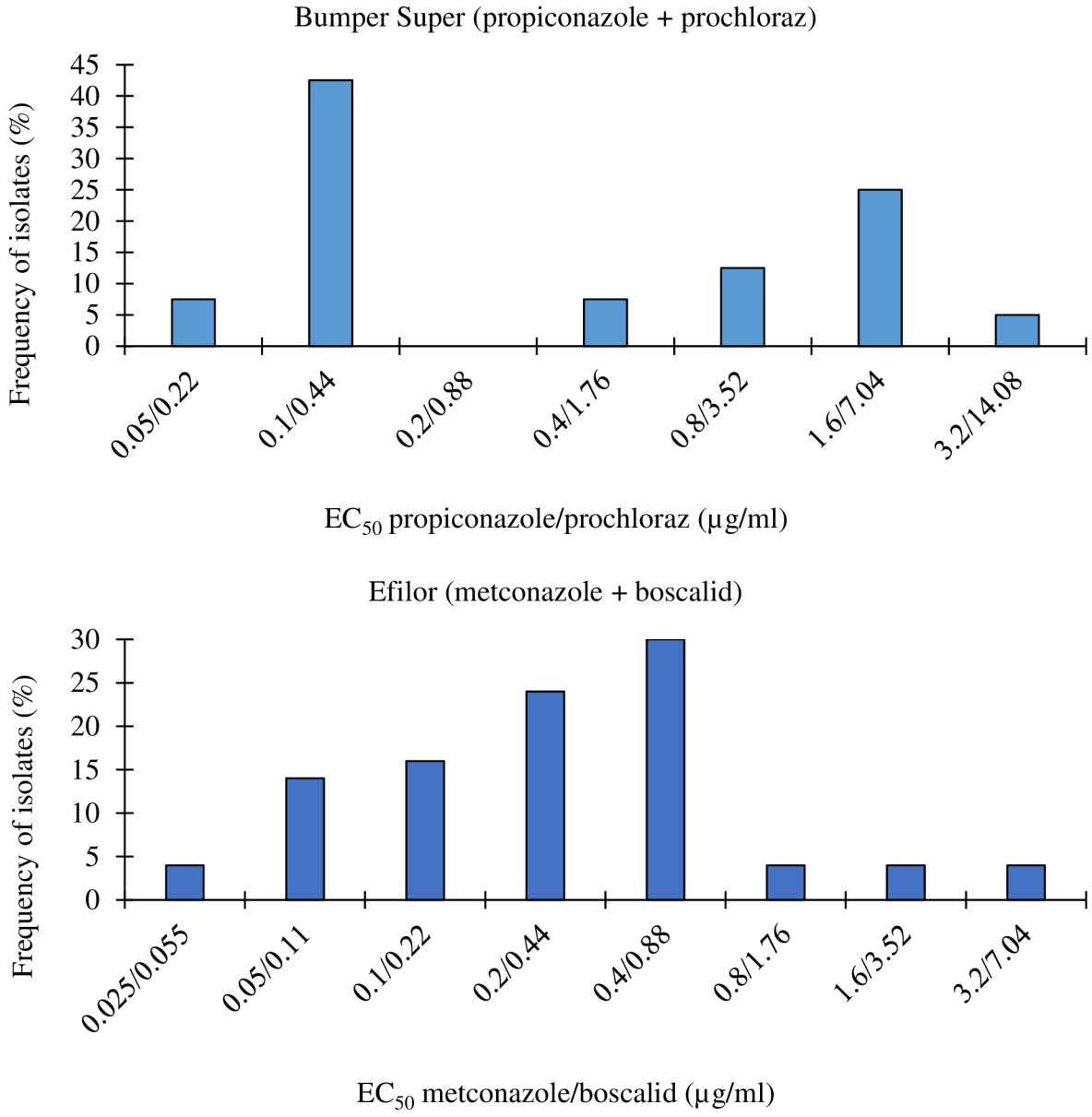


Figure 16. Frequency distribution of the sensitivity of 50 *Leptosphaeria maculans* isolates commonly used in the Czech Republic to 6 commercial mixtures collected between 2014 and 2017 from the Czech Republic.

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.

6.2.2 Microtitre plate assay

For this experiment, a representation of all fungicide groups was taken. For the 286 *L. maculans* isolates carried out with the microtitre plate assay, boscalid had an EC_{50} value range of 0.001496–0.8363 µg/ml, while those of dimoxystrobin had a range of 0.000727–2.801 µg/ml (variation factor: 3852.8). The mean EC_{50} value of boscalid was 0.029 µg/ml and did not statistically differ from the mean EC_{50} value of dimoxystrobin (0.02 µg/ml). Tetraconazole had EC_{50} values ranging from 0.00659 to 59.51 µg/ml with a variation factor of 9022.02 fold; metconazole had EC_{50} values ranging from 0.0136 to 70.69 µg/ml with a variation factor of 5197.80 fold, and prochloraz had EC_{50} values ranging from 0.00298 µg/ml to 56.04 µg/ml 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 respectively. Frequency distributions of the mean EC_{50} values were lognormally distributed for all five fungicides when tested using the D'Agostino-Pearson normality test (Figure 17).

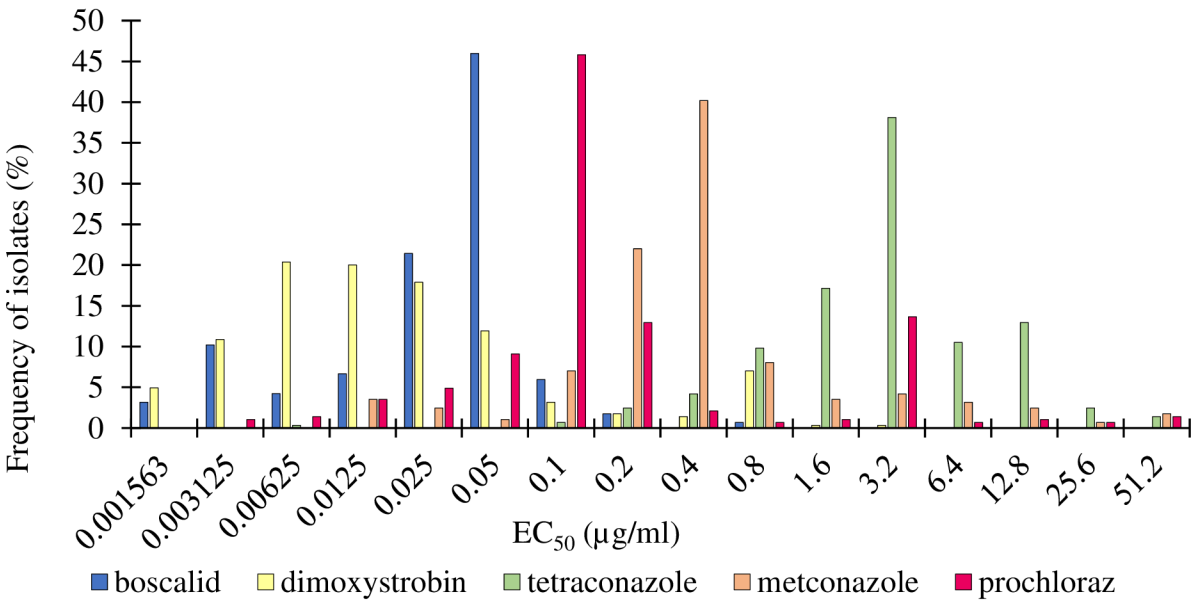


Figure 17. Frequency distribution of the sensitivity of 286 *Leptosphaeria maculans* isolates collected between 2014 and 2017 from the Czech Republic to boscalid, dimoxystrobin, tetraconazole, metconazole and prochloraz.
Individual isolates are grouped in class intervals when following interval is twofold the previous interval. Values on the x-axis indicate the midpoint of the interval.

6.2.3 Differences between fungicide sensitivity monitoring methods

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

6.2.4 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 (Figure 18). For tetraconazole, the mean EC_{50} for the sensitive *L. maculans* isolates was 2.4025 µg/ml, compared with 7.8981 µg/ml for the resistant isolates. The mean EC_{50} value of the metconazole-sensitive isolates was 0.8464 µg/ml and of metconazole-resistant isolates was 3.7139 µg/ml. For prochloraz, the mean EC_{50} values were 0.5637 and 1.6884 µg/ml for sensitive and resistant isolates, respectively.

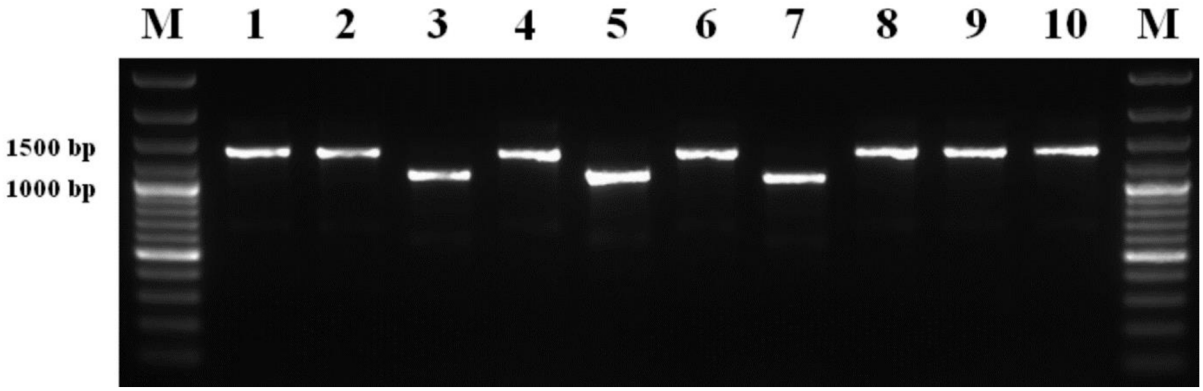


Figure 18. Amplification of the *ERG11* promoter region in *Leptosphaeria maculans* isolates using the primers EPS1 and EPS6.
Sensitive isolates of *L. maculans* are shown in lanes 3, 5, 7; resistant isolates of *L. maculans* are shown in lanes 1–2, 4, 6, 8–10, lane M: GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA).

6.2.5 Cross and multiple sensitivity between *L. maculans* isolates to SDHI, QoI and DMI fungicide classes

L. maculans isolates were tested for cross and multiple sensitivity using Spearman correlation analysis.

With the mycelium growth plate method, significant positive correlations were observed between log EC_{50} values for boscalid and trifloxystrobin (r = 0.32, P = 0.0008), boscalid and tetraconazole (r = 0.35, P =0.0002), boscalid and tebuconazole (r = 0.30, P =0.0459), boscalid and metconazole (r = 0.31, P = 0.031), dimoxystrobin and prothioconazole (r = 0.34, P = 0.0202), dimoxystrobin and metconazole (r = 0.40, P = 0.0043), dimoxystrobin and prochloraz (r = 0.51, P = 0.0001), trifloxystrobin and tetraconazole (r = 0.26, P = 0.0083), tetraconazole and prothioconazole (r = 0.38, P = 0.0088), tetraconazole and metconazole (r = 0.38, P = 0.0109), tebuconazole and prothioconazole (r = 0.45, P = 0.0084), tebuconazole and propiconazole (r = 0.48, P = 0.0004), tebuconazole and metconazole (r = 0.49, P = 0.0003), tebuconazole and prochloraz (r = 0.48, P = 0.0003), prothioconazole and propiconazole (r = 0.35, P = 0.008), prothioconazole and metconazole (r= 0.32, P = 0.0239), prothioconazole and prochloraz (r = 0.47, P = 0.0004), propiconazole and metconazole (r = 0.43, P = 0.0016), propiconazole and prochloraz (r = 0.38 P = 0.0052), and metconazole and prochloraz (r = 0.52, P = 0.0001) (Table 8, Appendix I–V).

With the microtitre plate method, significant positive correlations were observed between log EC_{50} values for tetraconazole and metconazole (r = 0.33, P = < 0.0001), tetraconazole and prochloraz (r = 0.33, P = < 0.0001), and prochloraz and metconazole (r = 0.45, P = < 0.0001) (Table 9, Appendix VI–VII).

Table 8 Spearman correlation between log EC_{50} values of active ingredients used in the mycelium growth plate method

Isolates showing a positive correlation are marked in red.

Active ingredients	Boscalid		Dimoxystrobin		Trifloxystrobin		Tetraconazole		Tebuconazole		Prothioconazole		Propiconazole		Metconazole		Prochloraz	
	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P
Boscalid	-	-	-0.01	0.9416	0.32	0.0008	0.35	0.0002	0.30	0.0459	0.13	0.3489	-0.004	0.9769	0.31	0.0310	0.02	0.8886
Dimoxystrobin	-0.01	0.9416	-	-	-0.04	0.7804	0.14	0.3692	0.28	0.0571	0.34	0.0202	0.27	0.0600	0.40	0.0043	0.52	0.0001
Trifloxystrobin	0.32	0.0008	-0.04	0.7804	-	-	0.26	0.0083	0.09	0.5664	-0.02	0.9103	-0.06	0.6585	0.20	0.2065	0.005	0.9727
Tetraconazole	0.35	0.0002	0.14	0.3692	0.26	0.0083	-	-	0.19	0.1988	0.38	0.0088	0.27	0.0555	0.38	0.0109	0.21	0.1616
Tebuconazole	0.30	0.0459	0.28	0.0571	0.09	0.5664	0.19	0.1988	-	-	0.45	0.0084	0.48	0.0004	0.49	0.0003	0.48	0.0003
Prothioconazole	0.13	0.3489	0.34	0.0202	-0.02	0.9103	0.38	0.0088	0.45	0.0084	-	-	0.35	0.0080	0.32	0.0239	0.47	0.0004
Propiconazole	-0.004	0.9769	0.27	0.0600	-0.06	0.6585	0.27	0.0555	0.48	0.0004	0.35	0.008	-	-	0.43	0.0016	0.38	0.0052
Metconazole	0.31	0.0310	0.40	0.0043	0.20	0.2065	0.38	0.0109	0.49	0.0003	0.32	0.0239	0.43	0.0016	-	-	0.52	0.0001
Prochloraz	0.02	0.8886	0.52	0.0001	0.005	0.9727	0.21	0.1616	0.48	0.0003	0.47	0.0004	0.38	0.0052	0.52	0.0001	-	-

Table 9 Spearman correlation between log EC_{50} values of active ingredients used in the microtitre plate method

Isolates showing a positive correlation are marked in red.

Active ingredients	Boscalid		Dimoxystrobin		Tetraconazole		Metconazole		Prochloraz	
	r	P	r	P	r	P	r	P	r	P
Boscalid	-	-	0.04	0.4893	-0.03	0.6280	-0.06	0.3339	-0.05	0.3741
Dimoxystrobin	0.04	0.4893	-	-	-0.05	0.4407	-0.10	0.0909	-0.07	0.2533
Tetraconazole	-0.03	0.6280	-0.05	0.4407	-	-	0.33	< 0.0001	0.33	< 0.0001
Metconazole	-0.06	0.3339	-0.10	0.0909	0.33	< 0.0001	-	-	0.45	< 0.0001
Prochloraz	-0.05	0.3741	-0.07	0.2533	0.33	< 0.0001	0.45	< 0.0001	-	-

6.3 Experiment 2: Frequency of avirulence genes and race structure

6.3.1 Virulence test

A total of 458 *L. maculans* isolates were characterized for 6 *Avr* genes: *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm7* and *AvrLm9*. *B. napus* genotype / *L. maculans* isolate interactions with mean rating scores between 0 and 4 showed a hypersensitive response, indicating an incompatible interaction between resistant genotype / corresponding *AvrLm* gene, while a mean rating score 5–9 determined those (susceptible genotype / no corresponding *AvrLm* gene) that had a compatible interaction (Figure 19).

Figure 19. Responses of plants after the cotyledon inoculation test showing an (a) incompatible interaction (resistant genotype / corresponding *AvrLm* gene), (b) compatible interaction without pycnidia (susceptible genotype / no corresponding *AvrLm* gene) and (c) compatible interaction with pycnidia and a grey-green tissue collapse (susceptible genotype / no corresponding *AvrLm* gene).

6.3.2 Frequencies of *AvrLm* genes of *L. maculans* isolates in the Czech Republic

AvrLm7 gene accounted for the highest proportion (94.3 %) of avirulence genes (85.4 % as a single gene, 8.9 % in combination with the other *AvrLm* genes). The percentage frequencies of the other *AvrLm* genes in isolates were not much different from one another. *AvrLm3* accounted for 5.2 % as a single gene and 0.9 % in combination with the other *AvrLm* genes. *AvrLm1* accounted for 0.2 % as a single gene, and 5.2 % in combination with the other *AvrLm* genes; *AvrLm2* in combination with the other *AvrLm* genes accounting for 3.1 %; and *AvrLm4* in combination with the other *AvrLm* genes accounting for 3.9 %. Of all the six avirulence alleles, *AvrLm9* had the lowest frequency. It accounted for only 1.7 % of the avirulence genes (Figure 20). The frequency of *AvrLm* alleles varied from region to region. The *AvrLm7* gene was also the most frequent allele in all regions. Results showed that *AvrLm1* ranged from 3.4 % to 10 %, *AvrLm2* ranged from 2.3 % to 20 %, *AvrLm3* from 4 % to 15 %, *AvrLm7* from 76.7 % to 100 %, and *AvrLm9* from 1.9 % to 5 % (Table 10).

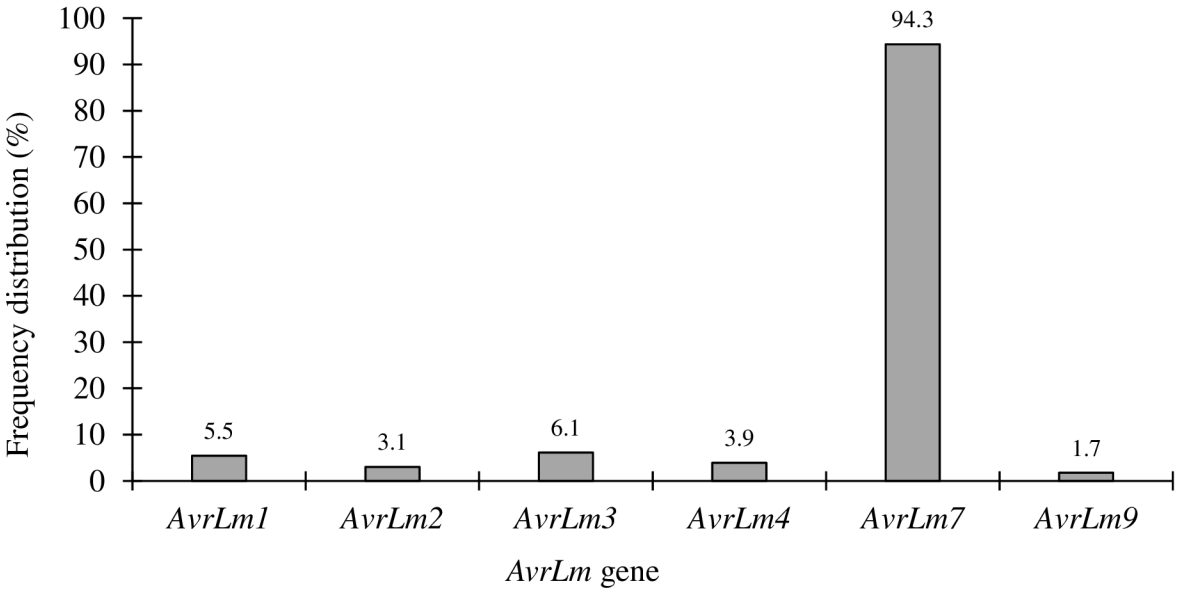


Figure 20. Frequency distribution of avirulence genes (*AvrLm*) in *Leptosphaeria maculans* populations from the Czech Republic.

Table 10. Percentage distribution of avirulence (*AvrLm*) genes in *Leptosphaeria maculans* across 10 regions in the Czech Republic.

Region	*AvrLm1*	*AvrLm2*	*AvrLm3*	*AvrLm4*	*AvrLm7*	*AvrLm9*
Central Bohemian	10.0	20.0	0.0	15.0	100.0	5.0
Hradec Králové	3.4	2.3	4.0	1.7	95.4	2.3
Karlovy Vary	16.7	0.0	23.3	0.0	76.7	0.0
Liberec	0.0	0.0	0.0	0.0	100.0	0.0
Moravian-Silesian	5.8	3.2	6.5	6.5	95.4	1.9
Olomouc	10.0	0.0	15.0	0.0	85.0	0.0
Pilsen	0.0	0.0	0.0	0.0	100.0	0.0
Praha	0.0	0.0	0.0	14.3	100.0	0.0
South Moravian	0.0	0.0	0.0	0.0	100.0	0.0
Zlín	0.0	6.3	0.0	6.3	100.0	0.0

6.3.3 Race structure of *L. maculans* isolates in the Czech Republic

A total of 17 races were found in 10 regions in the Czech Republic. The richness in races according to the Margalef index differed between the regions. Moravian-Silesian region had the highest race diversity with 12 races and a Margalef index of 2.18, while the Liberec, Pilsen, and South Moravian regions had the lowest race diversities with 1 race each and a Margalef index of 0.00. There were 8 races at Hradec Králové, 5 races in Central Bohemian, 3 races in Karlovy Vary, Olomouc and Zlín, two different races were found in Praha (Table 11).

Table 11. Number of *Leptosphaeria maculans* isolates and Margalef index showing the population diversity of *L. maculans* races in different regions in the Czech Republic.

Region	Number of isolates	Number of races	Margalef index
Central Bohemian	20	5	1.34
Hradec Králové	175	8	1.34
Karlovy Vary	30	3	0.59
Liberec	9	1	0.00
Moravian-Silesian	155	12	2.18
Olomouc	20	3	0.67
Pilsen	22	1	0.00
Praha	7	2	0.51
South Moravian	4	1	0.00
Zlín	16	3	1.08

Seventeen races were identified with frequencies ranging from 0.2 to 85.3 %. The race *AvrLm (5)-(6)-7-(8)* was the most predominant race (85.4 %) in all the regions. Frequencies of this race ranged from 60 % in Karlovy Vary to 100 % in Pardubice, Pilsen and Vysočina. However, in Pardubice and Vysočina regions there were sampled only one isolate per region. This shows most of the isolates were virulent to the major resistance gene *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4* and *Rlm9*. The second most abundant race (5.3 %) was *AvrLm3-(5)-(6)-(8)* showed virulence to the major genes *Rlm1*, *Rlm2*, *Rlm4*, *Rlm7* and *Rlm9* with frequencies of 23 % in Karlovy Vary, 6 % in Moravian-Silesian, and 14 % in Olomouc (Table 12). Following the nomenclature of Balesdent et al. (2005), only the avirulence genes carried by each race are listed and the genes in parentheses were not or could not be determined in these races.

Table 12. Frequency distribution of 17 *Leptosphaeria maculans* races from 10 regions in the Czech Republic collected between 2014 and 2020

Races are described based on the characterization of *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm7* and *AvrLm9*.

CB = Central Bohemian; HK = Hradec Králové; KV = Karlovy Vary; Lib = Liberec; MS = Moravian Silesian; Olm = Olomouc; Pil = Plzeň; Pha = Praha; SM = South Moravian; Zln = Zlín.

Race	CB	HK	KV	Lib	MS	Olm	Pil	Pha	SM	Zln	Frequency	Frequency (%)
AvrLm1-(5)-(6)-(8)	0.00	0.57	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.22
AvrLm1-2-4(5)-(6)-7-(8)-9	0.00	0.00	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00	1.00	0.22
AvrLm1-2-(5)-(6)-7-(8)	0.00	0.00	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00	2.00	0.44
AvrLm1-2-(5)-(6)-7-(8)-9	0.00	1.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.00	0.44
AvrLm1-3-(5)-(6)-(8)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.22
AvrLm1-3-4-(5)-(6)-7-(8)	0.00	0.00	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00	1.00	0.22
AvrLm1-3-(5)-(6)-7-(8)	0.00	0.00	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00	1.00	0.22
AvrLm1-4-(5)-(6)-7-(8)	5.00	0.57	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00	3.00	0.66
AvrLm1-(5)-(6)-7-(8)	0.00	1.14	16.67	0.00	2.58	10.00	0.00	0.00	0.00	0.00	13.00	2.84
AvrLm2-3-4-(5)-(6)-7-(8)	0.00	0.00	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00	1.00	0.22
AvrLm2-4-(5)-(6)-7-(8)	10.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.00	0.44
AvrLm2-4-(5)-(6)-7-(8)-9	0.00	0.00	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00	1.00	0.22
AvrLm2-(5)-(6)-7-(8)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.25	1.00	0.22
AvrLm2-(5)-(6)-7-(8)-9	5.00	1.14	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00	4.00	0.87
AvrLm3-(5)-(6)-(8)	0.00	4.00	23.33	0.00	4.52	15.00	0.00	0.00	0.00	0.00	24.00	5.24
AvrLm4-(5)-(6)-7-(8)	0.00	1.14	0.00	0.00	3.23	0.00	0.00	14.29	0.00	6.25	9.00	1.97
AvrLm(5)-(6)-7-(8)	75.00	90.29	60.00	100	84.52	75.00	100	85.71	100	87.50	391.00	85.37

6.4 Experiment 3: Mating type and genetic diversity of *L. maculans* populations

6.4.1 Distribution of mating type idiomorphs

Multiplex PCR primers were used to separate 430 *L. maculans* isolates into one of the two different mating type idiomorphs (MAT1.1 and MAT1.2). MAT 1.1 yielded a product size of 686 bp while MAT 1.2 yielded a product size of 443 bp. There was a total of 204 MAT1.1 isolates and 226 MAT1.2 isolates. Chi square analysis showed there was no significant deviation from the 1:1 ratio between the mating types (Figure 21).

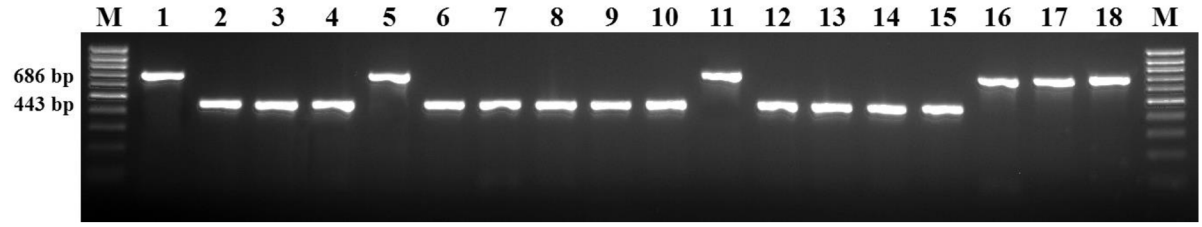


Figure 21. Distribution of mating type idiomorphs of *Leptosphaeria maculans* into MAT1.1 or MAT 1.2.
MAT 1.1 yielded a product size of 686 bp while MAT 1.2 yielded a product size of 443 bp.

6.4.2 Allele frequencies

The 10 minisatellite loci tested in this study were polymorphic in all 9 subpopulations. In all the examined loci, a total of 32 alleles were detected. The 10 minisatellites all had a variable number of alleles per loci. MinLm4, MinLm5, MinLm2452 and MinLm6 each had 2 alleles. The other six minisatellites (MinLm1, MinLm2451, MinLm8, MinLm935-2, MinLm555 and MinLm585) had 4, 4, 4, 6, 3 and 3 alleles, respectively (Table 13).

Table 13. Allele frequencies of nine subpopulations of *Leptosphaeria maculans* at 10 minisatellite loci from 9 regions in the Czech Republic.

Locus	[a]Allele frequency in population at:									
	Allele size (bp)	[b]CB (n = 44)	HK (n = 160)	KV (n = 36)	Lib (n = 10)	MS (n = 96)	Olm (n = 16)	Pha (n = 33)	SM (n = 17)	Zln (n = 18)
MinLm1	80	0.023	0.119	0.25	0.000	0.115	0.000	0.303	0.000	0.056
	100	0.091	0.05	0.083	0.000	0.073	0.000	0.000	0.000	0.111
	140	0.795	0.775	0.667	0.900	0.771	0.938	0.697	0.882	0.722
	170	0.091	0.056	0.000	0.100	0.042	0.062	0.000	0.118	0.111
MinLm2451	160	0.364	0.362	0.111	0.100	0.583	0.375	0.394	0.235	0.5
	180	0.364	0.431	0.528	0.900	0.208	0.25	0.333	0.647	0.167
	200	0.273	0.200	0.361	0.000	0.198	0.375	0.273	0.118	0.278
	300	0.000	0.006	0.000	0.000	0.01	0.000	0.000	0.000	0.056
MinLm2452	80	0.045	0.031	0.111	0.000	0.042	0.062	0.091	0.000	0.056
	100	0.955	0.969	0.889	1.000	0.958	0.938	0.909	1.000	0.944
MinLm4	290	0.023	0.013	0.000	0.000	0.125	0.000	0.091	0.000	0.000
	300	0.977	0.988	1.000	1.000	0.875	1.000	0.909	1.000	1.000
MinLm5	480	0.773	0.981	1.000	1.000	0.948	1.000	0.939	0.941	0.889
	500	0.227	0.019	0.000	0.000	0.052	0.000	0.061	0.059	0.111
MinLm6	100	0.568	0.488	0.194	0.900	0.479	0.438	0.758	0.412	0.444
	120	0.432	0.512	0.806	0.100	0.521	0.562	0.242	0.588	0.556
MinLm8	220	0.023	0.031	0.000	0.000	0.062	0.188	0.333	0.059	0.167
	240	0.409	0.406	0.083	0.100	0.24	0.438	0.121	0.471	0.278
	300	0.477	0.519	0.833	0.700	0.604	0.375	0.455	0.412	0.500
	330	0.091	0.044	0.083	0.200	0.094	0.000	0.091	0.059	0.056
MinLm585	200	0.182	0.138	0.417	0.000	0.052	0.125	0.121	0.000	0.056
	220	0.795	0.844	0.583	1.000	0.875	0.875	0.848	0.706	0.944
	260	0.023	0.019	0.000	0.000	0.073	0.000	0.03	0.294	0.000

Locus	[a]Allele frequency in population at:									
	Allele size (bp)	[b]CB (n = 44)	HK (n = 160)	KV (n = 36)	Lib (n = 10)	MS (n = 96)	Olm (n = 16)	Pha (n = 33)	SM (n = 17)	Zln (n = 18)
MinLm555	300	0.341	0.494	0.444	0.600	0.438	0.625	0.515	0.412	0.500
	320	0.659	0.506	0.472	0.400	0.51	0.375	0.485	0.588	0.500
	400	0.000	0.000	0.083	0.000	0.052	0.000	0.000	0.000	0.000
MinLm9352	180	0.114	0.188	0.000	0.000	0.062	0.188	0.121	0.529	0.111
	200	0.409	0.312	0.694	0.800	0.396	0.562	0.576	0.353	0.389
	220	0.023	0.194	0.139	0.200	0.365	0.125	0.152	0.118	0.056
	240	0.068	0.062	0.000	0.000	0.021	0.062	0.03	0.000	0.111
	260	0.000	0.000	0.000	0.000	0.000	0.062	0.000	0.000	0.000
	300	0.386	0.244	0.167	0.000	0.156	0.000	0.121	0.000	0.333

[a] Frequency of avirulence alleles; n, number of isolates analysed

[b] Subpopulations by region: CB= Central Bohemian; HK = Hradec Králové; KV = Karlovy Vary; Lib = Liberec; MS = Moravian Silesian; Olm = Olomouc; Pha = Praha; SM = South Moravian; Zln = Zlín.

6.4.3 Genotypic richness, diversity and clonal composition

In this study, 430 isolates were collected between 2015 and 2020 and tested for genetic variability. Out of the 430 isolates sampled, there were 236 unique MLGs (different genotypes). The eMLG ranged from 6.36 to 9.86. The genotype distribution among the samples within the tested groups was very close to equal abundance with E5 values > 0.772. The lowest Hexp value was found in the Liberec population. All other populations had values that ranged from 0.349 to 0.407. The genotypic diversity (λ) was lowest in SM (0.824), followed by Liberec (0.840) and then Olm (0.906). The highest diversity was in HK (0.987), which incidentally also had the highest number of isolates collected. The rbarD was used to assess the impact of clonal reproduction of *L. maculans* on our data analysis. It ranged from -0.038606 to 0.08. Lib and Olm had the highest and lowest LD (rbarD), respectively. This showed that all the groups belonged to one population (Table 14).

Table 14 .Genetic variability of the 9 *Leptosphaeria maculans* subpopulations.

Pop[a]	N[b]	MLG[c]	eMLG[d]	H[e]	λ [f]	E.5[g]	Hexp[h]	rbarD[i]
CB	44	41	9.86	3.69	0.974	0.966	0.412	0.016817
HK	160	101	9.69	4.47	0.987	0.866	0.377	0.000916
KV	36	15	7.65	2.56	0.914	0.890	0.349	0.042371
Lib	10	8	8.00	1.97	0.840	0.848	0.200	-0.038606
MS	96	64	9.47	3.99	0.977	0.812	0.392	0.017627
Olm	16	13	8.71	2.48	0.906	0.884	0.355	0.083431
Pha	33	29	9.60	3.31	0.961	0.919	0.405	0.000481
SM	17	9	6.36	1.95	0.824	0.772	0.361	0.067898
Zln	18	17	9.71	2.81	0.938	0.970	0.407	0.013128
Total	430	236	9.80	5.23	0.993	0.774	0.395	0.004120

[a] Sub populations by region: CB= Central Bohemian; HK = Hradec Králové; KV = Karlovy Vary; Lib = Liberec; MS = Moravian Silesian; Olm = Olomouc; Pha = Praha; SM = South Moravian; Zln = Zlín.
[b] Number of isolates
[c] Number of multilocus genotypes observed
[d] Number of expected MLGs at the smallest sample size on rarefaction
[e] Gene diversity
[f] Simpson's index
[g] Evenness
[h] Nei's unbiased gene diversity
[i] standard index of association

The genetic diversity at each locus across all populations was also determined. The average number of alleles across all loci was 3.2. Simpson's diversity index (1-D) ranged from 0.080 to 0.725. The highest diversity was observed at MinLm935-2 locus and the lowest at MinLm4. While comparing the genetic diversity contributed by each locus to the population within each region, MinLm4 had the smallest indices in all regions. The second less variable marker was MinLm5 (Table 15).

Table 15. Genetic diversity of *Leptosphaeria maculans* isolates across each locus.

Locus	Allele	1-D[a]	Hexp[b]	Evenness
MinLm1	4.000	0.384	0.385	0.537
MinLm2451	4.000	0.655	0.657	0.939
MinLm2452	2.000	0.089	0.089	0.470
MinLm4	2.000	0.080	0.080	0.459
MinLm5	2.000	0.101	0.101	0.486
MinLm6	2.000	0.500	0.501	1.000
MinLm8	4.000	0.592	0.593	0.764
MinLm585	3.000	0.295	0.296	0.569
MinLm555	3.000	0.517	0.518	0.920
MinLm9352	6.000	0.725	0.727	0.828
Mean	3.200	0.394	0.395	0.697

a Simpson's diversity index
[b] Nei's unbiased gene diversity

6.4.4 Genetic differentiation and structure of populations

Hierarchical AMOVA indicated that with clone correction, 3 % and 97 % of the variations were attributed to between and within subpopulations by region. AMOVA results with and without clone correction show there are more variations within samples than between samples.

6.4.5 Population structure and genetic relationships

The genetic structure of the *L. maculans* isolates was analysed using the principal coordinate analysis PCoA and STRUCTURE. The principal coordinate analysis (PCoA) based on the

pairwise distance matrix among all the 430 *L. maculans* isolates which showed there was only one clustered group (Figure 22).

The structure v 2.3.4 (Pritchard et al., 2000) was used to study the population structure and genetic relationship among the 430 *L. maculans* isolates that were collected from 9 regions in the Czech Republic. The K-value was used to estimate the number of clusters. In order to find the optimal K-value number, the number of clusters (*K*) was plotted against Δ*K,* which showed there was only one cluster at K=1 (Figure 23).

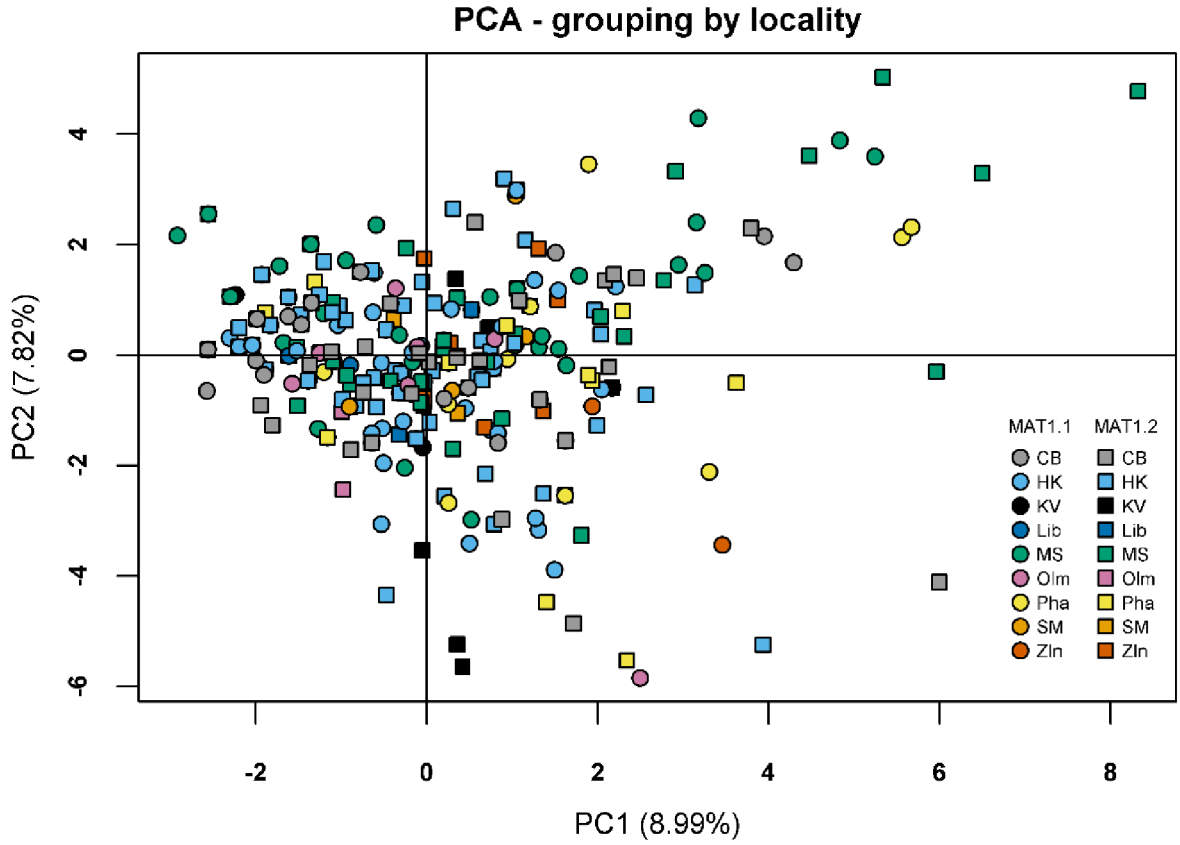


Figure 22. Two-dimensional plot of Principal coordinates analysis (PCoA) showing clustering of individual samples belonging to 9 populations of *Leptosphaeria maculans* based on 10 minisatellite loci.

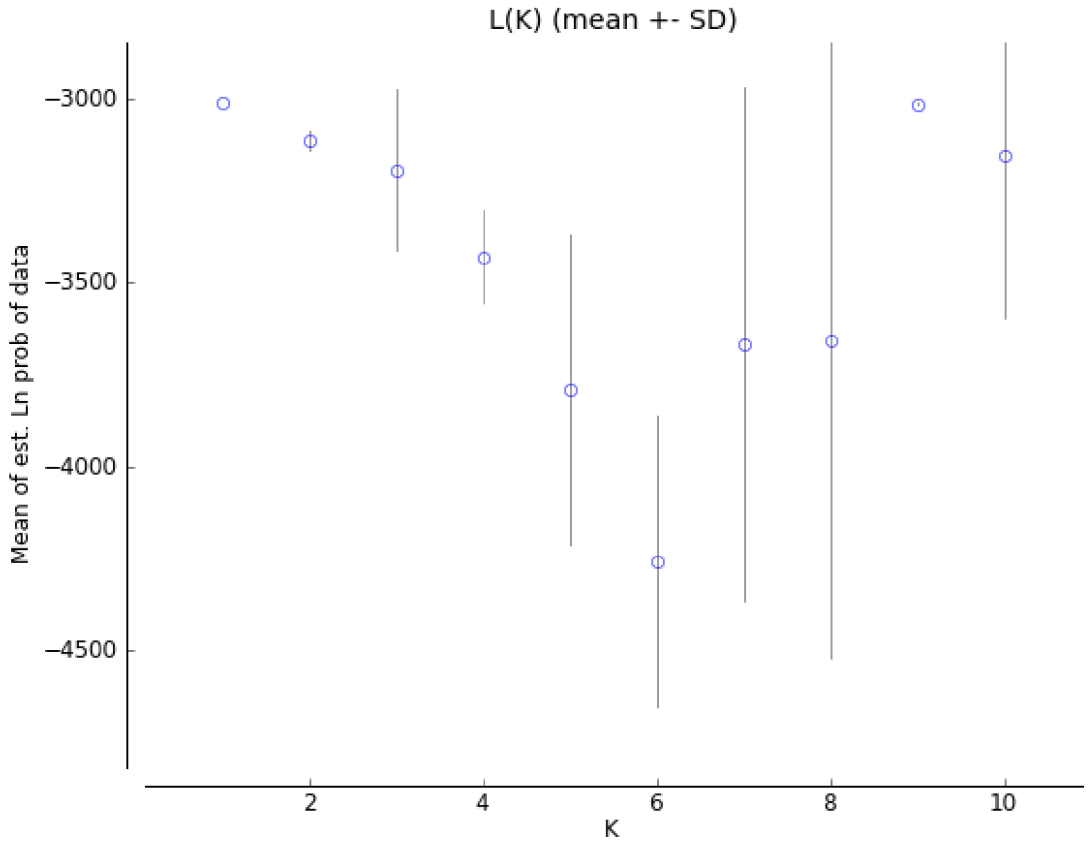


Figure 23. Estimated number of clusters obtained for *Leptosphaeria maculans* isolates with STRUCTURE for K values from 1 to 10 using minisatellite data.

Graphical representation of estimated mean L(K) values showing the clustering of different landraces.

7. Discussion

7.1 Fungicide sensitivity

In the Czech Republic, fungicides registered for the control of oilseed rape diseases include single-site fungicides from three major groups: SDHI, QoI, and DMI (Czech Institute for Supervising and Testing in Agriculture, 2022). Unfortunately, continuously using single-site fungicides often leads to resistance development over time (Brent & Hollomon, 2007). To prevent or delay the evolution of resistant populations, it, therefore, becomes important to monitor resistance development in pathogen populations. The first objective of this study focuses on the use of both the mycelium growth assay and the microtiter plate methods to test for the sensitivity of *L. maculans* isolates to nine fungicides (boscalid, trifloxystrobin, dimoxystrobin, tebuconazole, tetraconazole, metconazole, prochloraz, propiconazole, and prothioconazole) and six commercial mixtures (Caramba, Pictor, Horizon 250 EW, Bumper Super, Tilmor, and Efilor) registered for use in the Czech Republic for oilseed rape production that belong to the SDHI, DMI, and QoI fungicides classes.

Succinate dehydrogenase inhibitors (SDHIs)

Boscalid is an SDHI fungicide with excellent fungicidal activity against plant pathogenic fungi genera 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 µg/ml 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, 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 mycelial growth and conidia of *L. maculans* isolates. At the highest concentration of 10 µg/ml, mycelia of all 118 isolates

were completely inhibited. The EC_{50} range was between 0.014–0.989 µg/ml, with a mean EC_{50} of 0.26 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 *L. maculans* populations in the Czech Republic. However, because the unimodal curve tends towards the right, the potential of *L. maculans* developing resistance to boscalid is present.

Avenot & Michailides, (2007) and Stammer & Speakman, (2006) had results showing the mycelial growth of *Botrytis cinerea* isolates were more sensitive to boscalid than conidia. Nevertheless, Zhang et al. (2007) in their study showed that conidia of *B. cinerea* isolates were more sensitive to boscalid than mycelial growth. 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, EC_{50} values based on conidial growth assay was from 0.02 to 1.68 µg/ml with a mean of 0.42 µg/ml. *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 mycelium. This suggests boscalid is more effective at inhibiting conidial germination than mycelium.

Quinone outside inhibitors (QoIs)

QoI fungicides are well known site-specific fungicides that are known for their activities against a wide range of oomycete, ascomycete, basidiomycete and deuteromycete fungi (Bartlett et al., 2002). Although there have been no published reports of the sensitivity of *L. maculans* to either trifloxystrobin or dimoxystrobin, Wang et al. (2020) reported that there was a change in the sensitivity of Alberta *L. maculans* to another strobilurin, pyraclostrobin from 2011 to 2016.

Within the QoI group, the EC_{50} values may be highly variable for the same species in response to different fungicides, given the chemical properties of each fungicide is different. Thus, studies involving various QoI fungicides have often resulted in different EC_{50} values when tested on the same pathogen species (Patel et al., 2012). In this current study, the EC_{50} values for trifloxystrobin (mean 0.035 µg/ml) was much different from dimoxystrobin (mean 1.87 µg/ml). This suggests that trifloxystrobin is more effective in controlling *L. maculans* populations in the Czech Republic than dimoxystrobin. In addition, dimoxystrobin had a wide range of EC_{50} values, from 0.053–95.59 µg/ml for isolates tested for sensitivity to *L. maculans* isolates which could be a warning that some isolates have developed resistance to dimoxystrobin.

QoI fungicides are protective fungicides that are good inhibitors of spore germination and thus prevent fungal penetration into the host during the initial stages of the epidemic (Bartlett et al., 2002). Fungal spores are therefore more sensitive than mycelium to QoI fungicides. This was corroborated in this study, where the mean EC_{50} value of *L. maculans* conidia was 9.35 times (1.87/0.02 µg/ml) lower than mycelial growth sensitivity to dimoxystrobin. Spore germination inhibition assays are therefore considered a more appropriate and sensitive method for conducting sensitivity studies. Thus, continuous monitoring and additional management practices should be employed to avoid the selection of resistant isolates (Avenot & Michailides, 2007).

DeMethylation Inhibitors (DMIs)

DMIs are broad-spectrum fungicides that have been successfully used for many years in controlling plant pathogens, including causal agents of phoma stem canker. They have both preventive and curative properties and are widely used in agriculture to control plant diseases. Nevertheless, because they are categorized as medium-risk fungicides by FRAC, there is a risk of resistance developing in fungal populations (Brent & Hollomon, 2007).

There have only been a few studies on resistance to DMI fungicides, with resistance to DMI fungicides in *L. maculans* being documented in Australia (Van De Wouw et al., 2017; Yang et al., 2020). Yang et al. (2020) reported that *L. maculans* isolates sensitive to tebuconazole had a mean EC_{50} of 0.54 µg/ml, while *L. maculans* isolates resistant to prothioconazole had a mean EC_{50} of 0.07 µg/ml. These EC_{50} values were lower than those in this study where the mean EC_{50} for *L. maculans* isolates tested for their sensitivity to tebuconazole, and prothioconazole were 2.2 and 1.8 µg/ml respectively. For the other four DMI fungicides, it was difficult to determine the sensitivity of the isolates to these fungicides based on mean EC_{50} alone. This is because the mean EC_{50} was 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. 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. Resistance to DMI fungicides is quantitative, meaning it develops in a stepwise gradual progression. It results from the modification of many interacting genes. Therefore, depending on the number of gene changes, isolates exhibit a range of sensitivity to the fungicide (Brent & Hollomon, 2007). Our current study showed that when tested for mycelial growth

inhibition, *L. maculans* isolates had variation factors between 17.96 and 1176.888. With the microtiter plate method and a larger number of isolates, this variation factor was much higher, between 5197.80 and 18,824.32. In the Czech Republic, triazole fungicides are commonly used as both fungicides and growth regulators. However, Czech farmers have reported a decrease in the efficacy of triazole fungicides (personal communication). The high mean EC_{50} values and variation factors for isolates tested with triazole fungicides suggest high-intensity use of these fungicides may have led to the selection of resistant isolates in some pathogen population. The results in this study also explain 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.

Prochloraz is an imidazole fungicide registered in the Czech Republic against both oilseed rape and cereal diseases (Czech Institute for Supervising and Testing in Agriculture, 2022). Nevertheless, in oilseed rape plants, this fungicide is more commonly used in managing Sclerotinia stem rot (Czech Statistical Office, 2021). 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. *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. 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. 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.

Molecular mechanisms of DMI resistance have been studied where overexpression of the *ERG11* gene is sometimes associated with inserts upstream of the *ERG11* gene (Patel et al., 2007). The use of molecular markers for rapid PCR-based detection have been recently developed (Patel et al., 2007), where similar PCR tests have been used to find inserts in isolates of *Venturia inaequalis* and *Blumeriella jaapii* resistant to myclobutanil. Yang et al. (2020) 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.

Cross sensitivity

Cross sensitivity usually occurs between fungicides with similar modes of action while multiple sensitivity occurs between fungicides with different modes of action. 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 (Brent & Hollomon, 1998), as has been seen between DMI fungicides in *Cercospora beticola* isolates (Karaoglanidis & Thanassoulopoulos, 2003), and between tebuconazole and difenoconazole in *Didymella bryoniae* (Thomas et al., 2012). In addition, Ishii et al. (2021) demonstrated cross resistance between mefentrifluconazole and other DMI fungicides: propiconazole, difenoconazole, and tebuconazole. In this study, regardless of which sensitivity method was used cross sensitivity was observed between the DMI fungicides. However, with the QoI fungicides trifloxystrobin and dimoxystrobin no cross resistance was observed. This suggests that no two DMI fungicides should be used together. An absence of multiple resistance between boscalid and dimoxystrobin confirms that although both fungicide classes affect respiration, they act at different sites in the pathogen. Therefore, mixing two at-risk fungicides, without intrinsic positive cross resistance, is a useful resistance management tactic. With the mycelial growth plate method, multiple resistance was observed in this study between boscalid and three DMI fungicides (tetraconazole, tebuconazole, and metconazole); between boscalid and trifloxystrobin; and between dimoxystrobin and three DMI fungicides (prothioconazole, metconazole, and prochloraz), between trifloxystrobin and tetraconazole. The discrepancies in sensitivity methods could be because a smaller sample size was tested with this method.

Although fungicide mixtures do not prevent resistant strains from arising, they can slow down the evolution rate of resistance in the pathogen population (Brent & Hollomon, 2007; Brent, 1995; Staub, 1991). A proper mixing partner should provide satisfactory disease control when used alone on the target disease (Ma et al., 2006). Integrated disease management programs recommend using generation II SDHIs fungicides such as boscalid and fluopyram either as mixing or alternation partners to reduce the possibility of fungicide resistance. In this study, *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. Similarly, the six

fungicide mixtures tested for their efficacy against *L. maculans* isolates were more sensitive to the mixtures than the individual active ingredients.

The results shown here therefore demonstrate that there is a risk of development among *L. maculans* populations to boscalid and dimoxystrobin fungicides. This study has also shown that the DMI fungicides 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. The use of at-risk fungicides on a regular and exclusive basis increases the risk of resistance problems. Where repeated applications are required for disease control, such as with many foliar diseases, selection pressure is increased. Resistance management should also include cultural practices and optimal fungicide use patterns. This would reduce selection pressure by reducing the time of exposure or the size of the population exposed to the at-risk fungicide. 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 in the Czech Republic, 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. Future research is needed to test the efficacy of these fungicides in the greenhouse and on the field.

7.2 Frequency of avirulence genes

One of the most effective methods of controlling phoma stem canker is with the use of major genes which harbour resistance genes. However, the continuous use of the same cultivars can lead to a breakdown of genetic resistance by plant pathogen populations (Rouxel et al., 2011). This leads to an increase in the number of virulent isolates in *L. maculans* populations and subsequently severe yield losses during oilseed rape cultivation. Therefore, 457 *L. maculans* isolates collected from 10 regions in the Czech Republic between 2014 and 2020 were analysed to determine the frequency of avirulence alleles and race structure of *L. maculans* isolates in the Czech Republic, which will be crucial for the effective use of major resistance genes in sustainable disease management.

In this study, there was little variation among the frequency distribution of the avirulent alleles *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, and *AvrLm9*. The percentage frequencies of these alleles ranged from 1.75 to 5.46%. This suggests that the *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4* and *Rlm9* genes are not very effective in the Czech Republic, which could be as a result of a long history of using cultivars with these resistance genes. A similar occurrence was reported in France and Australia, where intensive oilseed rape cultivation harbouring *Rlm1* and *Rlm6* major resistance genes led to an increase in virulent isolates. Hence, both major genes were defeated after only a few years of commercial use (Rouxel et al., 2003; Sprague et al., 2006).

Unlike in Balesdent et al. (2005) where an absence of *AvrLm7* was found, the avirulence allele *AvrLm7* was carried by 94% of the isolates which indicates that cultivars with the corresponding *Rlm7* are resistant against 94 % of the isolates collected. This suggests that only the major gene *Rlm7* is still efficient. This is similar to a study carried out in Germany between 2014 and 2016 where 85 % of the isolates collected in Northern Germany also carried the *AvrLm7* (Winter & Koopmann, 2016). However, a recent study from Germany has already shown there is an increase in isolates virulent to the *Rlm7* gene (Alnajar et al., 2021). The increase in *Rlm7*-virulent isolates in Germany closely resembles what happened in France where Balesdent et al. (2001) found the frequency of resistant isolates to *Rlm7* was 0.06 % isolates from 2001 and 2002 and unfortunately by 2005, the frequency of resistance isolates had increased to between 0 and 60.4% (Balesdent et al., 2006). In this study, 6 % of the isolates were virulent to *Rlm7* in the Czech Republic. This is higher than what was seen in Germany where only 6 out of 644 isolates (0.9 %) were seen to be virulent to *Rlm7* (Winter & Koopmann, 2016). At least one

virulent isolate in this current study was found in 5 of the 13 regions. Interestingly, virulent isolates were mostly found in 2019 (24 out of 26). This suggests that this may be the beginning of resistance breakdown of *AvrRlm7* in the Czech Republic.

Epistatic effects are mechanism for evading recognition. This current study showed that there is an epistatic effect between *AvrLm3* and *AvrLm7*. Thus, the presence of a functional *AvrLm7* masked the recognition of *AvrLm3*. *AvrLm4* and *AvrLm7* are two distinct alleles of a single gene, named *AvrLm4-7*. A single base mutation, leading to the change of a glycine to an arginine residue in the avirulence protein, is responsible for the breakdown of *Rlm4* resistance whereas the *Rlm7* resistance is unaltered (Parlange et al., 2009).The epistatic mechanism of *AvrLm7* however does not stop the expression of *AvrLm3* or the physical interaction of the *Avr* effector proteins of *AvrLm3* and *AvrLm4-7* (Plissonneau et al., 2016).

Climate, sowing date, cropping history, and management practices did not differ between sampling regions. These agronomic practices are critical in the formation of *L. maculans* races (Balesdent et al., 2005; Guo & Fernando, 2005; Kutcher et al., 2013). Characterization of *L. maculans* isolates on the host differential set in this study confirmed the avirulence alleles for 6 genes, which have the potential to differentiate 2^6 or 64 races. Isolates showing the same avirulence pattern towards the different *Rlm* genes were grouped into the same race. Out of these potential 64 races, only 17 races were detected which suggests that variation on many resistance genes is low or that races with many virulence alleles may be less fit and consequently difficult to detect. The two dominant races were *AvrLm* (5)-(6)-7-(8) and *AvrLm1*-(5)-(6)-7-(8). This is consistent with studies that analysed the race spectra of *L. maculans* in northern Germany in 2002 and 2014 (Stachowiak et al., 2006; Winter & Koopmann, 2016).

Preserving and prolonging the *Rlm* mediated major gene resistance should be of main interest for oilseed rape breeders and growers. Several integrative management strategies such as burying plant debris to avoid sexual propagation and growing oilseed rape in rotation with other crops prolongs the durability of major genes (Daverdin et al., 2012; Kutcher et al., 2013). Furthermore, the rotation of oilseed rape cultivars with different major resistance genes is a suitable way to reduce the frequency of virulent isolates and may prolong resistance to blackleg (Marcroft et al., 2012). The future breeding strategy should therefore focus on the combination of specific major resistance genes with a quantitative background. This is because studies have shown that the durability of major gene resistance increased when major genes were combined with quantitative resistance (Delourme et al., 2014; Brun et al., 2010).

7.3 Mating Type and Genetic Variability

According to Dilmaghani et al., (2012), the migration patterns and biology of *L. maculans* worldwide collection of isolates suggests the USA as the origin of *L. maculans* and from there independent introductions into Eastern Canada (Ontario), Europe and Australia occurred. Pongam et al. (1999) also studied the genetic variation among *L. maculans* isolates collected from North Dakota, Georgia, Ontario, Western Canada, the United Kingdom, France, Germany, and Australia. Interestingly, the isolates from North Dakota, Western Canada, Georgia, and the UK formed one cluster while Georgia, Ontario, France, Germany and Australia formed the other cluster. The genetic diversity of *L. maculans* has also been studied independently by country using a variety of AFLP, RAPD, minisatellite and microsatellite markers. As the genetic variability of *L. maculans* in the Czech Republic is still unknown, *L. maculans* isolates were collected from commercial oilseed rape fields in the Czech Republic in this study to infer the genetic structure of phoma stem canker using 10 minisatellite markers previously designed by other researchers (Eckert et al., 2005; Attard et al., 2001; Jedryczka et al., 2010).

L. maculans has a mixed reproductive system. Ascospores are the major source of inoculum and are thought to initiate epidemics. Pycnidia on the other hand are produced throughout the growing season and cause secondary infections through pycnidiospores. Samples in this study were collected in autumn which corresponds to when epidemics are initiated. Ascospores can travel long distances through wind dispersal between regions, thus allowing for the presence of gene flow over large distances. Generally, the mating type idiomorphs of all 456 *L. maculans* isolates in this study did not deviate from the 1:1 distribution. This suggests there is high genotypic diversity within populations as a result of the presence of active sexual recombination within the Czech *L. maculans* population, which is characteristic of a randomly mating population.

In contrast to other studies carried out in Australia and Canada, where there were considerable differences between subpopulations (Barrins et al., 2004; Zou et al., 2018), the results in this study were most similar to the study from France (Gout et al., 2006). Here, there was more genetic variability within subpopulations than between subpopulations. The hierarchical analysis of molecular variance (AMOVA) also revealed a significantly low genetic variation (3 %) due to differences in isolates from different *L. maculans* population, whereas the isolates

from each individual population was responsible for the high genetic variation (97 %). In addition, the Bayesian population structure, PCoA results and the presence of linkage disequilibrium all suggest that similar to French *L. maculans* populations. *L. maculans* populations in the Czech Republic are likely coevolving into a panmictic population.

One reason why there was little variation among subpopulations could be through gene flow via infected plant material. Commercial seeds are routinely treated and there have been no reports of internal infection by *L. maculans*. However, seed transmission cannot be excluded as a mechanism of past gene flow because there is little information on bulk seed transportation, especially during the establishment of the industry. Also, many farmers retain seed from their own crops in preference to buying commercial seed. Another reason for little variation among subpopulations could be selection pressure. Avirulence alleles in *L. maculans* continuously evolve to adapt to resistance alleles in the host which might have had an influence on the population structure.

To our knowledge, this is the first report in the Czech Republic that sheds light on a genetically distinct population of *L. maculans* in the Czech Republic as well as possible reproduction patterns. The findings of this study will aid in the direction of the oilseed rape breeding program by screening breeding lines against a diverse population of *L. maculans*. At the same time, it contributes to better disease management practices by limiting the movement of infected plant materials within and across regions. Nonetheless, additional samples will be collected throughout the growing season to validate these findings and determine the role of asexual spores in the epidemiology of *L. maculans* in the Czech Republic.

8 Conclusions

The results suggest that boscalid which belongs to the SDHI fungicides class and trifloxystrobin which belongs to the QoI fungicide class are the most effective fungicides in controlling *L. maculans* populations, while DMI fungicides are the least effective fungicides, possibly because of the presence of inserts in the promoter region of the CYP51. Also observed in this study was the presence of cross sensitivity between the DMI fungicides, Therefore, it is important to limit the use of DMI fungicides in oilseed rape fields, whether as fungicides or as growth regulators. These fungicides may be rotated with other fungicide classes such as SDHI or QoI or may be used as tank mixes.

This study also provided insight into frequency and race structure of avirulence alleles of *L. maculans* populations where it was determined that the most frequent *AvrLm* allele is the *AvrLm7* allele. This suggests the most effective *Rlm* gene is the *Rlm7*. Other *Rlm* (*Rlm1*, *Rlm2*, *Rlm3*, *Rlm4* and *Rlm9*) genes in this study were found in low frequencies suggesting they may not be very effective anymore in the Czech Republic. Also noticed in this study was the epistatic mechanism of *AvrLm7* which masked the recognition of *AvrLm3* and the presence of 17 distinct *L. maculans* races. Therefore, to prolong the durability of major genes in the Czech Republic, rotation of oilseed rape crops with other crops and integrative management strategies such as burying plant debris to avoid sexual propagation should be encouraged. In addition, rotating oilseed rape plants with different major resistance genes and introgressing the *Rlm7* into a new breeding line could preserve this major gene.

In this study, the two mating type idiomorphs were evenly distributed suggesting there is high genotypic diversity within populations as a result of active sexual recombination within the *L. maculans* populations. Interestingly in this study, there was more genetic variability within subpopulations than between subpopulations. This could be because there was gene flow via infected seeds that may not have been treated and transported from region to region. Another reason could be the adaptation of avirulence alleles in *L. maculans* to resistance alleles of plant cultivars.

Therefore, the knowledge gained from this study would improve our understanding of fungicides sensitivity of *L. maculans* isolates to SDHI, QoI and DMI fungicides. Genetic resistance and the flow of genes in Czech *L. maculans* populations. Nevertheless, continuous

monitoring of fungicide sensitivity of *L. maculans* isolates including *in vivo* and field experiments should be carried out.

9. References

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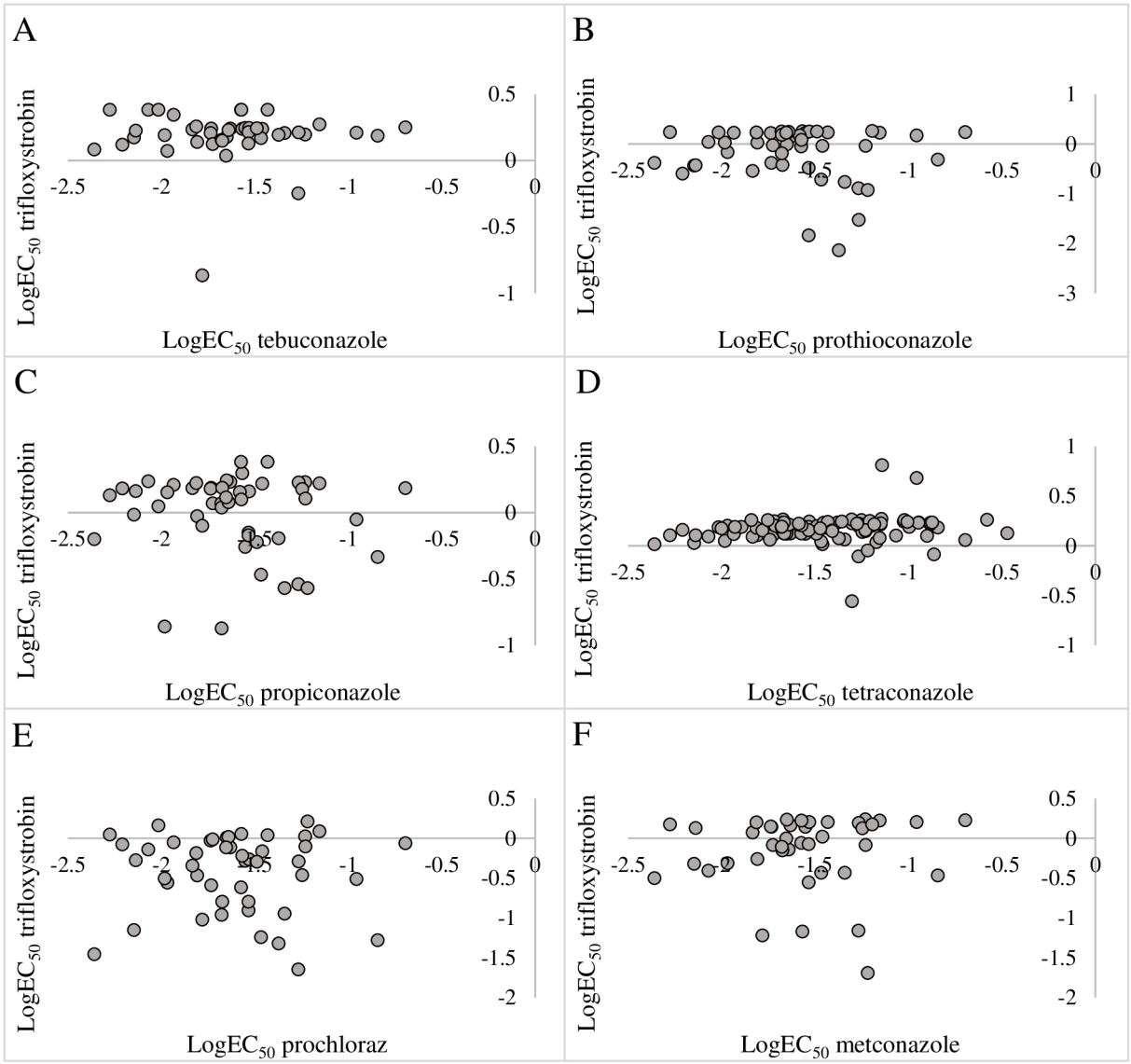
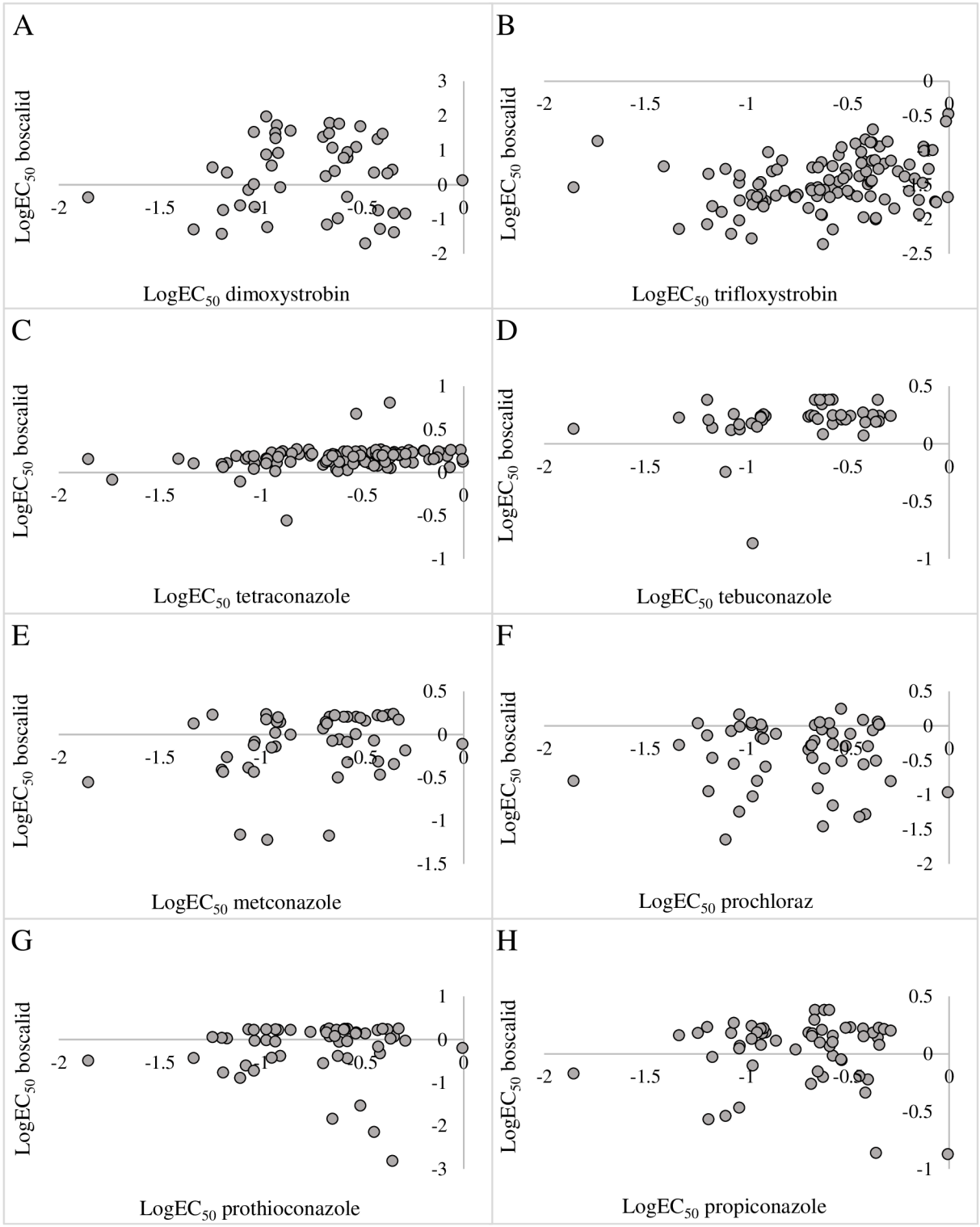
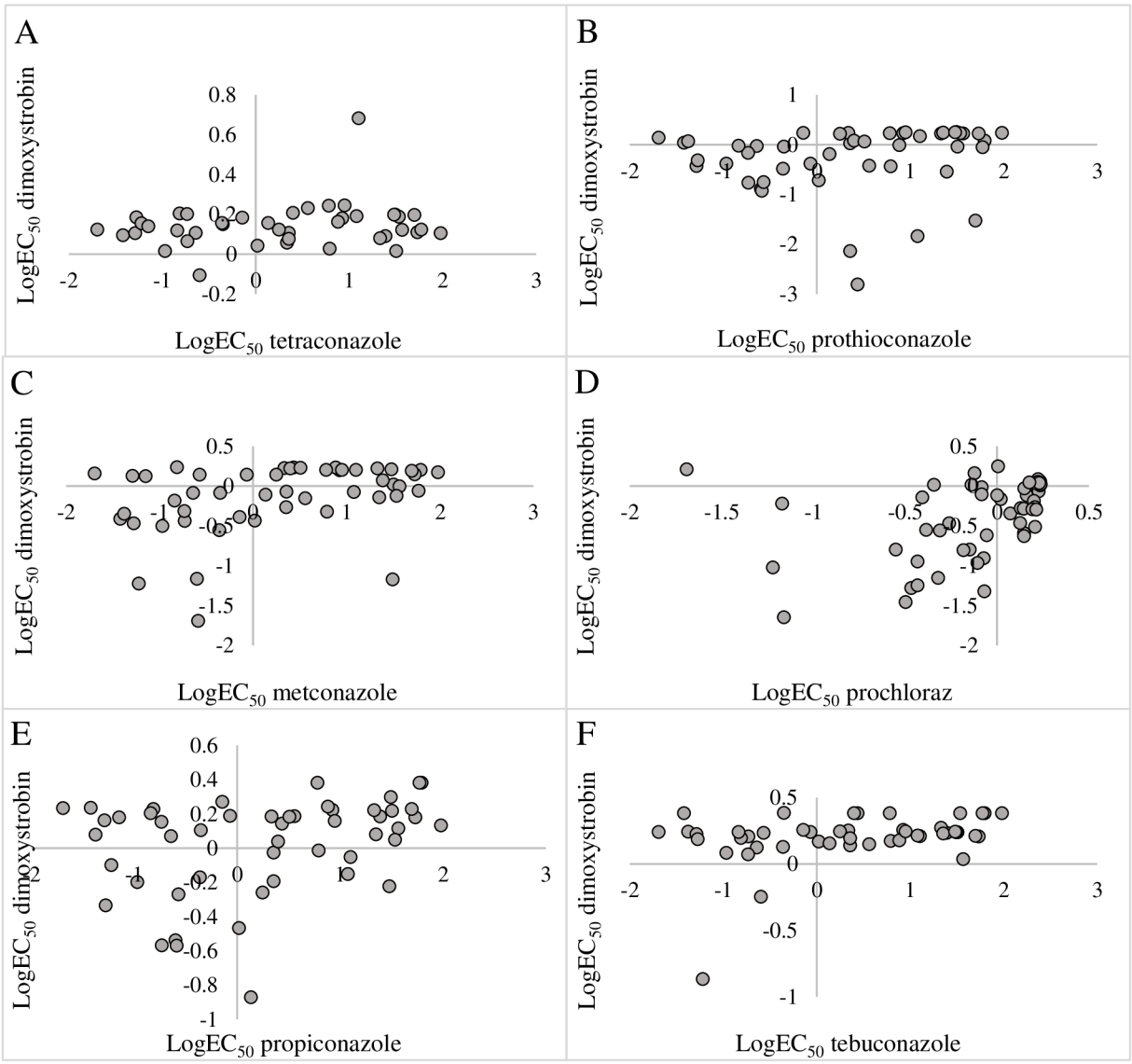
Appendix



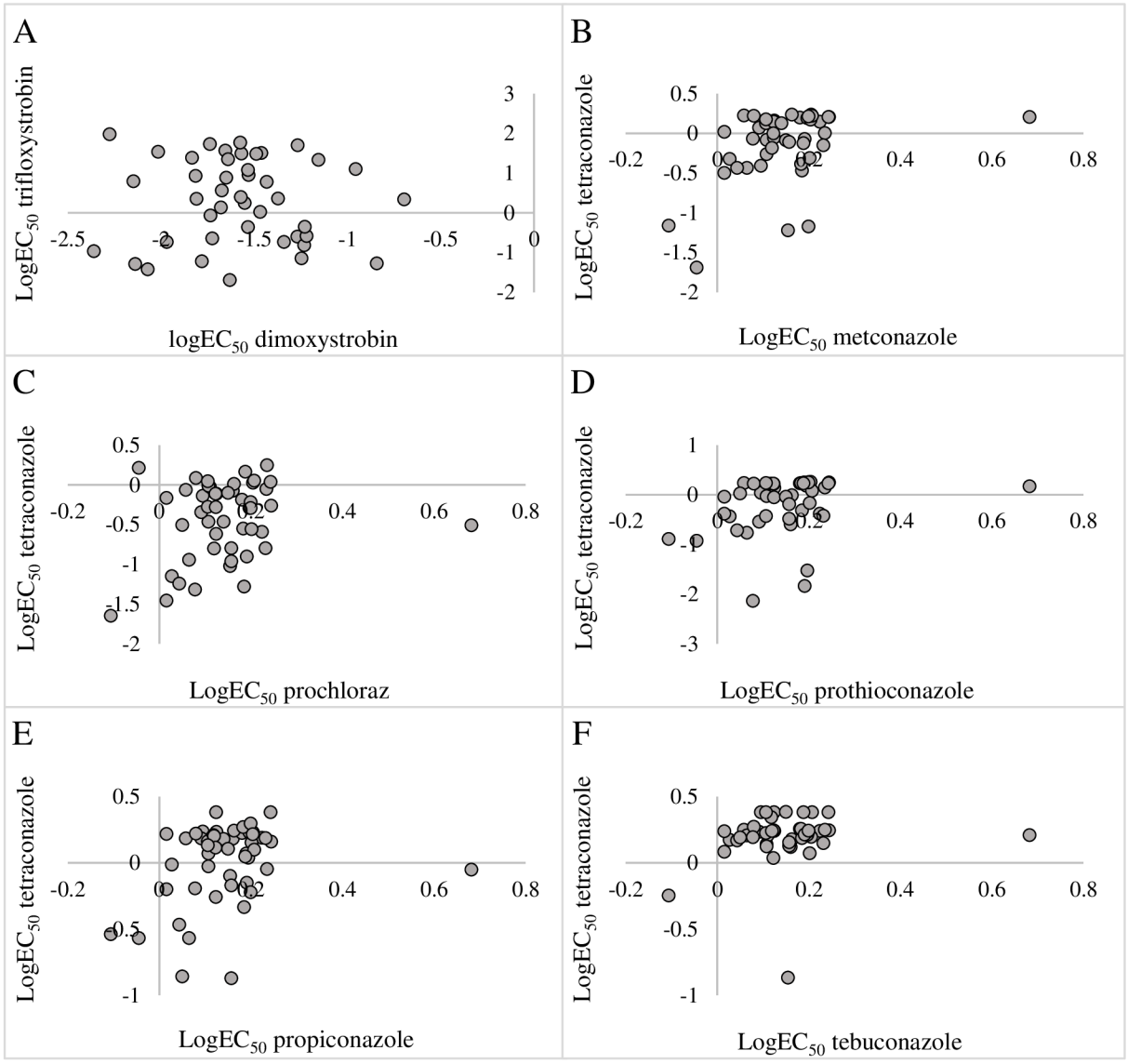
Appendix I. Multiple resistance between (A) trifloxystrobin and tebuconazole (r = 0.09, P = 0.5664), (B) trifloxystrobin and prothioconazole (r = -0.02, P = 0.9103), (C) trifloxystrobin and propiconazole (r = -0.06, P = 0.6585) (D) trifloxystrobin and tetraconazole (r = 0.26, P = 0.0083), (E) trifloxystrobin and prochloraz (r = 0.05, P = 0.9727) and (F) trifloxystrobin and metconazole (r = 0.20, P = 0.2065) of *Leptosphaeria maculans* mycelial isolates collected from oilseed rape plants obtained in the Czech Republic.



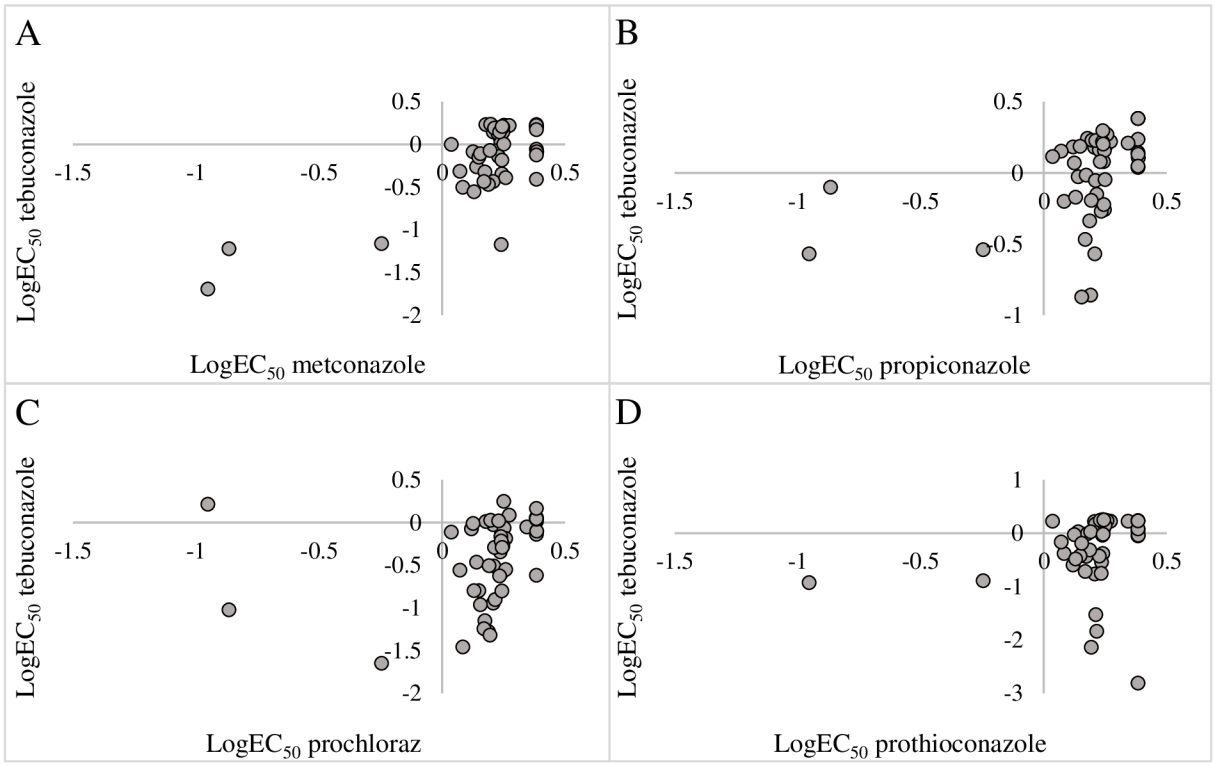
Appendix II. Multiple resistance between (A) boscalid and dimoxystrobin (r = -0.01, P = 0.9416), (B) boscalid and trifloxystrobin (r = 0.32, P = 0.0008), (C) boscalid and tetraconazole (r = 0.35, P = 0.0002) (D) boscalid and tebuconazole (r = 0.30, P = 0.0459), (E) boscalid and metconazole (r = 0.31, P = 0.0310) and (F) boscalid and prochloraz (r = 0.02, P = 0.8886) (G) boscalid and prothioconazole (r = 0.13, P = 0.3489), and (H) boscalid and propiconazole (r = -0.004, P = 0.9769) of *Leptosphaeria maculans* mycelial isolates collected from oilseed rape plants obtained in the Czech Republic.



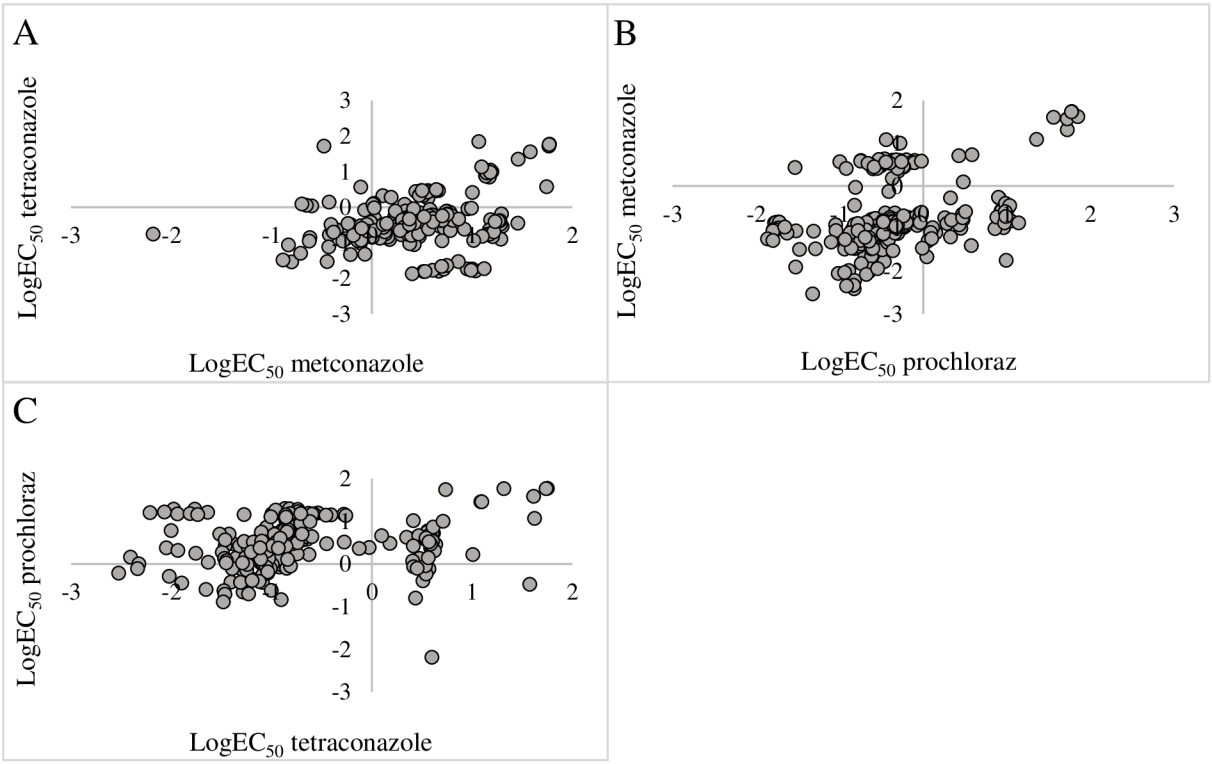
Appendix III. Multiple resistance between (A) dimoxystrobin and tetraconazole (r = 0.14, P = 0.3692), (B) dimoxystrobin and prothioconazole (r = 0.34, P = 0.0202), (C) dimoxystrobin and metconazole (r = 0.40, P = 0.0043) (D) dimoxystrobin and prochloraz (r = 0.52, P = 0.0001), (E) dimoxystrobin and propiconazole (r = 0.27, P = 0.0600) and (F) dimoxystrobin and tebuconazole (r = 0.28 P = 0.0571) of *Leptosphaeria maculans* mycelial isolates collected from oilseed rape plants obtained in the Czech Republic.



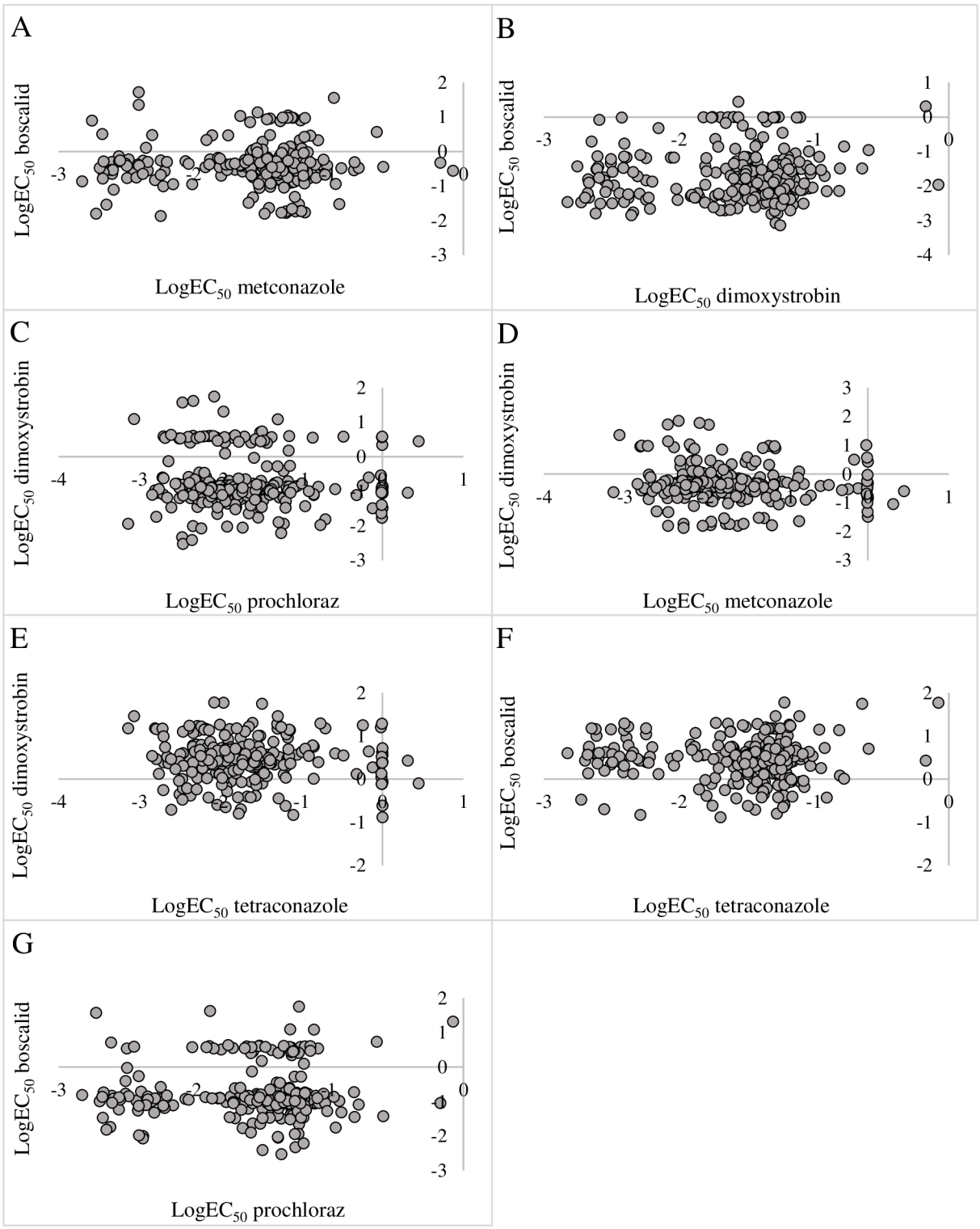
Appendix IV. Cross resistance between (A) trifloxystrobin and dimoxystrobin (r = -0.04, P = 0.7804), (B) tetraconazole and metconazole (r = 0.38, P = 0.0109), (C) tetraconazole and prochloraz (r = 0.21, P = 0.1616) (D) tetraconazole and prothioconazole (r = 0.38, P = 0.0088), (E) tetraconazole and propiconazole (r = 0.27, P = 0.0555) and (F) tetraconazole and tebuconazole (r = 0.19, P = 0.1988) of *Leptosphaeria maculans* mycelial isolates collected from oilseed rape plants obtained in the Czech Republic.



Appendix V. Cross resistance between (A) tebuconazole and metconazole (r = 0.49, P = 0.0003), (B) tebuconazole and propiconazole (r = 0.27, P = 0.0555), (C) tebuconazole and prochloraz (r = 0.48, P = 0.0003), and (D) tebuconazole and prothioconazole (r = 0.45, P = 0.0084) of *Leptosphaeria maculans* mycelial isolates collected from oilseed rape plants obtained in the Czech Republic.



Appendix VI. Cross resistance between (A) tetraconazole and metconazole (r = 0.33, P < 0.0001 0.9416), (B) metconazole and prochloraz (r = 0.45, P < 0.0001), (C) prochloraz and tetraconazole (r = 0.33, P < 0.0001) (of *Leptosphaeria maculans* conidial isolates collected from oilseed rape plants obtained in the Czech Republic.



Appendix VII. Multiple resistance between (A) boscalid and metconazole (r = -0.06, P = 0.3339), (B) boscalid and dimoxystrobin (r = 0.04, P = 0.4893), (C) dimoxystrobin and prochloraz (r = -0.07, P = 0.2533) (D) dimoxystrobin and metconazole (r = 0.30, P = 0.0459), (E) dimoxystrobin and tetraconazole (r = -0.10, P = 0.00909) and (F) boscalid and tetraconazole (r = -0.03, P = 0.6280), and (G) boscalid and prochloraz (r = -0.05, P = 0.3741) of *Leptosphaeria maculans* conidial isolates collected from oilseed rape plants obtained in the Czech Republic.