# SENSITIVITY TO FUNGICIDES AND ESSENTIAL OILS IN CZECH ISOLATES OF *PHYTOPHTHORA INFESTANS**

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A total of 235 *Phytophthora infestans* isolates were collected from five regions of the Czech Republic during the growing seasons 2012–2014 and 2016 and examined using the *in vitro* amended agar method for their sensitivity to metalaxyl-M (MFX), propamocarb-HCl (PCH), and dimethomorph (DMM). A majority of the isolates (50%) were sensitive to MFX. Resistant isolates were found in all four years of the survey; they represented 30% of the samples. The $EC_{50}$ values of PCH in inhibiting mycelial growth of 65% of the overall isolates were higher than 100 μg $ml^{-1}$, which indicates the occurrence of insensitivity to PCH in the Czech *P. infestans* populations. DMM was very effective, and the mycelial growth of all isolates tested was completely suppressed at the concentration of 0.1 μg $ml^{-1}$. Furthermore, the efficacy of 12 plant essential oils was tested against 20 isolates of *P. infestans* using the *in vitro* amended agar method. Essential oils of *Cymbopogon winterianus*, *Litsea cubeba*, *Mentha spicata*, *Pelargonium graveolens*, *Syzygium aromaticum*, and *Thymus vulgaris* were observed to have the highest antifungal activity against *P. infestans,* with minimal inhibitory concentrations less than or equal to 1 μl $ml^{-1}$.

dimethomorph, aromatic plants, fungicide resistance, late blight, metalaxyl-M, propamocarb-HCl

DE GRUYTER OPEN

doi: 10.2478/sab-2018-0011
Received for publication on October 12, 2017
Accepted for publication on December 2, 2017

## INTRODUCTION

A shift in sensitivity of plant pathogenic fungi and oomycetes to antifungal compounds is a common phenomenon occurring in populations of plant pathogens that are repeatedly and frequently exposed to the application of primarily systemic fungicides with a single-site mode of action (Brent, Hollomon, 2007). In view of the fact that the use of fungicides has become a critical element in effective integrated plant disease management, the evolution of fungicide resistance is a crucial threat to crop protection, especially against pathogens with airborne inoculum and polycyclic life cycle (Brent, 2012), such as the late blight pathogen *Phytophthora infestans* (Mont.) de Bary. Genetic plasticity of *P. infestans* is achieved through genomic flexibility (by mutation, mitotic and sexual recombination, and migration) and plays an important role in the adaptation of the pathogen to various factors. This genetic plasticity, in combination with frequent fungicide application, together cause the pathogen to develop fungicide resistance (Gisi, 2002).

The best known and most studied example of fungicide resistance has been in *P. infestans* to metalaxyl (MET), the active ingredient belonging to systemic phenylamides (PAs) with preventive and curative activity that is limited to oomycetes (Gisi, Cohen, 1996; Gisi, 2002). The first failure of MET was observed within *Pseudoperonospora cubensis* in Israel in 1979, only two years after introducing MET onto the market (Reuveni et al., 1980). Many reports on the insufficiency of MET to control other oomycetes followed (Gisi, 2002; Gisi, Sierotzki, 2008). The resistance to MET in *P. infestans* populations was first reported in Ireland and in the Netherlands where MET was used as a solo ingredient product that was applied curatively during high disease pressure (Davidse et al., 1981; Dowley, O'Sullivan,

* Supported by the Ministry of Agriculture of the Czech Republic, Projects No. QJ1210305 and No. QJ1310226.

1981). In 1996, metalaxyl-M (MFX, mefenoxam), the R-enantiomer (D-alaninate analogue) of MET, was launched as a compound active at the same level of efficacy as MET, but at half the application rate (Nuninger et al., 1996). Nevertheless, despite the reduction in the environmental risk associated with the use of MET, MFX-resistant isolates of *P. infestans* have also been detected (e.g. Lehtinen et al., 2008; Runno-Paurson et al., 2015).

The high risk of reinforcing the resistant strains in populations of oomycete pathogens has intensified the need for new oomycete-targeted compounds for disease control with varying modes of action. Currently in the Czech Republic, fungicides with four systemic active ingredients – benalaxyl, benalaxyl-M, metalaxyl-M (PAs), propamocarb-hydrochloride (PCH; carbamates) and five locally systemic active ingredients – azoxystrobin (QoIs; quinone outside inhibitors), benthiavalicarb, dimethomorph (DMM), mandipropamid, valifenalate (CAAs; carboxylic acid amides) and cymoxanil (cyanoacetamide oximes) are registered for potato late blight control and allowed to be marketed (Anonymous, 2017). All these chemicals are likely site-specific fungicides, and most of them bear a high intrinsic risk of causing the evolution of resistant pathogen sub-populations (Gisi et al., 2000; Gisi, Sierotzki, 2008). Unlike for MET or MFX, there are only several studies about the sensitivity of *P. infestans* to some of these compounds (e.g. Gisi et al., 1997; Cohen et al., 2007).

Despite several cultural practices and resistant cultivars that are implemented to manage late blight, the effective, preferred method of controlling the pathogen is heavily focused on fungicide application several times per growing season to prevent yield losses, especially in 'blight years'. At the same time, the growing pressure from social and political spheres to reduce pesticide use on crops increases the need for innovative and effective control alternatives if fungicide application is to be reduced or eliminated. Therefore, interest in natural products with antifungal properties, such as plant extracts and essential oils, as potential alternative compounds for use in crop protection has increased during the last years. There are few reports on the effects of essential oils obtained from various plants on the inhibition of mycelial growth of *P. infestans* (Quintanilla et al., 2002; Olanya, Larkin, 2006; Soylu et al., 2006).

In view of the abovementioned aspects, this study focused on the assessment and examination of changes in the sensitivity of Czech *P. infestans* isolates to MFX, PCH, and DMM. MFX represented a specific example of a compound against which resistance has been reported in most countries around the world. In contrast, until now, no DMM-resistant isolates of *P. infestans* have been detected in fields. PCH is the most widely used systemic active ingredient in the Czech Republic, and recently, failure of PCH has been observed repeatedly in field studies (Hausvater, Potato Research Institute Havlíčkův Brod, pers. comm.). In addition, the inhibitory effect of essential oils on *in vitro* growth of *P. infestans* was evaluated.

## MATERIAL AND METHODS

### Collection, isolation, and maintenance of *P. infestans* isolates

A total of 235 isolates of *P. infestans* were sampled from commercial growers' fields, research station fields, and gardens in five regions of the Czech Republic in 2012–2014 and 2016 (Table 1). Since the years of the survey were not considered to be typical late blight years in the Czech Republic, the geographical distribution of sampling sites and mean number of isolates sampled per site varied from year to year depending on the appearance and further development of infection in potato crops in a given year. Symptomatic potato leaves were deposited individually in a plastic bag, transported to the laboratory and then immediately prepared for isolation of the pathogen (Mazakova et al., 2010).

### *In vitro* testing of fungicide sensitivity

Sensitivity to MFX, DMM, and PCH was determined by assessing the growth of all 235 *P. infestans* isolates on amended agar plates. The active substances (Sigma-Aldrich, St. Louis, USA) were dissolved in dimethyl sulfoxide (DMSO) and added to cooled rye A agar. Using the classification criteria of Therrien et al. (1993), MFX was added at concentrations of 5 and 100 μg ml$^{-1}$. To calculate $EC_{50}$ values, a range of MFX concentrations was supplemented with concentrations of 0.1, 1, and 10 μg ml$^{-1}$. PCH sensitivity testing was performed using the concentrations corresponding to those of MFX (0.1, 1, 10, and 100 μg ml$^{-1}$). For DMM, only three different concentrations (0.1, 1, and 10 μg ml$^{-1}$) were used based on previous data (Mazakova et al., 2011). The control plate contained agar to which DMSO had been supplemented at 1 μl ml$^{-1}$. Mycelial plugs (9 mm diameter) were cut using a cork borer from the margin of colonies of *P. infestans* isolates that were actively growing for two weeks and then were placed mycelial side down in the centre of 9-cm Petri dishes containing amended agar. There were three replicate plates for each concentration and for each of the tested isolates. The plates were incubated at 15–18°C in darkness for approximately 10–20 days, until the mycelial growth of the control reached the edge of the plate. The colony diameters in the dishes were measured in two perpendicular directions on all plates and the average colony diameter was determined. Isolates were classified as sensi-

Table 1. Origin and characterization of *Phytophthora infestans* isolates evaluated for sensitivity to metalaxyl-M (MFX)

| Year/locality | Region and district code | Number of tested isolates | | | |
|---|---|---|---|---|---|
| | | MS | MI | MR | Σtotal |
| **2012 (*n* = 35 isolates)** | | | | | |
| Valečov (Okrouhlice) | CZ0631 | 4 | 6 | – | 10 |
| Olešnice (Okrouhlice) | CZ0631 | – | – | 3 | 3 |
| Lučice | CZ0631 | – | 3 | 1 | 4 |
| Malčín | CZ0631 | – | 3 | – | 3 |
| Frýdnava | CZ0631 | – | 1 | 2 | 3 |
| Veselý Žďár | CZ0631 | 1 | 1 | – | 2 |
| Jedouchov (Věž) | CZ0631 | 1 | – | – | 1 |
| Kamenice (Herálec) | CZ0631 | – | – | 3 | 3 |
| Bystřec | CZ0534 | – | 3 | 3 | 6 |
| **2013 (*n* = 40 isolates)** | | | | | |
| Valečov (Okrouhlice) | CZ0631 | 7 | 4 | 4 | 15 |
| Veselý Žďár | CZ0631 | 8 | – | – | 8 |
| Český Dvůr (Knyk) | CZ0631 | – | – | 4 | 4 |
| Rozsochatec | CZ0631 | – | 3 | 8 | 11 |
| Čachotín | CZ0631 | 2 | – | – | 2 |
| **2014 (*n* = 60 isolates)** | | | | | |
| Veselý Žďár | CZ0631 | 7 | 7 | 1 | 15 |
| Valečov (Okrouhlice) | CZ0631 | 10 | – | 1 | 11 |
| Pohled | CZ0631 | 1 | – | – | 1 |
| Modlíkov | CZ0631 | – | – | 3 | 3 |
| Nové Dvory | CZ0631 | – | – | 3 | 3 |
| Semice | CZ0208 | – | – | 20 | 20 |
| Čelákovice | CZ0209 | 1 | – | 6 | 7 |
| **2016 (*n* = 100 isolates)** | | | | | |
| Malý Bor | CZ0322 | 12 | 2 | – | 14 |
| Domanínek | CZ0635 | 14 | – | – | 14 |
| Praha-Suchdol | CZ0100 | 3 | – | – | 3 |
| Lukavec | CZ0633 | 8 | – | – | 8 |
| Želiv | CZ0633 | 6 | – | 1 | 7 |
| Lípa | CZ0631 | 10 | – | 1 | 11 |
| Valečov (Okrouhlice) | CZ0632 | 16 | 13 | – | 29 |
| Veselý Žďár | CZ0633 | 1 | – | – | 1 |
| Velhartice | CZ0322 | 0 | 1 | 6 | 7 |
| Únětice | CZ020A | 6[t] | – | – | 6[t] |

*n* = number of collected isolates, [t] = isolates collected from tomatoes, MS = MFX-sensitive, MI = MFX-intermediate, MR = MFX-resistant
region and district code are based on NUTS classification

tive, intermediate and resistant to MFX according to the criteria of Therrien et al. (1993). There is no similar classification range for separating isolates into sensitive, intermediate and resistant groups depending on their sensitivity to PCH and DMM, therefore the $EC_{50}$ values (concentration of active ingredients which inhibits mycelial growth by 50%) of PCH, DMM as well as MFX were calculated for each isolate from the regression of the probit of the percentage of growth inhibition against the logarithmic value of fungicide concentration.

***In vitro* testing of essential oil efficacy**

The antifungal activity of essential oils against 20 selected *P. infestans* isolates was evaluated using

Table 2. Inhibitory effect of essential oils on mycelial growth of *Phytophthora infestans* at a concentration of 1 µl ml$^{-1}$ and minimal inhibitory concentration of essential oils

| Essential oil of | MGI (%) | MIC (µl ml$^{-1}$) |
|---|---|---|
| *Cymbopogon winterianus* | 100 ± 0 | 1 |
| *Corymbia citriodora* | 25.98 ± 32.57 | n |
| *Foeniculum vulgare* | 14.15 ± 20.74 | n |
| *Lavandula angustifolia* | 23.67 ± 29.08 | n |
| *Litsea cubeba* | 100 ± 0 | 0.4–1 |
| *Mentha spicata* | 100 ± 0 | 0.6–1 |
| *Pimpinella anisum* | 8.32 ± 18.89 | n |
| *Pelargonium graveolens* | 100 ± 0 | 0.6–0.8 |
| *Rosmarinus officinalis* | 10.01 ± 18.65 | n |
| *Salvia officinalis* | 11.73 ± 22.89 | n |
| *Syzygium aromaticum* | 100 ± 0 | 0.2–0.4 |
| *Thymus vulgaris* | 100 ± 0 | 0.25–0.4 |

MGI = mycelial growth inhibition at 1 µl ml$^{-1}$, MIC = minimal inhibitory concentration, n = not performed

values are means ± standard deviation, mean for 20 isolates of *P. infestans*

the amended agar test, as described above. All essential oils (M+H, Míča a Harašta s.r.o., Prague, Czech Republic) from different plant species (Table 2) were diluted in DMSO in a 1 : 1 ratio and added to rye A agar at a concentration of 1 µl ml$^{-1}$. This concentration was selected as the initial concentration; the essential oils that did not inhibit the growth of isolates by 100% were excluded from subsequent testing. A variant with the application of DMSO (1 µl ml$^{-1}$) and another with 0.1 µg ml$^{-1}$ DMM were used as controls. Mycelial growth inhibition (MGI) was expressed as a percentage and calculated according to the formula:

MGI (%) = 100 – [(dT/dC) × 100]

where:

dT = colony diameter (mm) of the tested plate

dC = colony diameter (mm) of the control plate

Furthermore, the essential oils exhibiting 100% inhibition of mycelial growth were tested in a concentration range of 0.1–1 µl ml$^{-1}$. The minimal inhibitory concentration (MIC) of each essential oil was determined for each isolate as the lowest concentration that inhibited 100% of the growth of a pathogen isolate.

### Statistical analysis

Detailed statistical analyses were performed using STATISTICA software (Version 12 CZ for MS Windows) (StatSoft, Tulsa, USA). To compare the frequencies of isolates that were sensitive, intermediate, and resistant to MFX and the frequencies of isolates in different classes of $EC_{50}$ values for MFX and PCH (< 0.1, 0.1–1, 1–10, 10–100 and > 100 µg ml$^{-1}$) within the years of the survey, data were summarized into contingency tables and evaluated by Pearson's chi-square and Cramér's V. To measure the correlation between sensitivity to fungicides, the correlation coefficients ($r$) and probability ($P$) values were calculated using Pearson's correlation analysis. The significance level was set as $P < 0.05$.

## RESULTS

### Fungicide sensitivity

**Metalaxyl-M**. Among the tested isolates, all three types of reaction to MFX was found in each year of the survey (Fig. 1). On average 50, 20, and 30% of all tested isolates were sensitive, intermediate, and resistant to MFX, respectively. The frequency of isolates with all three different responses to MFX significantly varied with the year in which the isolates were collected ($\chi^2 = 74.87$, $P = 0$, $\phi_c = 0.399$). The highest percentage of sensitive isolates was found in 2016 (76%). By contrast, the percentage of sensitive isolates was the lowest in 2012 (17%). In 2013, the proportions of isolates that were sensitive (43%) and resistant (40%) to MFX were almost equal. The highest frequency of MFX-resistant isolates was observed in 2014 (57%), while in 2016, only 8% of the isolates were resistant. The frequency of intermediate isolates was found to be the highest in 2012 (49%), decreased to 18% in 2013, and to 12% in 2014 and then slightly increased to 16% in 2016 (Fig. 1). The proportion of isolates with different reactions also varied with sites from which the isolates were collected and ranged from 0 to 100%, depending on the site. Of the 31 sites sampled, sensitive isolates were detected at 19 sites, with 3, 3, 4, and 9 sites sampled in 2012, 2013, 2014, and 2016, respectively. Resistant isolates were observed within

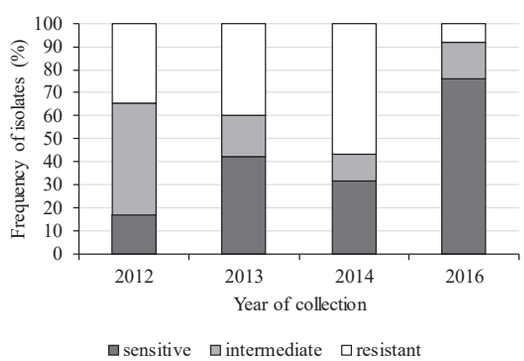


Fig. 1. Responses of *Phytophthora infestans* isolates to metalaxyl-M (years 2012–2014 and 2016)

isolates that were collected at 17 sites and intermediate isolates were observed at 12 sites (Table 1).

The percentage growth rates of all sensitive isolates on media with 5 and 100 µg ml$^{-1}$ of MFX relative to the control was 0% in each year. Of 118 isolates sensitive to MFX, 64% showed no growth at an MFX concentration of 0.1 µg ml$^{-1}$. Thus, an $EC_{50}$ value could not be estimated, and these isolates were grouped together with the other isolates (18%) having $EC_{50}$ values < 0.1 µg ml$^{-1}$ into the class with $EC_{50}$ values < 0.1 µg ml$^{-1}$. In total, 18% of the remaining sensitive isolates had $EC_{50}$ values ranging from 0.1 to 1 µg ml$^{-1}$. The mean effective concentration ($EC_{50}$) value for sensitive isolates was 0.33 ± 0.36 µg ml$^{-1}$, ranging from 8.77E-08 to 0.97 µg ml$^{-1}$. The intermediate isolates had mean percentage growth rates relative to the control on media with 100 µg ml$^{-1}$ of MFX ranging from 13 to 40%. The mean $EC_{50}$ value for the isolates with an intermediate reaction to MFX was 20.67 ± 16.74 µg ml$^{-1}$, ranging from 4.69 to 69.3 µg ml$^{-1}$. There were 38 and 62% of the intermediate isolates with the $EC_{50}$ values in the range of 1–10 and 10–100 µg ml$^{-1}$, respectively. The resistant isolates exhibited growth of 62 to 100% on MFX-amended (100 µg ml$^{-1}$) media when compared to the growth of the unamended control. The $EC_{50}$ value could not be estimated for all resistant isolates, and these isolates were placed into the class with $EC_{50}$ values > 100 µg ml$^{-1}$. Of the 70 MFX-resistant isolates, 83% showed no inhibition of growth at the highest concentration of MFX, and 17% showed less than 50% growth inhibition at the same MFX concentration. There was a significant positive relationship between the frequencies of isolates in different $EC_{50}$ classes across the years, with an observed $\chi^2$ value, *P*-value, and Cramér's V coefficient of $\chi^2 = 105.94$, $P = 0$, and $\phi_c = 0.387$, respectively. Correlation analysis showed a significant positive correlation between sensitivity of isolates to MFX and DMM ($r = 0.231$, $P = 0$).

**Propamocarb-HCl**. In total, 235 *P. infestans* isolates tested for their sensitivity level to PCH exhibited varying levels of response (Fig. 2). There was no effect of PCH at a concentration of 100 µg ml$^{-1}$ on 16 isolates of *P. infestans*. These isolates exhibited 100% mycelial growth on media with the highest concentration of PCH compared with their growth on control plates, and thus, their $EC_{50}$ values could not be determined. Accurate $EC_{50}$ values also could not be estimated for 137 isolates that were not 50 to <100% inhibited by PCH at the highest concentration, relative to the control. Within these isolates, the dose-response curve did not fit a sigmoidal shape, or the estimated $EC_{50}$ values were much greater than the highest concentration of PCH. These two groups of isolates (65%) were categorized as having $EC_{50}$ > 100 µg ml$^{-1}$. The mean $EC_{50}$ value for the remaining *P. infestans* isolates was 34.64 ± 26.02 µg ml$^{-1}$. The lowest calculated $EC_{50}$ value for PCH was 0.46 µg ml$^{-1}$, and the highest $EC_{50}$ value was 97.97 µg ml$^{-1}$. Only five isolates had $EC_{50}$ values < 1 µg ml$^{-1}$, and eight isolates belonged to the class with $EC_{50}$ values ranging from 1 to 10 µg ml$^{-1}$. The $EC_{50}$ values of 69 isolates ranged from 10 to 100 µg ml$^{-1}$ (Fig. 2). There was a statistically significant association between the frequencies of isolates in different $EC_{50}$ classes across years, with an observed $\chi^2$ value, *P*-value, and Cramér's V coefficient of $\chi^2 = 77.76$, $P = 0$, and $\phi_c = 0.3832$, respectively.

**Dimethomorph**. None of the isolates of *P. infestans* collected in 2012–2014 and 2016 was insensitive to DMM. All the 235 isolates exhibited a growth rate of 0% on all concentrations and thus, it was not possible to calculate an $EC_{50}$ value for DMM.

**Essential oils**. The essential oils had different antifungal effects on *P. infestans* at the tested concentration of 1 µl ml$^{-1}$. The percentage of MGI of the isolates ranged from 8.32 to 100% (Table 2). The mycelial growth of *P. infestans* was completely inhibited by essential oils of *C. winterianus*, *L. cubeba*, *M. spicata*, *P. graveolens*, *S. aromaticum*, and *T. vulgaris* at a concentration of 1 µl ml$^{-1}$. As seen in Table 2, the MIC values obtained for *T. vulgaris* and *S. aromaticum*, ranging from 0.25 to 0.4 µl ml$^{-1}$ and 0.2 to 0.4 µl ml$^{-1}$, respectively, indicate that these two essential oils totally inhibited the growth of *P. infestans* isolates at the lowest concentrations used in this study. The essential oils of *L. cubeba*, *M. spicata,* and *P. graveolens* exhibited 100% inhibitory effect against *P. infestans* at MIC values ranging from 0.4 to 1 µl ml$^{-1}$, 0.6 to 1 µl ml$^{-1}$, and 0.6 to 0.8 µl ml$^{-1}$, respectively. The MIC of *C. winterianus* was found to be the highest at a concentration of 1 µl ml$^{-1}$.

## DISCUSSION

The results presented here indicate that changes in the level of sensitivity to fungicides have occurred in the Czech *P. infestans* populations in the field over

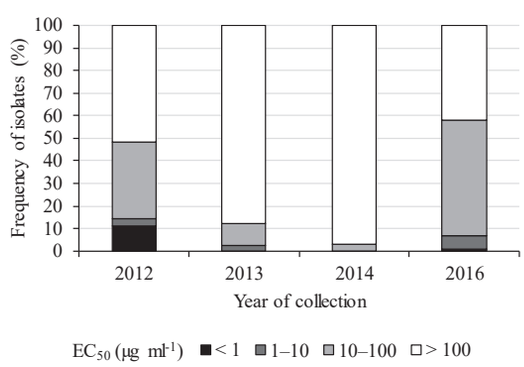


Fig. 2. Responses of *Phytophthora infestans* isolates to propamocarb-HCl (years 2012–2014 and 2016)

the past fourteen years. In the Czech Republic, the decreased efficiency of PAs in potato crops was first observed in 1986 (Hausvater, Rasocha, 2000), although the MET-resistant isolates were first detected using *in vitro* and *in vivo* assays of response to MET in 2003 (58%), subsequently in 2008 (29%) (Mazakova et al., 2011), and again in 2009 (31%) (unpublished data). In the Czech Republic, the use of MFX-based fungicides is limited to a maximum of three applications per season at 10- to 14-day intervals. Although the proportion of MFX that is used mixed with mancozeb has remained under 2% of all the active ingredients used against late blight in the Czech Republic, MFX-resistant isolates are still present in *P. infestans* populations, including in fields untreated by PAs. Moreover, 83% of MFX-resistant isolates were extremely resistant and exhibited enhanced growth on media amended with MFX at 100 µg ml$^{-1}$, and the $EC_{50}$ values could not be calculated for all resistant isolates, suggesting that the $EC_{50}$ values for these isolates could reach or exceed the doses of MFX recommended in the field (250 µg ml$^{-1}$ in a spray volume of 400 l ha$^{-1}$; the concentration value depends on fungicide spray coverage ranging from 50 to 60%). This indicates that prevalence of MFX-resistant isolates in *P. infestans* population may cause disease control failure in the MFX-treated field. On the other hand, in total, 64% of sensitive isolates showed an extreme response to MFX, with 100% inhibition of growth already observed at 0.1 µg ml$^{-1}$.

Since MET and MFX have the same specific mode of action but different compositions, comparing levels of sensitivity to MFX in the collection of *P. infestans* isolates analyzed in this study to previous data showing levels of sensitivity to MET is difficult. However, in general, the shifts in proportions of isolates with different responses to an active ingredient may be considered with respect to PAs as a group. Comparison of data from the present study with those from previous years showed that the level of sensitivity of *P. infestans* isolates to PAs in the Czech Republic is currently in a completely different range than in 2003–2009. It is especially evident in regards to the proportion of MFX-intermediate isolates that ranged from 12 to 49% (2012–2014, 2016), while only a very low frequency of MET-intermediate isolates was found in 2004 (6%) (Mazakova et al., 2011) and in 2009 (4%) (unpublished data). Additionally, in contrast to previous findings, the MFX-resistant isolates of *P. infestans* were found in each year of the survey at a frequency that was higher than (2012, 2014) or almost equal to (2013) that of the sensitive isolates. In contrast, only 8% of isolates were resistant to MFX in 2016. This decrease was likely due to the uncommon weather conditions during the summer of 2015. High temperatures and no rainfall did not allow the development and spread of *P. infestans* in potato crops. No occurrence of *P. infestans* as well as no fungicide selection pressure reduced the source of pathogen inoculum, including resistant isolates that could overwinter to the following season. Thus, the majority (76%) of isolates sampled from potato crops originating from seed produced in the Czech Republic were sensitive to MFX.

Recent studies in Baltic countries found predominant proportions of isolates that were sensitive to MFX in Estonian (Runno-Paurson et al., 2016), Latvian (Aav et al., 2015), and Lithuanian (Runno-Paurson et al., 2015) populations of *P. infestans*. A survey performed in three regions of Poland during 2010–2012 also showed in total a high proportion of MFX-sensitive isolates (66%) (Brylinska et al., 2016). However, Brylinska et al. (2016) showed that there were significant differences in the proportions of sensitive, intermediate, and resistant isolates between a region where large production fields under intensive chemical control were sampled and the other regions in which gardens prevailed. This phenomenon is well known because of the selection pressure of single-site fungicides under which fungicide-resistant isolates may survive and become dominant in pathogen populations (Gisi, Cohen, 1996; Gisi et al., 2000).

In this study, there were several observations that agreed with findings summarized by Gisi, Cohen (1996) and Gisi, Sierotzki (2008). As mentioned above, some MFX-resistant isolates were sampled in fields with an absence of fungicide application. Such resistant isolates can compete with or be fitter than sensitive isolates (Kadish, Cohen, 1992). These isolates could overwinter in infected potato seed tubers, despite the better survival of sensitive isolates (Kadish, Cohen, 1992) or migrate from fields treated with MFX. The frequency of MFX-resistant isolates fluctuated from 34% (2012) to 40% (2013) to 57% (2014) to 8% (2016). In Valečov, in isolates taken from the same field throughout the growing season during the three years of the survey, changes in the frequency of sensitive, intermediate, and resistant isolates were observed, with increased proportions of intermediate and resistant isolates in 2013 and an increased proportion of only intermediate isolates in 2016. However, only one MFX-resistant isolate was detected in Valečov in the third sampling period. The proportion of MFX-resistant isolates was higher in fields treated with MFX than in untreated fields. The high prevalence of MFX-resistant isolates was found in Semice (100%) and Čelákovice (86%) in October when infected potato leaves used for pathogen isolation were collected from plants remaining after harvest. These results are consistent with the findings that the frequency of resistant isolates increases at the expense of sensitive isolates during the season (Gisi, Cohen, 1996).

Not much data are available about the range of PCH sensitivity within *P. infestans*. Lehtinen et al. (2008) screened Nordic *P. infestans* isolates for sensitivity to PCH using a floating leaf-disc bioas-

say and detected two isolates from Sweden and two isolates from Finland that were able to sporulate in the presence of PCH at a concentration of 1000 µg ml$^{-1}$. In Serbia, *P. infestans* isolates exhibited $EC_{50}$ values ranging from 12.1 to 31.1 µg ml$^{-1}$ and were classified as sensitive according to their resistance factor (1–2.6) (Rekanovic et al., 2011). In the present study, the $EC_{50}$ values of PCH for *P. infestans* isolates showed distribution across a wide range (from 0.46 to >100 µg ml$^{-1}$); moreover, the $EC_{50}$ values could not be estimated for 65% of all tested isolates. In Germany, the majority (82%) of isolates from 1999 had $EC_{50}$ values from 100 to 1000 µg ml$^{-1}$ and were grouped as partially resistant or intermediate, while 8 and 10% of isolates were classified in groups with $EC_{50} \leq 100$ µg ml$^{-1}$ (sensitive) and $EC_{50} \geq 1000$ µg ml$^{-1}$ (resistant), respectively (Moller et al., 2009). Using the same classification scheme as these authors, 65% of the Czech *P. infestans* isolates may be considered intermediate or resistant. However, a valid discriminatory concentration for PCH that distinguishes among sensitive, intermediate, and resistant isolates is not clearly defined; therefore, the *P. infestans* isolates analyzed in this study were not grouped. Present data indicate that a shift towards high insensitivity to PCH has probably started in recent years because the mycelial growth of all isolates tested from 2003 to 2008 was suppressed even at a PCH concentration of 1 µg ml$^{-1}$ (Mazakova et al., 2011). Furthermore, the failure of PCH to control late blight has been observed in the fields in recent years (Hausvater, Potato Research Institute Havlíčkův Brod, pers. comm.). PCH is generally applied at a recommended concentration of about 2188 µg ml$^{-1}$ in a spray volume of 400 l ha$^{-1}$. This concentration is approximately 20-fold higher than the highest concentration of PCH used for *in vitro* testing. Since most (65%) isolates of *P. infestans* had $EC_{50} \geq 100$ µg ml$^{-1}$, a modification of the concentration range of PCH should be considered for further testing the sensitivity to PCH. There were also several reports on the sensitivity to PCH in other oomycetes, e.g., *Phytophthora capsici* (Qi et al., 2012) and *P. cubensis* (Pavelkova et al., 2014).

Compared with the level of sensitivity to MFX and PCH, DMM remains a highly effective compound used to control potato crops against late blight. All tested *P. infestans* isolates were uniformly controlled by the lowest concentration of DMM (0.1 µg ml$^{-1}$) which was much lower than those applied in the field (450 µg ml$^{-1}$ is a recommended concentration of DMM in a spray volume of 400 l ha$^{-1}$). This finding agrees with a previous survey conducted in the Czech Republic during 2003–2008 (Mazakova et al., 2011). These results are also consistent with previous studies (Zhang et al., 2005; Elansky et al., 2007; Zhu et al., 2008) that reported no presence of DMM-resistant isolates in *P. infestans*. Some studies also showed through several attempts that mutant isolates resistant to DMM with good fitness and stable resistance are difficult to generate (Bagirova et al., 2001; Stein, Kirk, 2004; Rubin et al., 2008). Considering the cross-resistance among all CAA active ingredients that was demonstrated for *Plasmopara viticola* (Gisi et al., 2007), our current results suggested that the risk of resistance developing in *P. infestans* populations was low for the CAAs used in the Czech Republic. Resistance to DMM also has not been found in *P. capsici* (Qi et al., 2012). In contrast, CAA-resistant isolates have been documented in *P. viticola* populations in some European countries (CAA FRAC working group, www.frac.info) and in *P. cubensis* populations in Israel (Lebeda, Cohen, 2011) and the Czech Republic (Pavelkova et al., 2014).

The results of the present study showed that six (*C. winterianus*, *L. cubeba*, *M. spicata*, *P. graveolens*, *S. aromaticum*, and *T. vulgaris*) out of twelve tested essential oils had complete inhibitory effects on the mycelial growth of *P. infestans* at a concentration of ≤ 1 µl ml$^{-1}$. Essential oils of *T. vulgaris* and *S. aromaticum* exhibited the highest inhibitory activity with the lowest values of MIC (0.25(0.2) –0.4 µl ml$^{-1}$). The high efficacy of thyme oil against *P. infestans* was also documented in previous studies (Quintanilla et al., 2002; Olanya, Larkin, 2006; Soylu et al., 2006). Several studies have linked the essential oil of *T. vulgaris* to antifungal activities against other *Phytophthora* spp., e.g., *P. capsici* (Mueller-Riebau et al., 1995) and *P. cactorum* (Kim et al., 2008). The essential oil of *S. aromaticum* has been reported as an active compound inhibiting other plant pathogenic fungi (e.g. Beg, Ahmad, 2002; Velluti et al., 2004). Our first screening of the antifungal activity of essential oils against *P. infestans* indicates that they could be useful in controlling late blight in potato and tomato crops. Although using essential oils may not be a fully adequate substitute for 'common' chemical control in agriculture, known for their bactericidal, virucidal, fungicidal, and insecticidal properties, essential oils are considered to be effective alternatives or complements to synthetic pesticides (Bakkali et al., 2008). There has been considerable interest shown by especially ecologically conscious growers to have natural substances available and of course, by consumers to know which compounds were used to control plant health. However, further research (*in vivo* tests, field trials, etc.) is needed to obtain information regarding the practical effectiveness, phytotoxicity, synergism, costs, and formulation of essential oils before they can be incorporated into an integrated pest management.

## CONCLUSION

The findings presented here show that there is a risk of resistance development in the Czech *P. infestans*

populations to PCH resulting from the selection of insensitive isolates. While MFX-resistant isolates of *P. infestans* were detected each year of the survey, all isolates were sensitive to DMM. Although the risk of resistance to DMM is low to moderate, *P. infestans* is a medium-risk pathogen, and the possibility of resistance developing cannot be ignored. It may be beneficial to develop natural products based on essential oils that are usable as alternatives to conventional fungicides in the future.

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