Sensitivity to fungicides in the isolates of *Phytophthora infestans* (Mont.) de Bary in the Czech Republic from 2003 to 2008

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Abstract

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In the growing seasons from 2003 to 2008, 547 isolates of *Phytophthora infestans* from five regions in the Czech Republic were collected and examined for their sensitivity to the active ingredients (metalaxyl, dimethomorph and propamocarb-HCl) of frequently used fungicides. The response of the isolates to each of these substances was examined using the *in vitro* amended-agar method; in 352 of these isolates, the sensitivity to metalaxyl was also assessed by the floating leaf-disc assay. The majority of the isolates were sensitive (89.8%) to metalaxyl. Resistant isolates were found only in two of the sample years (2003 and 2008); they represented 58% of the samples in 2003 and only 29% in 2008. Four isolates from 2004 were found to be intermediate for their level of resistance. All the isolates that were tested were sensitive to dimethomorph and propamocarb-HCl; these particular substances completely suppressed mycelial growth at 1 µg a.i. per ml.

Keywords: late blight; fungicide resistance; metalaxyl; dimethomorph; propamocarb-hydrochloride

Phytophthora infestans (Mont.) de Bary (1876), an oomycete pathogen that causes late blight in potatoes (*Solanum tuberosum* L.) and tomatoes (*Solanum lycopersicum* L.), belongs to one of the most important and best-known classes of plant pathogens – the *Oomycetes* class. Under favourable moisture and temperature conditions, the pathogen can spread very rapidly from the primary focus to other plants, causing massive attacks on potato and tomato foliage as well as on potato tubers and on tomato fruits.

In potato, the steps that are required for the prevention of the late blight infection include minimising the primary sources of the pathogen, using cultural methods, selecting resistant cultivars and fungicide applications. Specific criteria regarding the use of chemical substances under integrated disease management programmes (e.g. minimum effective doses, forecasting models and anti-resistance strategies) are required to avoid the potential risk to health, environment and the risk of the development of pathogen resistance to the applied fungicides, especially to systemic compounds (Brent & Hollomon 2007).

Resistance to metalaxyl in *P. infestans* populations is well known and well documented in many

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countries (Gisi & Cohen 1996). Metalaxyl belongs to phenylamides, a group of systemic fungicides with preventive and curative activity that is limited to *Oomycetes*. All of the phenylamide fungicides inhibit RNA polymerisation within the synthesis of ribosomal RNA, resulting in the inhibition of mycelial growth, haustorium formation and sporulation. Phenylamide resistance originates from a very small proportion of resistant strains in the pathogen populations that are present before exposure to the fungicide treatment. Selection pressures result in an increased frequency of resistant strains (Gisi 2002). The occurrence of resistant isolates of *P. infestans* was first observed in potato crops grown in Ireland and in the Netherlands where metalaxyl was used as a single ingredient product that was applied curatively and during high disease pressure (Davidse *et al*. 1981; Dowley & O'Sullivan 1981). Reports of resistant isolates appeared together with discovery of the A2 mating type of *P. infestans* in Europe (Hohl & Iselin 1984). Until the early 1980s, a single clonal lineage of A1 mating type, known as the old population and designated US-1, was spread worldwide (Goodwin *et al*. 1994). Since then increases in the frequency of resistant isolates and the occurrence of the A2 mating type of *P. infestans* have been reported in many countries. Consequently, development of resistance to phenylamides may have been associated with the distribution of A2 mating type. However, no correlation existed between the resistance level and the proportion of mating types (Gisi & Cohen 1996) and the A2 mating type has not posed a greater threat than that of the A1 mating type. The resistance to metalaxyl may be connected with changes in *P. infestans* populations relating to the occurrence of new genotypes composed of the A1 and A2 mating types (Cooke *et al*. 2009) which were introduced from Mexico, the probable centre of the coevolution of the *P. infestans*–*Solanum* spp. pathosystem (Goodwin *et al*. 1992). The replacement of previous genotypes by new, more aggressive populations and the possibility of sexual reproduction in *P. infestans* have increased the genetic variability of *P. infestans*. As a consequence, it allowed the new populations to overcome the current efficiency of commonly used fungicides. Furthermore, Daggett *et al.* (1993) reported that the first metalaxyl resistant isolates were observed among German *P. infestans* isolates that were collected in 1977, prior to the use of phenylamides in Germany. It is also hypothesised that the resistance of *P. infestans* to metalaxyl in the United States and Canada originated from the migration of resistant isolates, while resistance to metalaxyl in Europe may have developed from the selection of resistant mutants (Goodwin *et al*. 1996).

Changes in *P. infestans* populations have necessitated a different approach to applying phenylamide fungicides; a modification of the previous chemical control practices, and the release of other fungicides (e.g. dimethomorph and propamocarb-hydrochloride) with novel modes of action required for the effective control of late blight (Gisi 2002). Dimethomorph, a cinnamic acid derivative, has protective, curative and antisporulation activity toward the genus *Phytophthora* and the family *Peronosporaceae* (Albert *et al.* 1988; Cohen *et al*. 1995). In spite of attempts at generating dimethomorph resistance in *P. infestans* using various mutagenic agents (Bagirova *et al*. 2001; Stein & Kirk 2004), such resistant mutants had lower fitness, higher lethality and morphological anomalies. Naturally dimethomorph-resistant isolates have not been detected in field populations yet (Zhu *et al*. 2004; Elansky *et al*. 2007).

To determine the presence and/or degree of naturally occurring resistance to fungicides, we assessed the sensitivity of Czech *P. infestans* isolates to metalaxyl, dimethomorph and propamocarb-HCl. This report summarises the results obtained in the growing seasons between 2003 and 2008.

MATERIALS AND METHODS

Source of isolates. Samples of potato leaves, stems and tubers as well as of tomato leaves, stems and fruits that were naturally infected by *P. infestans* were collected in five regions in the Czech Republic during 2003–2008. The collection of samples from the potato crops was particularly focused on the main potato-growing areas and involved growers' fields, research stations, as well as variety testing institutes. In the first year of the survey, sampling was done randomly from individual plants showing the symptoms of late blight. During 2004–2008, the samples were systematically taken from infected plants of the potato varieties available. The tomato samples were taken from individual plants that originated from private gardens; this was also the case of some of the potato samples. Samples with freshly sporulating blighted lesions were transported in

plastic bags to the laboratory and then either immediately used for the pathogen isolation or stored in a refrigerator for subsequent usage. Segments of potato and tomato leaves with the abaxial side up and the stems, slices of potato tubers and tomato fruits were placed in a humid chamber (a Petri dish with a moistened filter paper or an inverted Petri dish with 1.5% water agar) to support the abundant formation of the pathogen sporangiophores. Following the isolation, the sporangiophores were transferred into a drop of sterile distilled water put onto a surface-sterilised potato (potato cv. Ditta) tuber piece (5 × 5 × 3 mm). The tuber piece was transferred into a 6-cm Petri plate with rye A or B medium. The isolates were incubated at 15–18°C in darkness, which allowed the pathogen to grow, then were transferred every 4–6 weeks and were maintained on the rye A medium until further isolate characterisation. Since 2005, a quicker and easier approach of the isolation was carried out. This approach involved trapping the sporangia on a small piece of agar with an inoculating needle under a stereomicroscope and replacing into a Petri dish with rye A medium. Then the pure cultures of *P. infestans* were maintained by transferring small mycelial plugs to a number of Petri dishes.

In vitro assay of responses to metalaxyl, dimethomorph and propamocarb-HCl. The metalaxyl sensitivity of *P. infestans* isolates was determined using *in vitro* testing on amended agar plates. The metalaxyl active substance was dissolved in dimethylsulphoxide (DMSO) and added into the rye A agar at concentrations of 1 µg, 10 µg, and 100 µg metalaxyl per ml of agar. The control plate contained 1 ml DMSO per l of agar. There were three replicate plates for each concentration and for each of the tested isolates. A 9-mm mycelial plug was cut using a cork borer from the margin of the colonies of *P. infestans* isolates that were actively growing for two weeks and was transferred in the middle of 9-cm Petri dishes containing amended agar. After incubation for approximately 10 days at 15–18°C in darkness, the colony diameters in the dishes were measured at two perpendicular directions on all plates when the mycelial growth of the control reached the edge of the plate or was 30 mm in diameter at least. The sensitive, intermediate and resistant isolates were defined as exhibiting < 10%, 10–60% and > 60% growth, respectively, on 100 µg metalaxyl per ml amended agar, relative to that on the control plate (Shattock 1988). The reaction of the *P. infestans* isolates to dimethomorph and propamocarb-HCl was evaluated using the amended-agar test, as mentioned above, but with 1 µg and 10 µg per ml of agar.

In vivo assay of the response to metalaxyl. The response to metalaxyl of *P. infestans* isolates from 2003–2006 was determined by means of the floating leaf-disc assay (Sozzi & Staub 1987). Leaf discs (15 mm diameter) were cut using a cork borer from fully expanded leaflets of the potato cv. Ditta, which was grown in the greenhouse. Six leaf discs were floated with the abaxial surface pointing up in 6-cm Petri dishes. Each dish contained 10 ml of one of the three metalaxyl concentrations (1, 10, 100 µg/ml) or distilled water as the control. The assay was performed in three replications. Since 2005, the test was carried out in tissue culture test plates with 24 wells (Orange scientific), each containing 1 ml of one of the different concentrations of metalaxyl or distilled water. Six leaf discs in four replications were used for each concentration. The sporangia were suspended into distilled water, the concentration of which was adjusted to a 2.5-5 × 10^4 per ml. The inoculum was incubated for 2 h at 4°C to induce the zoospore release. Twenty microlitre drops of suspension containing 500 to 1000 sporangia and zoospores were placed in the centre of each disc. The plates were incubated in a thermostat at 15–18°C under light period with 16-h illumination and 8-h darkness. After incubation for 7 days, the leaf discs were evaluated using a stereomicroscope to estimate the area with *P. infestans* sporulation. The isolates were considered to be resistant if they sporulated on the leaf discs at 100 µg/ml metalaxyl. Those sporulating only at metalaxyl concentrations of 1 or 10 µg/ml were considered to be intermediate, and those sporulating only in water were considered to be sensitive (Hermansen *et al*. 2000).

RESULTS

Collection and isolation of *P. infestans*

Over the six survey years, 1304 samples of the blighted plant tissue were collected from potato and tomato crops. A total of 547 isolates were maintained in pure cultures, with 31, 64, 177, 80, 76, and 119 isolates taken in each year, chronologically from 2003 to 2008. The majority of the isolates were from potato leaves; six of them were from stems and one was from a tuber. Four of the isolates originated

Table 1. Origin and characterization of *Phytophthora infestans* isolates evaluated for fungicide resistance

Year/locality	Number of tested isolates – Response to								
	metalaxyl			dimethomorph			propamocarb–HCl		
	S	I	R	S	I	R	S	I	R
2003 (*n* = 31 isolates)									
Lysá nad Labem	9	–	–	9	–	–	9	–	–
Olešná	–	–	18	18	–	–	18	–	–
Želiv	1	–	–	1	–	–	1	–	–
Okřesaneč	2	–	–	2	–	–	2	–	–
unknown[tu]	1	–	–	1	–	–	1	–	–
2004 (*n* = 64 isolates)									
Vitice	11	–	–	11	–	–	11	–	–
Vepřová	8	1	–	9	–	–	9	–	–
Sojovice	9	1	–	10	–	–	10	–	–
Valečov	4	2	–	6	–	–	6	–	–
Blatnice pod Sv. Antonínkem	1	–	–	1	–	–	1	–	–
Pacov	3	–	–	3	–	–	3	–	–
Prague-Suchdol	5	–	–	5	–	–	5	–	–
Sojovice[g]	6	–	–	6	–	–	6	–	–
Unětice[g]	3	–	–	3	–	–	3	–	–
České Budějovice[g]	6	–	–	6	–	–	6	–	–
Holubov[g]	3	–	–	3	–	–	3	–	–
Český Krumlov[g,t]	1	–	–	1	–	–	1	–	–
2005 (*n* = 177 isolates)									
Semice	13	–	–	13	–	–	13	–	–
Kolinec-Vlčkovice	10	–	–	10	–	–	10	–	–
Keřkov	4	–	–	4	–	–	4	–	–
Žabčice	21	–	–	21	–	–	21	–	–
Lípa	66	–	–	66	–	–	66	–	–
Valečov	26	–	–	26	–	–	26	–	–
Horažďovice	27	–	–	27	–	–	27	–	–
Holubov[g]	2	–	–	2	–	–	2	–	–
Prague Suchdol[t]	3	–	–	3	–	–	3	–	–
Blatnice pod Sv. Antonínkem	5	–	–	5	–	–	5	–	–
2006 (*n* = 80 isolates)									
Horažďovice	46	–	–	46	–	–	46	–	–
Lípa	12	–	–	12	–	–	12	–	–
Valečov	22	–	–	22	–	–	22	–	–
2007 (*n* = 76 isolates)									
Humpolec[g]	3	–	–	3	–	–	3	–	–
Lípa	1	–	–	1	–	–	1	–	–
Horažďovice	40	–	–	40	–	–	40	–	–
Valečov	24	–	–	24	–	–	24	–	–
Černý Dub[g]	8	–	–	8	–	–	8	–	–
2008 (*n* = 119 isolates)									
Semice	20	–	–	20	–	–	20	–	–
Olešná	–	–	1	1	–	–	1	–	–
Valečov	–	–	33	33	–	–	33	–	–
Horažďovice	21	–	–	21	–	–	21	–	–
Lípa	42	–	–	42	–	–	42	–	–
České Budějovice[g]	1	–	–	1	–	–	1	–	–
Holubov[g]	1	–	–	1	–	–	1	–	–

S – sensitive; I – intermediate; R – resistant.; *n* – number of collected isolates; [g]isolates collected in gardens; [t]isolates collected from tomatoes; [tu]isolates collected from a potato tuber

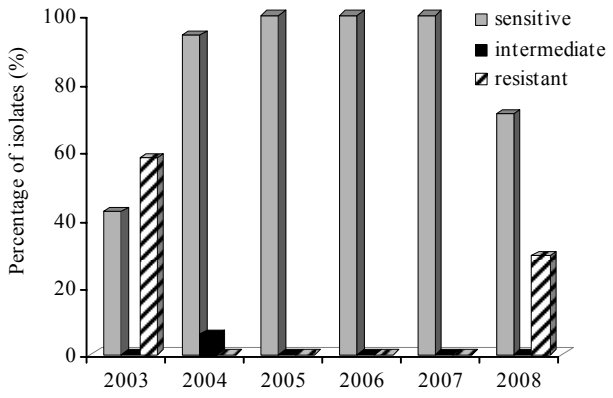

Figure 1. Responses to metalaxyl of *Phytophthora infestans* isolates from the years 2003–2008

from tomato plants; three of them were from leaves and one isolate was from a fruit.

In vitro assay of the response to metalaxyl, dimethomorph and propamocarb-HCl

Of the 547 isolates, 491, 4 and 52 were scored as sensitive, intermediate and resistant to metalaxyl, respectively. The majority of the isolates were sensitive (89.8%), and on average 9.5% of all tested isolates were resistant. The resistant isolates were found only in 2003 with a frequency (58%) that was higher than that of sensitive isolates and in 2008 (29%). Four intermediate isolates were observed among those collected in 2004 (Figure 1, Table 1). In all the 547 isolates that were tested for dimethomorph and propamocarb-HCl sensitivity, mycelial growth was suppressed even at 1 µg a.i. per ml and these were considered as sensitive (Table 1).

In vivo assay of the response to metalaxyl

Among the 352 analysed isolates, 95%, 1% and 4% of the isolates were sensitive, intermediate and resistant, respectively. The same results were obtained using both the floating leaf-disc method and the metalaxyl-amended agar method.

DISCUSSION

Since the first report on *P. infestans* resistant isolates to metalaxyl in potato crops in Ireland (Dowley & O'Sullivan 1981), an increased frequency of resistant isolates has been observed in *P. infestans* populations in many countries (Gisi & Cohen 1996). Two years before in 1979, the first metalaxyl-resistant isolates of *Pseudoperonospora cubensis* were detected in Israel, followed by many reports on the insufficiency of metalaxyl to control *Plasmopara viticola*, *Bremia lactuca*, and others (review Gisi 2002).

In the Czech Republic, the first serious problem with the decreased efficiency of phenylamide-based fungicides in potato crops was observed in 1986. During the 1990's, efficacious results were achieved with phenylamide treatments again (Hausvater & Rasocha 2000). This trend was observed e.g. in Ireland and was most likely caused by the rational application of phenylamides and the declining frequency of resistant isolates in comparison with the frequency that occurred in the 1980's and at the beginning of the 1990's (Carlisle *et al*. 2001). In Scotland during 1996–1997, the frequency of resistant isolates decreased, but the proportion of intermediate isolates increased (Cooke *et al*. 2003). The same results of decreasing resistant isolates and increasing intermediate isolates between 1995 and 1998 in Great Britain are presented by Day *et al*. (2004).

The present study summarises the results obtained over six years of surveying the occurrence of sensitive and resistant isolates to metalaxyl, dimethomorph and propamocarb-HCl as a phenotypic marker from different localities of the Czech Republic. Over the years of the survey, the metalaxyl-resistant isolates were detected only in 2003 (58%) and in 2008 (29%) and the majority of the isolates were sensitive. Based on Eucablight data between 2003 and 2008, the resistant isolates predominated in Northern Ireland (2003, 2007), Estonia (2004, 2005), and Slovakia (2004) (Anonymous 2009). In Estonia, the metalaxyl-resistant, intermediate and sensitive isolates were recorded at frequencies of 37.1%, 45.4% and 15.1%, respectively (Runno & Koppel 2006). In 2003–2004, the ratio of sensitivity to resistance was higher among Polish isolates (Śliwka *et al*. 2006).

The high frequency of metalaxyl-sensitive isolates in the Czech collection may have been caused by the uncommon summer weather. Adverse temperature and moisture conditions decreased late blight development, numbers of fungicide applications, and consequently, the fungicide selection

pressure imposed on *P. infestans* populations. In 2003, the conditions for the pathogen development and distribution among the host plants were not sufficient. The infection caused by *P. infestans* originated from seed tubers from the previous year that was characterised by a very strong infection pressure. The resistant isolates likely originated from the overwintering mycelium on tubers infected by both the sensitive and the resistant pathogen strains at the end of the previous potato-growing season. In subsequent years (2004–2007), almost 99% of the isolates were sensitive. The late blight symptoms in the crops were detected at later stages because of the relatively dry weather and low or moderate disease pressure in those years. Since 2008, the disease pressure was higher again and resistant isolates were identified (2009 data not presented). The evaluation of the pathogen resistance to a fungicide is influenced by the time of sampling since the level of resistance changes in different periods throughout the growing season. The frequency of resistant isolates increases when the fields are treated with phenylamide fungicides. The resistance level is lower at the beginning of the next season than at the end of the previous season. In addition to the selective pressure of the fungicide, the fitness of both the resistant and the sensitive strains, and the overwintering phase of pathogen, may result in increasing/decreasing the proportion of resistant isolates (Gisi & Cohen 1996).

The resistant isolates detected in 2003 and 2008 originated from fields in Olešná and Valečov, where the potato cultivar and the fungicide effectiveness were tested, thus the presence of resistant isolates may have been induced by the selective pressure of phenylamide that was applied in various treatment regimes as well as by the migration of the pathogen among crops in fields. Many studies suggest that the resistance level has no relationship with the occurrence of either mating type (Gisi & Cohen 1996; Hermansen *et al*. 2000; Cooke *et al*. 2003; Lehtinen *et al*. 2008). In our observations, the resistant isolates from 2003 corresponded to the A1 mating type, while each resistant isolate from 2008 was detected as the A2 mating type.

The floating leaf-disc method was used for determining the response to metalaxyl in isolates collected between 2003 and 2006. Among the 352 analysed isolates, 95%, 1% and 4% were sensitive, intermediate and resistant, respectively. Sozzi & Staub (1987) used various methods for measuring the sensitivity of *P. infestans* to metalaxyl, including potato leaf discs, detached leaves, whole plants and the rye agar amended test. Their results obtained from the *in vitro* assay showed similar resistance factors like *in vivo* assays. Differences in the pathogen reaction were observed within the different concentrations of metalaxyl. In contrast to the *in vivo* tests, the sensitive isolates had a higher minimal inhibitory concentration, while the resistant isolates were already inhibited by low fungicide concentrations. Although the amended-agar assay has been widely used to determine pathogen sensitivity to metalaxyl, Goodwin *et al*. (1998) recommended using the leaf-disc assay as it eliminates some of the variance seen in the *in vitro* test and may predict the response to metalaxyl in the field. Peters *et al*. (1998) presented the same results from both assays and Reis *et al*. (2005) found no correlation between these methods. In our study there were no differences in the results that were observed among the 352 isolates analysed using the amended agar assay and the leaf-disc test.

Sensitivity to dimethomorph and propamocarb-HCl was investigated in all of our isolates. The results demonstrated that mycelial growth was inhibited at the lowest concentration of each active ingredient. The fact that the selection for fungicide resistance in *P. infestans* has not been detected with the use of dimethomorph (Zhu *et al*. 2004; Elansky *et al*. 2007) does not mean that resistance could not evolve as it did in *Plasmopara viticola* in a number of vineyards in France and Germany (Brent & Hollomon 2007). Mutants of *P. infestans* that are resistant to dimethomorph were induced in the laboratory using ethidium bromide/UV light mutagenesis and repeated culturing on dimethomorph-amended medium (Stein & Kirk 2004) or nitrosomethyl urea mutagenesis (Bagirova *et al*. 2001), but low fitness was observed among these isolates. Accordingly, based on the lack of practical resistance, stable mutants and co-applications of dimethomorph with protectant fungicides, there is a small risk of development of dimethomorph resistance in *P. infestans*. Although no specific recommendations have been made yet for use against *P. infestans*, users should follow the manufacturers' recommendations (Brent & Hollomon 2007).

The propamocarb-resistant isolates of *P. infestans* were detected in four isolates from Sweden; these isolates were presented as being capable of sporula-

tion when exposed to 1000 mg/l propamocarb-HCl. However, the risk of accumulation of propamocarb-HCl resistance in the Nordic population is low and associated with the infrequent use of products containing propamocarb-HCl (Lehtinen *et al*. 2008). Möller *et al*. (2009) detected ten isolates as propamocarb resistant among isolates collected in southern Germany. As for other oomycete species, Urban and Lebeda (2007) demonstrated variation in the fungicide resistance of *Pseudoperonospora cubensis* in the Czech Republic between 2001 and 2004. The isolates collected between 2001 and 2003 were uniform in their sensitivity to propamocarb and belonged to highly sensitive strains; in the year 2004 the strains were characterised by a certain level of tolerance.

The areas under potatoes in the Czech Republic over the past nineteen years have decreased. Since the beginning of our survey, potatoes have been grown for various purposes on average on 40 000 ha each year. The average consumption of fungicides based on metalaxyl, metalaxyl-M, dimethomorph and propamocarb-HCl in these fields has been approximately 300, 2000, 3000 and 15 000 kg/year, respectively. Considering that *P. infestans* is classified as a high risk pathogen for the RNA polymerase target and as a medium risk pathogen for all other modes of action (FRAC classification), yearly monitoring of fungicide sensitivity is essential for selecting optimal use guidelines for the application of fungicides.

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