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Use of metalaxyl against some soil plant pathogens of the class Peronosporomycetes – A review and two case studies

Matěj Pánek[1], Asad Ali[2], Štěpán Helmer[1]*

[1]*Crop Research Institute, Prague, Czech Republic*
[2]*Czech University of Life Sciences Prague, Prague, Czech Republic*

**Corresponding author: panek@vurv.cz*

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Abstract: Upon its discovery and implementation in plant protection, metalaxyl became one of the most important fungicides against *Phytophthora infestans*, but its efficiency has also been proven against other soil pathogens of the class Peronosporomycetes. The most important genus – *Phytophthora* comprises more than 150 plant pathogens, which cause significant losses in crop production or damage to natural plant associations. Many species of related genera *Pythium*, *Phytopythium* and *Globisporangium* have a similar ability as the species of *Phytophthora*. Those pathogens are able to quickly spread in wet soils by actively movable zoospores or in the air by means of zoosporangia; they are able to persist in an environment for long periods once they are introduced into the locality, having durability from their resting structures (chlamydospores, hyphal swelling and oospores). Metalaxyl has proven to be very efficient against these pathogens. However, shortly after its release, the rapid development of resistance against this compound was recorded in many species of the class Peronosporomycetes. Such easily developed resistance is due to the monogenic nature of the resistance, which also determines any anti-resistant strategies. The solution of this issue rests in the cautious use of metalaxyl, with consideration given to these strategies, and should be based also on precise information about the environment and the present pathogenic agents.

Keywords: phenylamide fungicide; resistance; *Phytophthora*; strawberry

REVIEW: METALAXYL (MEFENOXAM), HISTORY OF USE, MODE OF ACTION, RESISTANCE IN PERONOSPOROMYCETES AND ANTI-RESISTANT STRATEGIES

History of the use of metalaxyl against pathogens of Peronosporomycetes

In 1845 the plant pathogen *Phytophthora infestans* de Bary, which originated in Mexico, was introduced by human activity to Europe (Bourke 1964) and subsequently spread worldwide (Fry et al. 1993). This pathogen caused several agricultural disasters such as the Great Famine in Ireland from 1845–1852 (Goodwin et al. 1994). Until the mid-1970s, biocidal sterilants like vapam or methylbromide were used for the control of Ooomycetes. Since the end of the 1970s, the inhibitory effect of some acylalanins against plant pathogens has been observed (Schwinn et al. 1977; Fuller & Gisi 1985), including metalaxyl against Perenosporales (Schwinn et al. 1977; Urech et al. 1977; Bruck et al. 1980; Malajczuk et al. 1983; Mackrill et al. 2020). However, resistance to phenylamides also came soon after their distribution (Morton & Urech 1988; Schwinn &

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Staub 1995; Gisi et al. 2000). The first case of resistance to metalaxyl was observed in Israel on *Pseudoperonospora cubensis* as early as 1979 (Reuveni et al. 1980). It was later found that samples of *Phytophthora infestans* collected in 1977 from the surroundings of North Berlin contained isolates resistant to phenylamids (Daggett 1993); soon, other species, such as *Plasmopara viticola*, *Peronospora tabacina*, *Bremia lactucae* and many others, were revealed to be resistant to this compound (Gisi 2007; Gisi & Sierotzki 2008). Such a rapid development of resistance suggests that the resistance capability was already present before the commercial use of metalaxyl (Gisi & Cohen 1996). The innate presence of resistance genes has been confirmed in the natural population of *P. infestans* in the Toluca Valley in Mexico (Grünwald et al. 2006). In 1982, the PA-FRAC Working Group (Phenylamide-Fungicide Resistance Action Committee), focused on the development of anti-resistant strategies, was established.

Mode of action of metalaxyl

Metalaxyl contains two enantiomers (R and S) which differ in their activity. The R-enantiomer, called mefenoxam, is used in agriculture. Mefenoxam is around 1 000 times more efficient *in vitro* and 3–10 times more efficient *in vivo* than S-metalaxyl (Zadra et al. 2002). This compound is also less biodegradable than S-metalaxyl and, therefore, works for a longer time (Wang et al. 2014). Both isomers of metalaxyl inhibit the synthesis of ribosomal RNA (Davidse 1995; Gisi & Sierotzki 2008) by disrupting the incorporation of uridine into the RNA chain (Davidse et al. 1988). Resistance to phenylamides is most probably based on a target site change; the gene for RNA-polymerase I has been proposed as a target (Wollgiehn et al. 1984).

Cross-resistance to the various phenylamides, such as benalaxyl or oxadixyl, is an important attribute of the resistance to metalaxyl (Joseph & Coffey 1984; Davidse et al. 1988; Davidse 1995). Metalaxyl is a highly effective inhibitor of mycelial growth (Farih et al. 1981) and sporangial formation, but a lower effectiveness in the inhibition of encysted zoospore germination has been documented (Matheron & Porchas 2000). This ascertainment correlates with the fact that metalaxyl does not inhibit the penetration inside a host plant, but does effectively inhibit the mycelial growth in a host plant (Cohen et al. 1979; Staub et al. 1980).

Genetic background of resistance to metalaxyl in some soil pathogens of the class Peronosporomycetes

The formation of resistance against fungicides in a pathogen population consists of three phases: emergence, evolutionary selection, and finally fixation in a population (Chen et al. 2018). In the first phase, resistant genes can be naturally present in some individuals in the population (Grünwald et al. 2006), and initially those genes are present at a very low frequency (Gisi et al. 2000); in some circumstances, mutations also lead to their development. Such development of resistance to metalaxyl was documented in laboratory conditions on *Phytophthora citricola*, whose zoospores were exposed to ultraviolet radiation or chemical mutagens while under parallel exposure to metalaxyl (Joseph & Coffey 1984). Alternatively, the resistance of a population can be established as a result of gene flow (Raymond et al. 1991; Garcia-Rubio et al. 2017; Chen et al. 2018). In cases where resistance to the fungicide is coincidentally associated with the higher fitness of the bearer of both those properties, the fungicide resistance may also occur in populations which were not treated by a particular compound (Gisi et al. 2000).

Once resistant genes are present, the continuous use of a particular fungicide poses permanent selection pressure favouring the resistant individuals, whose frequency in the population increases unless there is a break in the selection cycle (Cooke 1992). A directional selection toward metalaxyl-resistant individuals was experimentally discovered in natural populations of *Phytophthora infestans* exposed to metalaxyl in the Toluca Valley in Mexico. This selection was accompanied by a decrease in the genotypic diversity (Grünwald et al. 2006). In many members of the class Peronosporomycetes, the described mechanism of increasing the frequency of resistant individuals in a population is strengthened due to the predominantly clonal spreading. After the frequency of resistant individuals become dominant, the efficiency of the fungicide used is significantly reduced compared to its earlier use (Gisi et al. 2000).

The development and durability of resistance to fungicides in microorganisms depends on a few basic attributes of the particular microorganism's population. On the genetic level, it is determined by the number of gene loci involved in resistance against a particular fungicide, the extent of the al-

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lelic variation of the involved loci, the occurrence of additivity in the participating genes, the occurrence of epistasis (the occurrence of a mutation of the modifier genes), and the importance of a potential pleiotropic effect (the influence of a particular gene on seemingly unrelated phenotypic features) on the fitness of its bearer (Crute 1992). In contrast to quantitative (polygenic) resistance, qualitative (monogenic) resistance is controlled by a single gene of major effect (Georgopoulos 1985; Adaskaveg et al. 2017). Qualitative resistance is associated with a high risk of resistance development, since the single mutation confers full protection against the fungicide used (Vincelli 2014). Vincelli (2014) stated that if a pathogen has the genetic potential to develop resistance, then there is no way to prevent the development of the quantitative type of resistance; it is only possible to slow it down.

The genetic background of resistance to metalaxyl was historically studied predominantly on *Phytophthora infestans* due to its absolute incomparable importance for agriculture and food production security. The inheritability of resistance to metalaxyl of *P. infestans* was revealed as matching the Mendelian mode of heritability (Shattock 1988; Gisi et al. 2000); similar results were also achieved in *P. sojae* and *P. capsici* (Lucas et al. 1990; Bhat et al. 1993). Such a mode of heritability led to the conclusion that resistance to metalaxyl is single-gene based (Shattock 1988; Gisi et al. 2000). According to those findings, the resistance gene is incompletely dominant (Gisi & Cohen 1996; Gisi et al. 2000).

The probable molecular mechanism of resistance to metalaxyl in *P. infestans* was suggested only recently. The mutation in the *RPA190* gene contributes to a decrease in the sensitivity to this compound according to Randall et al. (2014). In contrast, Matson et al. (2015) ascertained that the presence of a mutation in this locus did not correlate with the sensitivity to metalaxyl. They concluded that genes other than *RPA190* are involved in the resistance, and suggested the *RPA135* gene as an addition. Using an analysis of up- and down-regulated genes, Wang et al. (2021) recently demonstrated that the *RPA190* gene is the key to resistance for both the *in vitro* and *in vivo* growth of *P. capsici* in the presence of metalaxyl. This gene, which encodes a large subunit of the RNA polymerase I, seems to be under positive selection. The RNA polymerase activity is significantly inhibited by metalaxyl in sensitive strains in comparison to those resistant to this compound, in which only slight inhibition was recorded (Wang et al. 2020; 2021). Similar conclusions were also reached by Chen et al. (2018). It was also considered that the analogical gene *RPA190-pc* was associated with the development of oospores and mycelial growth, and that mutations on this gene also have an impact on changes in the pathogenicity (Wang et al. 2021). Multiple point mutations were revealed in the locus of this gene; a total of 35 SNPs (single nucleotide polymorphisms) were found in the *RPA190* locus in *P. infestans*, whose total length is 5 433 bp (Chen et al. 2018). Although the revelation of this single gene conforms to the Mendelian mode of heritability, some other genes are also considered as possibly involved in the metalaxyl resistance (Montes et al. 2016; Chen et al. 2018). As follows, metalaxyl is a site-specific inhibitor having only a single target in the metabolism of the species of Peronosporomycetes, which is considered a cause of the quick formation of resistance in members of this group against this compound (Davidse et al. 1983; Gisi & Sierotzki 2008; Hu & Li 2014; Wang et al. 2021). However, except for the main target – RNA polymerase I, in the metabolic profiling of *P. infestans*, another 49 metabolites were differently expressed during cultivation of resistant isolates in the presence of metalaxyl in comparison to sensitive ones (Maridueña-Zavala et al. 2017).

Resistance to metalaxyl in some soil pathogens of the class Peronosporomycetes

Resistance to fungicides is characterised by the ability of an individual to grow, or to sporulate, in the presence of an effective concentration of a particular chemical compound more quickly in comparison to a sensitive individual (Porter et al. 2009).

Resistance to metalaxyl has been documented in many *Phytophthora* species. Considering the importance of *P. infestans*, resistance was first documented in this species (Davidse et al. 1981; Davidse et al. 1983; Rekanović et al. 2012); other examples of developed resistance from the most important species were documented in *P. cinnamomi* (Darvas & Becker 1984), *P. ramorum* (Wagner et al. 2007), *P. cactorum* (Utkhede & Smith 1993; Hill & Hausbeck 2008), *P. nicotianae* (Chabane et al. 1996; Timmer et al. 1998), *P. sojae* (Bhat et al. 1993) and *P. capsici* (Jackson et al. 2012). Examples of species of the related genera *Pythium* and *Phytopy-*

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thium with documented resistance to metalaxyl are abundant as well: resistant strains have been documented in *Pythium dissotocum* (Broders et al. 2007), *Py. irregulare* (Huzar-Novakowiski & Dorrance 2018), *Py. graminicola*, *Py. aphanidermatum*, *Py. sulcatum*, *Py. arrhenomanes*, *Py. vanterpolii* (Titone et al. 2009), *Phytopythium litorale*, *Phy. helicoides*, and *Phy. chamaehyphon* (Choudhary et al. 2016), but the full list is undoubtedly much longer. In many species, resistance was repeatedly documented and in different parts of the world as well.

The frequency of resistant individuals varies over time and in different populations, but usually does not decrease (Goodwin & McGrath 1995). The frequency of resistant isolates in some populations of *P. cactorum* reached a high value of 80% as early as eight years after the commercial release of metalaxyl in 1980 (Utkhede & Gupta 1988). Similarly, the number of resistant isolates of *P. erythroseptica* grew from 2.9% to 36.2% in a three-year period between 1997 and 2000; the frequency of resistant *Pythium ultimum* isolates also grew from 1% to 6.9% during a period of three years, but, before 1997, no resistant isolates of this species were recorded in study of Taylor et al. (2002). The rapid development of metalaxyl resistance in a three-year period was also documented in *Py. aphanidermatum* (Sanders 1984), in which 75% of the tested individuals were resistant to this compound.

Biologically meaningful levels of sensitivity to metalaxyl and other phenylamide fungicides vary on the intra- as well as inter-specific level; even isolates originating from one population can range widely (Brantner & Windels 1998; Mazzola et al. 2002; Taylor et al. 2002). Differences in the EC_{50} values (which represents the compound concentration inhibiting 50% growth of the concerned isolate) of samples of different *Pythium* species were between 0.01 and 5 µg/mL (White et al. 1988; Mazzola et al. 2002), and the EC_{50} values of *Phytophthora erythroseptica* were even between 0.05 µg/mL and 98.9 µg/mL (Taylor et al. 2002). In *P. infestans*, a correlation between the sensitivity to metalaxyl and membership in a particular clonal lineage was demonstrated (Goodwin et al. 1996). In *P. ramorum*, only European isolates were sensitive to this substance (Wagner et al. 2007).

In species of the genus *Pythium*, differences were revealed on the level of the morphological groups. Species with filamentous sporangia (*Py. graminicola*, *Py. dissotocum*, *Py. inflatum*, *Py. torulosum*), together with those with proliferating sporangia (morphological group 3 and group 6), were less sensitive to mefenoxam and other fungicides than species with globose sporangia (*Py. irregulare*, *Py. ultimum* var. *ultimum*, *Py. sylvaticum*, *Py. attrantheridium*, *Py. echinulatum*) (Kato et al. 1990; Broders et al. 2007). Although the diversity of the *Pythium* responses was remarkable, the separation of *Pythium* species from species of *Phytophthora* was obvious (Kato et al. 1990).

The association between the sensitivity to metalaxyl and the population structure of the pathogens remains unclear. Some works revealed an important association between the sensitivity of *Py. aphanidermatum* and the genetic background (Garzón et al. 2005); by contrast, another author did not find a similar structure in *Py. irregulare* (Huzar-Novakowiski & Dorrance 2018).

The use of metalaxyl poses the problem of affecting microbial communities, including non-target oomycetes. The results of Mazzola et al. (2002) show an increase in the resistance in such non-target organisms. In relation to use of this compound, the increasing resistance to metalaxyl of *Pythium* spp. was recorded in ten potato fields treated primarily against potato late blight caused by *Phytophthora infestans* (Porter et al. 2009); resistance against metalaxyl was revealed in *Pythium* species, both pathogenic and non-pathogenic for apple roots after treatment against *Phytophthora cactorum* (Mazzola et al. 2002). The use of fungicides, including metalaxyl, thus impacts the whole microbial community in the treated area. The systematic use of a fungicide against one particular *Phytophthora* species causes the development of resistant populations across the whole spectrum of relative and non-relative species, which may pose a problem when growing another crop. This problem is worsened by the fact that even isolates originating from the same locality, and representing different *Pythium* species, differ in their sensitivity to diverse fungicides (Broders et al. 2007).

Anti-resistant strategies

Since the nature of mefenoxam/metalaxyl is quantitative, the risk of its development is considered high (Adaskaveg et al. 2017). Strategies have been formulated to deal with the development of resistance in oomycetes, which these were summarised by the FRAC (Urech & Staub 1985). The strategies mentioned below should not be ap-

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plied schematically, but rather should be modified according to pathogen and host peculiarities as well as environmental conditions. Resistance management must be based on information about fungal population genetics and dynamics, and also on the molecular mechanisms of fungicide action (Crute 1992). Since the risk of the development of resistance against phenylamide fungicides is considered high in oomycetes, and this process could be rapid (Gisi & Sierotzki 2008), the maintenance of basic recommendations is necessary. The following basic recommendations comprise the main anti-resistant strategies.

The use of pre-packed mixtures of metalaxyl with other fungicides. For phenylamide fungicides, which are considered systemic, the use of pre-packed mixtures with non-systemic fungicides (such as dithiocarbamates like mancozeb), or the rotation of different mixes, is recommended (Adaskaveg et al. 2017). The total use of any single-site fungicide should ideally be limited to one or two treatments per growing season (Adaskaveg et al. 2017). This strategy also has the potential to deal with the development of cross-resistance. Broad spectrum control was observed by the application of this method. Experimental data revealed that mixtures clearly reduced the risk of disease, even in the presence of resistant strains (Staub & Sozzi 1984), and that the delay in the development of the resistance depends on the activity of the accompanying compound (Urech & Staub 1985). Van den Bosch et al. (2014) demonstrated that the mixture of an at-risk fungicide with an appropriate mixing partner, will significantly reduce the risk of the resistance development and, thus, enhance the effective life of the at-risk fungicide and maintain its effective control ability even at its simultaneous dose reduction. This effect was verified on sporangia of oxadixyl-resistant *Phytophthora infestans* isolates, whose numbers were decreasing after treatment with one-, two- and three-way mixtures of fungicides, respectively (Cohen & Samoucha 1990). On potatoes, these strategies for *P. infestans* are all based on using phenylamides only in combination with a low-risk partner, usually mancozeb at 3/4 of the full rate, and on restricting the number of applications (Russell 1995). The reason for using mixtures is to broaden the spectrum of the antifungal activity and provide insurance against pathogen resistance, i.e., in case one component of the mixture fails, the other component with a different mode of action can ensure the effective control (Van den Bosch et al. 2014). Two-way metalaxyl + mancozeb mixtures provide control of highly metalaxyl-resistant populations which is only equivalent to that achieved by mancozeb alone (Cooke 1992). Exploiting the synergistic interactions among fungicides with a multisite mode of action increases the overall efficiency of the fungicides, as well as their effective life, by slowing the rate of the resistance evolution in pathogen populations (Jansen et al. 2017).

Application intervals. The limited application of phenylamide fungicides (PAF) is recommended by FRAC. Each crop can receive two to four applications, with a maximum of two consecutive applications. The application intervals of PAFs should not exceed 14 days, and may be shorter if the disease incidence rate is high. If the application intervals are reduced, the total amount of PAFs used per season should not exceed that of the full rate, and the exposure time should also remain the same (Urech et al. 1977). Bergquist (1974) concluded that effective control of *Phytophthora* leaf blight, which is closely related to the development of resistance, is directly proportional to the frequency interval of the fungicide application. If the intervals between fungicide applications are increased, then the concentration of the fungicides available for the pathogens may decrease due to the environmental conditions like flushing by rain or denaturation by UV radiation. The repeated exposure of pathogens to this sub-lethal concentration, sometimes called training or forced selection, poses a practical risk in the resistance (Brent & Hollomon 2007). The timing of the fungicide application should also respect the host, pathogen and stage of the epidemics; treatment of large pathogen populations (for example, after an epidemic had already developed) increases the probability of the presence of resistant individuals (Adaskaveg et al. 2017). The use of multi-site fungicides is recommended for the beginning of the treatment programme in order to reduce the total pathogen population, and use of a single-site compound should be considered only after the population has been reduced. The use of a single-site fungicide is not recommended when the pathogen population is large, since the probability of the occurrence of resistant individuals is also high, and the use of a quantitative fungicide creates selection pressure, which favours resistant individuals and eliminates intra-specific competition (Adaskaveg et al. 2017).

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Maintain manufacturer's dose. The proper rate is crucial for the effective application of a fungicide; too low a dosage results in some pathogenic individuals surviving (Adaskaveg et al. 2017), while a higher dosage poses an increased environmental burden. Resistance may develop after multiple contacts between microbial individuals and a compound used in a sub-lethal dosage (Davis & Dennis 1981; Utkhede & Gupta 1988). High dosages of fungicide result in the suppression of the part of the pathogen population that is sensitive to the fungicide, which leads to the survival of resistant pathogens through reduced competition; a resistant population is then established by the resistant individuals capturing the entire niche (Hobbelen et al. 2014). This risk is high in the quantitative type of resistance, such as in the case of metalaxyl; however, it is argued that the use of low dosages also increases the number of sensitive survivors, which reduces the selection for resistant forms (Brent & Hollomon 2007).

Avoid the eradicant use. The use of metalaxyl/mefenoxam should be restricted to preventive treatments, because this compound is supplied exclusively in a mixture with multi-site fungicides. In case of use as an eradicant, the multi-site partner compound in the mixture is inefficient as an eradicant and, thus, the systemic fungicide acts alone; after several exposures, this leads to the development of resistance (Davidse 1985). As mentioned above, metalaxyl is not very effective at inhibiting zoospore cyst germination and the penetration of the pathogen into the host apoplast; on the other hand, mycelial growth in the plant is inhibited quite effectively (Cohen et al. 1979). These two factors account for the recommendation that this compound should be used only for preventive use.

Avoid the use of a mixture, designated against foliar pathogens, as a soil application. This recommendation is based on the fact that only a low dosage is available per soil volume after an improper soil application. As a consequence, non-controlled dosages of the acylalanine fungicide are present for an unspecified period during its degradation. Such a situation presents a high risk of resistance development, particularly for the quantitative type of resistance, because even only a very small probability of an emerging resistant individual is enough for the establishment of a resistant population; drench applications, thus, create long-lasting selection pressure (Urech & Staub 1985). The use of phenylamide fungicides as seed treatments is recommended only in mixtures designated for this type of use (Russell 1995). Drenching the soil with metalaxyl is effective against soil *Phytophthora* spp. (Ellis et al. 1982; Thomidis 2002), but a single mixture should be used. The use of metalaxyl, even in appropriate formulations, against soil pathogens should be considered thoroughly, because the entire microbial community is affected by this compound. The fungal community is negatively affected, while some actinomycetes and bacteria could be temporarily stimulated (Wang et al. 2019). Selection pressure favouring resistant individuals in all microbial groups is present.

Integrated disease management. To delay the development of resistance against metalaxyl and other fungicides, integrated management practices are recommended, including crop rotation (Rani & Sudini 2013), soil solarisation (Patel et al. 2014), removing diseased parts from crops, destroying plant debris, using biological control agents (alone or in a mixture with fungicides), using pathogen-resistant cultivars, and keeping cultivated plants in an environment optimal for their growth. Good implementation of these practices has the potential to decrease infectious pressure, regardless of the particular genotype of the pathogen.

TWO CASE STUDIES

Introduction

Strawberry plants (*Fragaria* × *annanasa*) in culture are hosts of an important and notorious pathogen of a rather wide range of host species – *Phytophthora cactorum* (Lebert & Cohn 1870; Erwin & Ribeiro 1996). This soil pathogen primarily attacks the roots and causes their necroses, as well as the necroses of crowns. The attacked plant wilts, its leaves turn yellow or brown, and the whole plant often gradually dies (Eikemo et al. 2000). This pathogen possesses a considerable capability to spread rapidly via zoospores (Delmas et al. 2014), thus it is able to attack a large number of plants in the field in a short time, especially during wet periods that favour its spread. Once introduced into a locality, this pathogen usually persists there, thanks to long-term survival structures such as oospores and chlamydospores (Jeffers & Aldwinckle 1987). Although *P. cactorum* is undoubtedly of great importance, there are other members of the genus *Phy-*

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tophthora and especially of its related genera *Pythium*, *Globisporangium* and *Phytopythium*, whose importance for strawberries is unclear. Since these pathogens often induce symptoms in strawberry plants which are similar to those caused by *P. cactorum* (Ishiguro et al. 2014), determination of the cause that relies solely on the symptoms may lead to their misidentification, and to the mismatched attribution of damage exclusively to *P. cactorum*.

Members of the mentioned *Pythium*, *Globisporangium* and *Phytopythium* genera are commonly widespread pathogens of agricultural crops, as well as of plants in natural stands. The genus *Phytopythium,* which currently includes 29 formally characterised species, was recently separated from the genus *Pythium* (Bala et al. 2010; Tao et al. 2011; Ishiguro et al. 2014; de Cock et al. 2015; Benfradj et al. 2017). The genus *Pythium* comprises more than 250 species, usually plant pathogens; some species originally included in this genus have recently been assigned to the genus *Globisporangium* (Uzuhashi et al. 2010). Species of those genera also participate in strawberry damage known as black root rot (Martin 1999; Marin et al. 2019), which is a complex disease caused by some *Pythium* spp. (*Py. irregulare*, *Py. ultimum* and many others) and *Phytopythium* members (*Phy. helicoides* and others), together with members of the genera *Rhizoctonia*, and *Fusarium* (Martin 1999; Fang et al. 2011), and the nematode species *Pratylenchus penetrans*. Many *Pythium* and *Phytopythium* species are known to be frequent pathogens of field crops and are characterised by low host specificity (Middleton 1943). Thus, in addition to strawberry plants, they are also able to attack other host species and maintain a high level of inoculum in the soil during crop rotations in the field.

The chemical protection of strawberry plants against members of the mentioned genera is focused mainly on *P. cactorum*, which constitutes a substantial and systematic problem for growers. Metalaxyl, dimethomorph, mancozeb and fosetyl-Al are among the most-used chemical substances worldwide. Since the presence of the *Pythium*, *Globisporangium* and *Phytopythium* species is quite often hidden or confused with *P. cactorum*, chemical treatments are less specific to them; this poses the risk of resistance development against the chemical compounds used.

The objective of our two studies was to evaluate the level of resistance to metalaxyl. To better represent the differences in the development of resistance between particular species, or genera, dimethomorph was used as a fungicide with a dissimilar mode of action. The variability of resistance was tested on the intra-specific level of *Phytophthora cactorum* (Case study 1) and on the inter-specific level of some species of *Phytophthora*, *Pythium*, *Phytopythium* and *Globisporangium* (Case study 2). All the isolates originated from strawberry plants in fields in the Czech Republic.

Material and methods

The used strains (Table 1) were isolated from strawberry plants during the years 2017–2019 in fields in the Czech Republic. Twenty-one isolates of *P. cactorum* were used in Case study 1 to infer the intra-specific variation of the metalaxyl resistance, while, in Case study 2, fourteen isolates of members of different related species were used to compare the inter-specific variation (Table 1). The isolation, from the strawberry plants, was performed using the leaf baiting method and subsequent cultivation on the semi-selective media PARPNH V8 (Tsao 1983); the cultures were then maintained on V8 plates. The species determination, after the preliminary morphological evaluation, was performed by DNA barcoding of the internal transcribed spacer (ITS) region of the ribosomal DNA gene using primers according to White et al. (1990). The amplification of the ITS region was performed in a polymerase chain reaction (PCR) reaction detailed in Pánek et al. (2016). The identity of each strain was checked by comparing the given sequence to the National Center for Biotechnology Information database using the BLAST algorithm.

Metalaxyl-M and dimethomorph were the tested substances (only in Case study 2). The test was performed on a V8 agar medium (200 mL V8 juice, 15 g of agar, 3 g of $CaCO_3$ in a total of 1 000 mL H_2O, autoclaved for 15 min at 121 °C) in Petri dishes, 9 cm in diameter. The cultivating medium was amended by the tested substances in the concentrations of 0.001, 0.01 (only for Case study 1), 0.1, 1.0 and 10 µg/mL (metalaxyl-M and dimethomorph). Metalaxyl dissolved in autoclaved water or dimethomorph dissolved in acetone were used to amend the cultivating medium. The tested substances were added to the V8 agar medium after the medium was cooled to 45 °C after autoclaving.

The growth inhibition was determined using the method of Parra & Ristaino (2001). A disk,

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Table 1. The list of isolates used in the case studies

Species ID	Isolate ID	CS	GenBank ID
Phytophthora cactorum	17_03_12	1	MW646877
Phytophthora cactorum	17_03_24	1	MW646878
Phytophthora cactorum	17_04_10	1	MW646879
Phytophthora cactorum	17_12_1b	1	MW646880
Phytophthora cactorum	17_12_5a	1	MW646881
Phytophthora cactorum	17_12_6a	1	MW646882
Phytophthora cactorum	17_07_25	1	MW646883
Phytophthora cactorum	17_07_27a	1	MW646884
Phytophthora cactorum	18_07_12a	1	MW646885
Phytophthora cactorum	18_10_17a	1	MW646886
Phytophthora cactorum	18_10_18c	1	MW646887
Phytophthora cactorum	18_10_11	1	MW646888
Phytophthora cactorum	17_37_13	1	MW193101
Phytophthora cactorum	18_07_2_S1	1	MW646889
Phytophthora cactorum	18_12_1b	1	MW646890
Phytophthora cactorum	17_11_19	1	MW646891
Phytophthora cactorum	18_37_7c	1	MW646892
Phytophthora cactorum	18_33_3	1	MW646893
Phytophthora cactorum	17_30_6	1	MW646894
Phytophthora cactorum	18_10_12	1	MW646895
Phytophthora cactorum	17_30_12b	1	MW646896
Phytophthora cactorum	17_12_20	2	MW193105
Phytophthora cactorum	17_15_10	2	MW193108
Phytophthora cactorum	17_60_26	2	MW193098
Phytophthora citrophthora	17_07_9	2	MW193110
Phytophthora citrophthora	17_57_1P	2	MW646897
Phytophthora lacustris	19_28_7	2	MW646898
Phytophthora plurivora	17_99_1	2	MW646899
Phytopythium litorale	17_26_8	2	MW193111
Phytopythium litorale	17_04_13	2	MW193112
Phytopythium mercuriale	17_07_22	2	MW646900
Phytopythium montanum	18_48_1	2	MW646901
Pythium dissotocum	18_10_7a	2	MW193116
Globisporangium heterothallicum	(19)18_62_5b	2	MW193094
Globisporangium heterothallicum	(19)18_43_1	2	MW646902

The use of isolates in Case study (CS) 1 or 2 is indicated by the number. All the tested isolates were isolated from symptomatic strawberry plants in the Czech Republic. The GenBank accession numbers for sequences of the ITS region of the DNA used in the species determination are listed

7 mm in diameter, cut from the margin of an actively growing colony developed on a V8 plate, was placed into the centre of a fresh plate amended by a chemical compound. Clear V8 agar plates were used as the control. Each combination of the isolate/concentration of the corresponding substance was repeated three times (Case study 1) or twelve times (Case study 2). After two days of cultivation in the dark at a temperature of 25 °C, the diameter of the developed colony was measured in two orthogonal directions and the average value was computed. The diameter of the inoculating disk was subtracted; the resulting value was divided by two days of cultivation and again by two to acquire the daily radial growth. The inhibition rate was calculated by comparing the mycelial growth at a particular concentration of the active substance to that on a non-amended V8 medium.

The determination of the EC_{50} value in Case study 1 was calculated by the Graph pad prism software (version 8.0.1). The metalaxyl concentrations were transformed into a logarithmic form and the responses were normalised into percentages followed by a non-linear regression analysis to calculate the EC_{50} value (probit analysis).

In Case study 2, the significance of differences in the inhibition rate values at a particular concentration of a given substance for each isolate was tested using the Kruskal-Wallis test. This analysis was performed by the software Statistica (version 13.3) (TIBCO Software Inc.). In case that significant differences were found between the growth at the different compound concentrations, a non-linear regression Probit analysis using a logarithmic transformation of the data was performed to determine the EC_{50} value.

Case study 1 – Results: Differences in the metalaxyl resistance in a population of *Phytophthora cactorum* on strawberry plants in the CR

Isolates of *Phytophthora cactorum* displayed variation in the inhibitory indices against metalaxyl. On the basis of the EC_{50} values, the resistance level was divided into three groups. Isolates having EC_{50} values in the range of 0.02 to 1.25 µg/mL are considered sensitive, while isolates which have EC_{50} values greater than 100 µg/mL are considered resistant (Parra & Ristaino 2001). EC_{50} values between 5 and 70 µg/mL are somehow at a high risk of resistance. In this study, 66.6% of the *Phytophthora* population was sensitive to metalaxyl, 14% of the population showed a high level of resistance, while 19% of the population was on the edge of building up resistance (Figures 1 and 2). Three *P. cac-*

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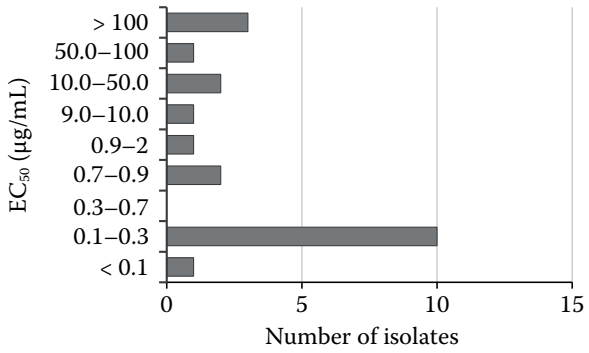

Figure 1. Distribution of the metalaxyl EC_{50} values of the *Phytophthora cactorum* isolates that originated from symptomatic strawberry plants in the Czech Republic

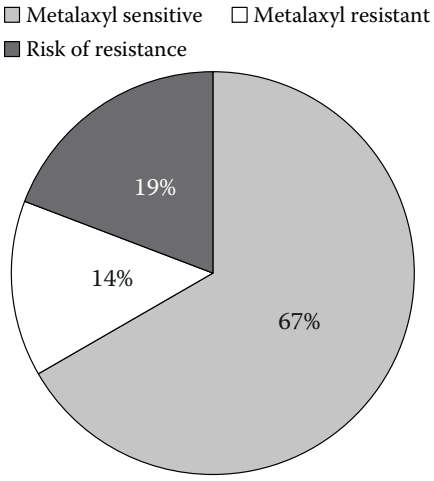

Figure 2. The percentage distribution of the resistant and sensitive isolates of *Phytophthora cactorum* to metalaxyl All 21 tested isolates originated from strawberry plants in the fields of the Czech Republic

torum isolates were evaluated as highly resistant, with the calculated EC_{50} values as high as hundreds of thousands of µg/mL. On the other hand, the EC_{50} values of some sensitive isolates were calculated as low as 0.13 and 1.25, respectively (Table 2, Figure 3). The growth inhibition percentage was also calculated to analyse the degree of efficiency of the metalaxyl at each concentration. For the resistant isolates, the growth inhibition at the highest metalaxyl concentration, i.e., 10 µg/mL, was between 6% and 8%, while in the case of the sensitive isolates at the highest concentration, metalaxyl inhibited 75–100% of the growth of *Phytophthora cactorum.* Such results present the risky situation of the resistance to metalaxyl spreading, since about one-third of all the isolates evince some measure of resistance, and even some sensitive isolates are not completely inhibited by this compound in all the tested concentrations. Resistance is present in the population, although this compound is not currently registered for use in strawberry protection against *P. cactorum* in the Czech Republic.

Case study 2 – Results: Differences in metalaxyl resistance of some *Globisporangium*, *Pythium*, *Phytopythium* and *Phytophthora* species isolated from strawberry plants in the CR

Metalaxyl was evaluated as being efficient in at least some measure against the isolates of all the tested species, although obvious differences were recorded between the *Phytophthora*, *Phytopythium*, *Globisporangium* and *Pythium* samples (Table 3, Figure 4). Differences were obvious in both the growth inhibition rate and the EC_{50} values. Differences were also obvious between the isolates of one species, although the numbers of tested isolates of each species were not high enough for more general conclusions. In all the tested *Phytophthora* isolates, the concentration of 0.1 µg/mL already inhibited the growth significantly in comparison to the control; the inhibition rate was between 30% and 72%. A similar effect was obvious for the two tested isolates of *Globisporangium heterothallicum*, but not in the representatives of the genera *Pythium* and *Phytopythium*, where the inhibition rate was between 7% and 13% at this concentration. The EC_{50} values of the two isolates of *Phytoypthium litorale* and the two isolates of *Phytopythium mercuriale* were three orders of magnitude higher than in the other isolates. In these four isolates, the inhibition rate was between 39% and 47%, even at the highest tested concentration of this compound. Only two of the tested isolates were inhibited at a rate higher than 95%, at least at the concentration of 10 µg/mL – isolates of *Phytophthora citrophthora* and of *Globisporangium heterothallicum* (Figure 4). These results show the effectiveness of this compound against the *Phytophthora* species, although there are some differences at the inter- and intra-specific levels.

The efficiency of dimethomorph was rather different for the *Phytophthora* isolates, on one hand, and the *Globisporangium*, *Pythium* and *Phytopythium* isolates, on the other. For six out of seven *Phytophthora* isolates, an inhibition rate higher than 90% was already reached at the concentration 1 µg/mL. On the contrary, and as the only exception, one *P. cactorum* isolate was inhibited only by 50%, even

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Table 2. Comparison of the resistance of the *Phytophthora cactorum* isolates against metalaxyl

Isolates ID	Metalaxyl concentration (µg/mL)						EC_{50}
	0.000	0.001	0.010	0.100	1.000	10.000	
17_3_12	0.00	1.35	2.67	5.33	6.67	8.00	7 887 596.00
17_3_24	0.00	1.75	2.52	4.50	5.96	7.28	24 643 476.00
17_4_10	0.00	1.00	4.91	11.59	51.33	75.57	1.25
17_12_1b	0.00	2.66	4.56	9.36	27.46	41.32	21.90
17_12_5a	0.00	1.64	2.39	3.71	4.89	6.34	90 648 578.00
17_12_6a	0.00	1.10	7.15	12.82	29.80	33.54	67.20
17_7_25	0.00	0.51	6.41	37.35	100.00	100.00	0.13
17_7_27a	0.00	0.25	6.70	7.06	57.40	88.86	0.81
18_07_12a	0.00	0.31	6.77	11.54	23.04	37.64	41.57
18_10_17a	0.00	0.41	10.85	35.44	100.00	100.00	0.14
18_10_18c	0.00	2.33	3.01	13.60	100.00	100.00	0.15
18_10_11	0.00	1.77	7.02	10.09	19.61	52.12	9.57
17_37_13	0.00	0.37	6.78	60.76	100.00	100.00	0.07
18_07_2_s1	0.00	0.07	9.56	34.13	67.43	100.00	0.28
18_12_1b	0.00	3.19	9.15	35.71	100.00	100.00	0.14
17_11_19	0.00	1.17	11.22	36.19	100.00	100.00	0.14
18_37_7c	0.00	1.83	3.74	29.36	91.78	100.00	0.19
18_33_3	0.00	1.84	6.48	34.25	95.76	100.00	0.15
17_30_6	0.00	0.78	4.37	39.26	95.94	100.00	0.13
18_10_12	0.00	1.64	7.27	32.49	95.57	97.48	0.15
17_30_12b	0.00	0.30	4.62	12.34	56.64	100.00	0.73

For each isolate, the values of the mycelial growth inhibition rate (%) at the particular metalaxyl concentrations in relation to the control variant are given; their EC_{50} values are indicated

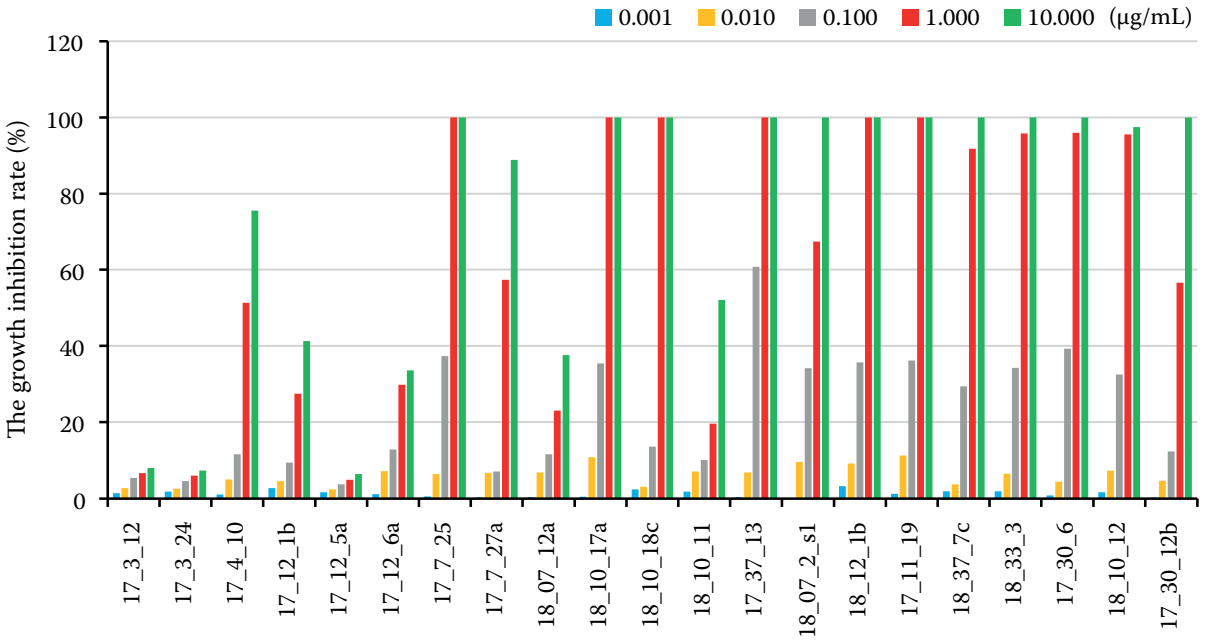

Figure 3. The mycelial growth inhibition rates (%) of 21 isolates of *Phytophthora cactorum* at the particular metalaxyl concentrations (µg/mL)

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Table 3. Comparison of the resistance to metalaxyl and dimethomorph of the *Phytophthora*, *Phytopythium*, *Pythium* and *Globisporangium* isolates

Species ID	Isolate ID	Dimethomorph					Metalaxyl				
		0.000	0.100	1.000	10.000	EC_{50}	0.000	0.100	1.000	10.000	EC_{50}
Pca	17_12_20	$0^{\backslash}$	$14^{\backslash}$	99^{a}	100^{a}	0.160	0^{a}	$55^{a,b}$	$69^{b,c}$	74^{c}	0.527
Pca	17_15_10	0^{a}	12^{a}	100^{b}	100^{b}	0.132	0^{a}	60^{a}	90^{b}	87^{b}	0.159
Pca	17_60_26	0^{a}	-10^{a}	-6^{b}	50^{b}	97.685	0^{a}	48^{b}	$67^{b,c}$	74^{c}	0.430
Pci	17_07_9	$0^{\backslash}$	$43^{\backslash}$	100^{a}	100^{a}	0.118	0^{a}	$75^{\backslash}$	100^{b}	97^{b}	0.068
Pci	17_57_9	$0^{\backslash}$	$45^{\backslash}$	100^{a}	100^{a}	0.151	0^{a}	52^{b}	$82^{b,c}$	88^{c}	0.232
Pl	19_28_7	0^{a}	6^{a}	100^{b}	100^{b}	0.344	0^{a}	$56^{\backslash}$	91^{b}	93^{b}	0.094
Pp	17_99_1	$0^{a,b,d}$	3^{a-d}	91^{b}	100^{b}	0.413	0^{a}	$30^{a,b}$	$66^{b,c}$	82^{c}	0.485
Phl	17_26_8	0^{ns}	1^{ns}	-2^{ns}	1^{ns}	\	0^{a}	$10^{a,b}$	37^{b}	$47^{\backslash}$	11.373
Phl	17_04_1	0^{ns}	-2^{ns}	0^{ns}	-1^{ns}	\	$0^{\backslash}$	8^{a}	30^{b}	43^{b}	27.289
Phm	17_07_22	$0^{a,b}$	$2^{a,b}$	$1^{a,b}$	4^{a}	\	0^{a}	7^{a}	31^{b}	46^{b}	19.829
Phm	18_48_1	$0^{a,b}$	0^{a-c}	-3^{a-c}	$1^{b,c}$	\	0^{a}	10^{a}	27^{b}	39^{b}	56.699
Pyd	18_10_7a	$0^{\backslash}$	-6^{a}	-6^{a}	-6^{a}	\	0^{a}	13^{a}	55^{b}	74^{b}	1.233
Gh	(19)18_62_5b	0^{ns}	0^{ns}	1^{ns}	2^{ns}	\	0^{a}	55^{b}	$97^{b,c}$	100^{c}	0.099
Gh	(19)18_43_1	0^{ns}	3^{ns}	2^{ns}	2^{ns}	\	0^{a}	71^{b}	47^{b}	56^{b}	0.830

Gh – Globisporangium heterothallicum; *Pca – Phytophthra cactorum*; *Pci – P. citrophthora*; *Phl – Phytopythium litorale*; *Phm – Ph. mercuraile*; *Pl – P. lacustris*; *Pp – P. plurivora*; *Pyd – Pythium dissotocum*

$^{a-d}$Values significantly indistinguishable according to the Kruskal-Wallis test; ^{ns}cases of very low inhibition at all concentrations, where no significant differences were found; $^{\backslash}$for nonsignificant difference, the EC_{50} value was not calculated
The median values of the mycelial growth inhibition (%) and EC_{50} values are given

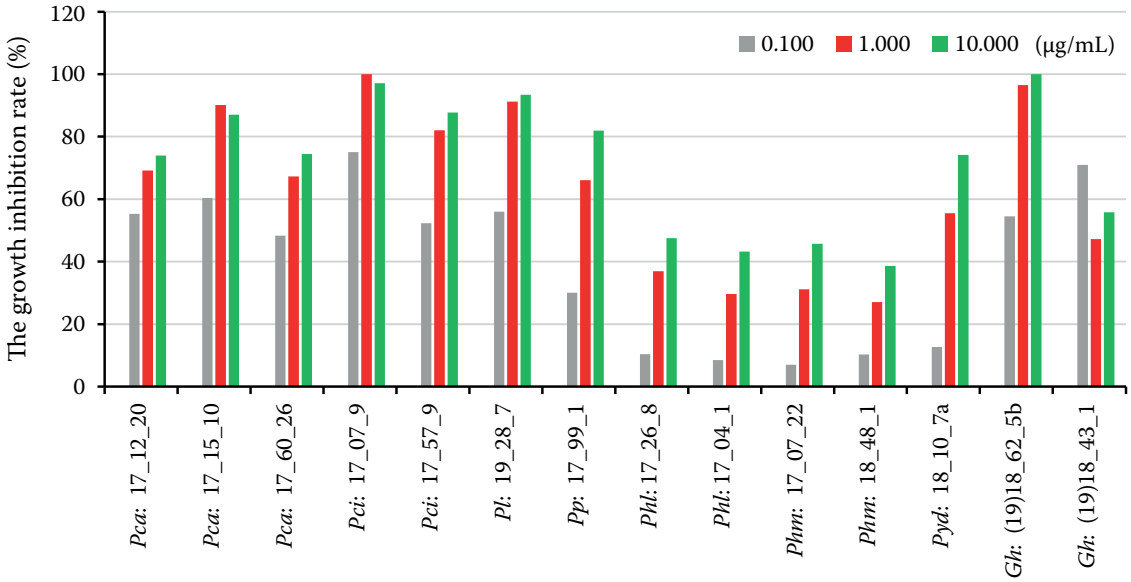

Figure 4. The mycelial growth inhibition rates (%) of the isolates of the Peronosporomycetes class at the particular metalaxyl concentrations (µg/mL)

Gh – Globisporangium heterothallicum; *Pca – Phytophthora cactorum*; *Pci – P. citrophthora*; *Phl – Phytopythium litorale*; *Phm – Ph. mercuraile*; *Pl – P. lacustris*; *Pp – P. plurivora*; *Pyd – Pythium dissotocum*

at a concentration of 10 µg/mL (Table 3), which means that this isolate has a significantly increased resistance to dimethomorph in comparison to the other *Phytophthora* isolates. The EC_{50} value of this isolate was 97.685 µg/mL, while the values of the other tested *Phytophthora* isolates were between

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0.118 and 0.413 µg/mL, regardless of their exact species classification. For members of *Globisporangium*, *Pythium* and *Phytopythium*, the differences between the inhibition rate at all the tested dimethomorph concentrations and the unenriched control were evaluated as non-significant, thus none of the isolates of those two genera can be considered sensitive to this compound.

A comparison of these two compounds provides an example of the dissimilar level of resistance to the chemical compounds developed in each particular isolate. The difference is visible, e.g., in a comparison of two isolates of *P. cactorum* 17_12_20 and 17_60_26. The first mentioned isolate was 100% inhibited by dimethomorph at a concentration of 10.0 µg/ml, while only it was 74% inhibited by metalaxyl at the same concentration. The second isolate was inhibited by metalaxyl at a similar level, but the inhibition caused by dimethomorph was only 50% (Table 3).

DISCUSSION AND FUTURE PROSPECTS

As pathogens of an extremely wide host spectrum, many soil species of the class Peronosporomycetes present a substantial problem for crop production, as well as for many natural formations (van der Plaats-Niterink 1981; Erwin & Ribeiro 1996). In agriculture, the solution to this problem relies on the use of chemical compounds, including metalaxyl; the production of some crops is currently hardly imaginable without the use of this compound, although the development of resistance makes this use complicated. The results of our two studies on isolates from strawberry fields reveal a new insight taking in consideration that, in practice, more than one pathogen species is often present in the field. Our example of pathogens of the class Peronosporomycetes on strawberries demonstrates the wide spectrum of more or less related members of this class participating in the damage to the plants (Watanabe 1977; Watanabe et al. 1977; Suzui et al. 1980; Eden & Hill 1998; Irzykowska et al. 2005; Abad et al. 2008; Rytkönen et al. 2012; Ishiguro et al. 2014; Rahman et al. 2014; de Cock et al. 2015; Mouden et al. 2016; Barboza et al. 2017; Toljamo et al. 2017; Shennan et al. 2018), which evince high differences in the sensitivity to one chemical compound. Many of these pathogens could be present together in the same field at the same time. The different sensitivity of members of various species of Peronosporomycetes to metalaxyl, similarly as the intraspecific variability in the *P. cactorum* species itself, can lead to the inefficiency in the use of this compound in strawberry protection, and to the increase in the resistance in part of the microbial population. The improper identification of the cause of the damage to the plants, relying only on the symptoms on the plants, can also lead to the choice of incorrect protection methods, having a similar effect, and thus underestimate the risk of resistance development. Since, in strawberry plants, the symptoms caused by different members of the genera *Phytophthora*, *Pythium*, *Phytopythium* and *Globisporangium* are easily mistakable, this hazard is not negligible. The variability in the resistance at the intra- and inter-specific levels, thus, contributes to the general development of resistance in both target and non-target organisms (Utkhede & Gupta 1988; Mazzola et al. 2002; Jeffers et al. 2004; Reeleder et al. 2007; Hill & Hausbeck 2008).

The results show that *P. cactorum* itself has high resistance variability, despite the fact that our case studies only comprise isolates of the genetic lineage "S", one of a total of five lineages discovered only recently (Pánek et al. 2021a); its total level of resistance variability can thus be assumed to be even higher. This finding together with previous findings looks important, since they make the choice of the proper protection method for strawberries even more complicated. A comparison with the dimethomorph results, whose risk of resistance formation was considered low (Bagirova et al. 2001), reveals that the resistance to this compound is also present in the *P. cactorum* population, although we revealed it to only be in a low percentage. On the contrary, all the members of the other tested genera – *Pythium*, *Phytopythium* and *Globisporangium* – were resistant to dimethomorph. Thus, pathogens resistant to some of the most important compounds used in plant protection are probably present together in one locality. Generally, the isolates of the whole *Phytophthora* spp. and of their relative genera show different levels of resistance against various compounds, thus the resistance to many compounds is theoretically present in some members of the Peronosporomycetes community, or on a wider scale in the local microbial community. This example poses the essential problem for the use of chemical compounds in plant protection. Since some degree of resistance

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to particular compounds is very probably present in some members of these populations, the continual use of each of these compounds presents the potential risk of extending the resistance regardless of the original frequency of the resistant genotypes. Moreover, the successful suppression of some metalaxyl-sensitive pathogen species could enable the rise of metalaxyl-resistant species, originally competitively restricted by this metalaxyl-sensitive one. Some threat also lies in the possibility of gene flow via horizontal gene transfer between different microbial species, which has already been observed in some members of the Peronosporomycetes class (Richards & Talbot 2007; Richards et al. 2011), but the most important threat is the hybridisation in this family (Olson & Stenlid 2002; Ioos et al. 2006; Bertier et al. 2013; Nagel et al. 2013; Burgess 2015; Van Poucke et al. 2021). This phenomenon presents a threat via changes in the host spectrum or virulence (Brasier et al. 1999) as a consequence of the gene flow between different species, however, in the context of practical plant protection, the threat of transferring resistance genes to chemical compounds is of similar importance.

As can be seen in our example of some Peronosporomycete pathogens on strawberry plants, beside the use of anti-resistant practices already commonly accepted, the judicious use of fungicides seems to be necessary, which should be based on the precise identification of the causes of the plant damage. Such precise identification of a particular species or pathotype is gradually becoming possible, even for practical use in the field, based on markers developed using methods of molecular genetics and through close cooperation between growers and research institutions.

The issue of pathogen resistance against fungicides could be solved by their gradual replacement by other protection methods. Except for the obligatory use of basic anti-resistant strategies, some alternatives to fungicides are based on the utilisation of biological research results detailing the knowledge of relationships and linkages in the microbial community. The use of a wide spectrum of biological control agents is under examination, although the current results are variable (Pánek et al. 2021b). Another direction is based on the targeted influencing of signalisation between soil microorganisms by their secondary metabolites, pheromones, autoinducers, or aggregating molecules (Kong et al. 2010; Rieger et al. 2010; Mir et al. 2015; Villa et al. 2016; Barriuso et al. 2018; Jeong et al. 2020). As these substances are not lethal to pathogens, they did not impose selective pressure on the pathogens for the onset of resistance (Molina et al. 2003; Villa et al. 2017).

As for the example of the strawberry, soilless cultivation has become common (Takeda 1999), which enables one to avoid any contact of the plants roots with the soil pathogens in the field. Although the use of such cultivating strategies is not possible for many other host species of pathogens of Peronosporomycetes, the total need of fungicides can be decreased in this way and, thus, the method could be considered as an anti-resistant strategy. Similar strategies decreasing the use of fungicide treatments in plant protection could potentially extend the time of resistance development to compounds and to decrease their total consumption.

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