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AGGRESSIVENESS AND METALAXYL SENSITIVITY OF *PHYTOPHTHORA INFESTANS* STRAINS IN BELARUS

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Abstract

A total of 50 *P.infestans* strains, collected in all Belarusian regions, were evaluated for aggressiveness on potato and metalaxyl sensitivity. About 90% of isolates tested were found to have intermediate or even high resistance level to metalaxyl. Samples isolated from organic potato fields were more metalaxyl-sensitive and aggressive as regards “sporulation intensity” and “incubation period” indices than strains collected from conventional potato crops with common fungicide use. Aggressiveness of Belarusian *P. infestans* strains varied greatly but differed insignificantly between the regions of collection. Samples with various levels of sensitivity to metalaxyl were nearly identical regarding all aggressiveness components and competitive ability that determines their co-existence in pathogen field populations.

Key words: potato, late blight, aggressiveness, metalaxyl, sensitivity

Introduction

Late blight caused by oomycete *Phytophthora infestans* (Mont.) de Bary still remains one of the most serious diseases affecting potato crops worldwide. Since the mid 40’s of XIX century, when its first devastating epidemics resulted in severe famine in Europe, study of pathogen biology and improvement of disease management has been relevant for scientists in different countries. Certain achievements in late blight control, conditioned by creation of relatively resistant potato cultivars and effective fungicides were replaced with long-lasting failures, when yield losses, caused by this disease, constituted millions of tons. According to Duncan /Филиппов, 2005/, annual net losses of potato, caused by the late blight, and costs for its control are evaluated at 3 billion of US dollars. In Belarus during epidemic years, when haulm and tubers are seriously affected, yield can be reduced twice and nowadays late blight epidemics can be observed nearly annually (during the period from 1960 till 2007 28 years of 47 (60%) were epidemic /Иванюк и др., 2005/. The most effective late blight managing practices are fungicide use and cultivation of resistant varieties. Cultivar resistance, however, is rather comparative due to its low durability. Lowering of variety resistance can be explained by the lack of knowledge of host protective mechanisms, on the one hand, and high pathogen variability, on the other. So, appearance and omnipresent spread of the “new” *P. infestans* strains and displacement of the “old” strains resulted in steady increase of pathogen harmfulness /Fry et al., 1993; Fry, Goodwin, 1997/. Sexual recombination regularly takes place in present populations of *P. infestans* /Flier et al., 2004/. It promotes the rise of genetic variability, pathogenicity and adaptability and, as a consequence, easier overcoming of protective mechanisms of previously resistant varieties.

Aggressiveness of parasitic organism is quantitative characteristic of its pathogenicity, major constituent of its short-term viability and competitive ability /Смирнов, Кузнецов, 2006/. “Aggressiveness” can be defined as capability to cause epidemic – heavy lesions of susceptible plants /Дьяков и др., 2001/. According to the theory, which is widely acknowledged by the most scientists dealing with *P. infestans*, “new” populations of this pathogen, originating from Mexico, displaced the indigenous, “old” populations worldwide /Fry et al., 1993/. It can probably be explained by increased aggressiveness of new strains of pathogen. Some works / Fry, Goodwin, 1997; Mizubuti, Fry, 1998; Flier et al., 2004/ have approved it. Nevertheless, these data are contradictory. Some researchers /Смирнов, 2007/ do not reveal significant advantages in pathogen aggressiveness when compared “new” and “old” genotypes. Moreover, the lack of unified protocols and technical approaches of aggressiveness assessment only worsen existing situation. Even more challenging problem with late blight control is the appearance and widespread distribution of field isolates with highly resistant response to phenylamides – systemic fungicides of extensive application /Kadish, Cohen, 1988; Fry et al., 1993/. These compounds, particularly, metalaxyl, serve as selective agent for pathogen field populations. Most probably, the reversion (restoration of population sensitivity) could be possible only in the case of lower competitive ability and fitness of resistant strains /Трус, Козловский, 1992/.

The present investigation was designed to study aggressive features and metalaxyl sensitivity of *P. infestans* strains from all Belarusian regions, purposely to compare parasitic activity of sensitive and resistant forms of this pathogen.

Materials and Methods

A total of 50 isolates of *P. infestans* obtained from naturally infected foliage of potato were collected from 6 administrative regions of Belarus in August, 2006 and 2007 (Figure). Most were sampled from commercial fields (after 3–5 fungicide sprays), but some were isolated from potato plants grown in home gardens without fungicide application.

Figure. Sites of origin of *P. infestans* isolates (marked with rectangles)

Single leaflets with freshly sporulating lesions of *P. infestans* were collected and placed between two tuber halves held together with rubber bands. These were packed individually in paper bags and placed in cardboard boxes for shipment. Tuber halves were separated in the lab and infected leaflets were removed and placed between the fresh potato slices of susceptible variety Delphin in the moist chambers (glass 90 mm Petri dishes containing moistened filter paper at the top). Petri dishes were incubated for 3–4 days at 18° C in the dark to promote sporulation on potato slices. Freshly formed sporangia were washed from infected tissue (bulked from three replicates) and adjusted to 1.5×10^4 ml^{-1} (mean of 10 counts using Goryaev chamber) in distilled water. The sporangial suspension (bulk sample) was chilled for 2 h in refrigerator (4° C) before inoculation to promote zoospore release. Then it was used immediately to infect tuber discs of susceptible cultivar Delphin to assay aggressiveness on potato and sensitivity to metalaxyl. Discs (30 mm-diameter) were cut from healthy surface-sterilized tubers with the aid of pinch, washed immediately with running tap water, exposed for 1 hour in distilled water with ampicillin (200 mg l^{-1}) to avoid bacterial infection and diagnostic concentrations of metalaxyl (during sensitivity assessment), than dried with filter paper and after inoculation with 20µl-droplet of inoculum were incubated in moisture-saturated atmosphere at 20° C in the dark. 10 slices were used for testing each isolate.

Sensitivity of isolates to metalaxyl was tested according to the VIZR (All-Russian Institute of Plant Protection) protocol /Мелоян, Тютерев, 1990/. Ridomil 25% w. p. (a. i. metalaxyl, 25%) was used to prepare diagnostic solutions. Concentrations used were 1, 10 and 100 ppm (µg ml^{-1}). Distilled water was used as control. Sensitivity of each strain was determined visually after 5 days of incubation using five-point scale: 0 – sporulation is absent; 1 – up to 10% of slice surface is covered with sporulation; 2 – 10–30%; 3 – 30–60%; 4 – 60–90%; 5 – 90–100% of slice surface is covered with sporulation. ED_{50} (concentration of metalaxyl at which sporulation capacity is 50% of control) was determined graphically /Мелоян, Тютерев, 1990/. Isolates were classified as sensitive when ED_{50} was less than 5 ppm, ED_{50} of resistant isolates was more than 50 ppm, intermediate isolates ED_{50} was more than 5 and less than 50 ppm.

Aggressiveness assessment was carried out according to Smirnov /Смирнов, 2007/. The following aggressiveness components (AC) were measured: (a) infection frequency (IF), the proportion of inoculated tuber discs infected; (b) lesion area (LA), the area of lesion produced 5 days after inoculation of tuber slices; (c) sporulation capacity (SC), concentration of sporangia (in 1 ml of distilled water) produced per square centimeter of lesion on tuber slices 5 days after inoculation; (d) latent period (LP), time (in days) from infection to initial sporulation; (e) incubation period (IP), time (in days) from infection to the onset of first late blight symptoms (necrotic lesions or sporulation); (f) composite aggressiveness index (CAI) was calculated as described by Smirnov (Смирнов, 2007): $CAI = IF \times LA \times SC / IP \times LP$.

To assess AC special five-point scale was used (Table 1).

Five days after inoculation IF and LA were measured. SC was assessed as follows. Two 5 mm-diameter tuber slices were cut from sporulating 30 mm-diameter slices with the pinch of lesser crosscut. They were transferred into plastic container with 5 ml of distilled water. Sporangia were dislodged by vortexing of containers for 2 min and counted (10 counts) with the aid of a Goryaev chamber. To estimate LP and IP discs were assessed every 24 hours following inoculation (on day 0) for 7 days.

Table 1. Determination of *P. infestans* strains aggressiveness components using five-point scale

	1	2	3	4	5
IF	1–2 infected slices	3–4 infected slices	5–6 infected slices	7–8 infected slices	9–10 infected slices
LA	up to 10% of surface affected	11–30% of surface affected	31–60% of surface affected	61–90% of surface affected	91–100% of surface affected
SC	Up to 10000 sporangia/ml	10000–25000 sporangia/ml	25000–50000 sporangia/ml	50000–75000 sporangia/ml	more than 75000 sporangia/ml

CAI numbers were also ranked to divide 3 groups of isolates. If LN(CAI) of isolate tested was less than 3, this strain was ranked as weakly aggressive; more than 4 – highly aggressive; from 3 to 4 – with intermediate level of aggressiveness.

All statistical analyses were performed using the MS Excel 2003 and Statistica 6.0 package. The data were analyzed by ANOVA and nonparametric Kruskall-Wallis ANOVA (H-test). Post-hoc analysis was made with the help of Duncan's range test. Significance of differences between the distribution of aggressiveness and metalaxyl sensitivity ranges was determined using χ^2 – test. Spearman correlation was calculated to show association between aggressiveness components. All accounts were made at $p = 0.05$.

Results and Discussion

Aggressiveness and metalaxyl sensitivity levels of *P. infestans* isolates from different Belarusian regions are revealed to have a broad range of variation. Strains tested proved to contain a considerable proportion of isolates with low and intermediate metalaxyl sensitivity (29.0% and 61.4% accordingly). The share of sensitive strains did not exceed 10% (Table 2).

Table 2. The proportion (%) of *P. infestans* isolates with different levels of aggressiveness and metalaxyl sensitivity in six Belarusian regions

Region	Aggressiveness			Metalaxyl sensitivity		
	High	Intermediate	Low	High	Intermediate	Low
Brest	50.0	30.0	20.0	0.0	60.0	40.0
Vitsebsk	25.0	25.0	50.0	16.7	66.7	16.7
Gomel	40.0	30.0	30.0	10.0	60.0	30.0
Grodna	–	75.0	25.0	0.0	75.0	25.0
Minsk	22.2	66.7	11.1	11.1	66.7	22.2
Mahiliou	40.0	20.0	40.0	20.0	40.0	40.0
Average	29.6	41.1	29.3	9.6	61.4	29.0

Insensitivity of *P. infestans* to phenylamides eventually resulted in failure to control late blight was first recorded in 1980 after several years of wide application of these fungicides /Cadish, Kohen, 1988; Bradshaw, Vaughan, 1996/. Monitoring of *P. infestans* metalaxyl sensitivity carried out in Belarus from 1989 to 1999 has shown that share of resistant isolates varied considerably between years from 10% to 94% /Иванюк, Авдеу, 2000/. Resistance level of *P. infestans* still remains high (Table 2). Phenylamides continue to be used widely for disease control and though they are marketed today only in pre-pack mixtures with contact fungicides such as mancozeb, it has not changed the situation established. The share of insensitive strains seems to be directly proportional to the rates of phenylamide application /Иванюк, Авдеу, 2000/. Metalaxyl sensitivity assessment of *P. infestans* strains collected in home gardens without fungicide application has supported that data. Isolates from unsprayed plots were significantly more sensitive, than samples from conventional fields with intensive spraying programs including phenylamide ingredients ($p = 0.01$, χ^2 – test) (Table 3).

Table 3. The proportion (%) of *P. infestans* isolates with different levels of metalaxyl sensitivity collected in home gardens and conventional fields

Field type	Metalaxyl sensitivity		
	High	Intermediate	Low
Home gardens (no phenylamides)	25.0	75.0	0
Conventional fields (with phenylamides)	7.2	54.7	38.1

No significant difference was observed in the sensitivity of strains collected in different geographic regions of the country ($p = 0.39$, χ^2 – test). No metalaxyl resistant forms were found among isolates sampled in home gardens, though, the percentage of strains with intermediate sensitivity was high there (75 %). It is conceivable that such forms with moderate insensitivity serve as adaptive potential enabling the population to respond quickly to phenylamide application or to fungicide pressure removal /Трус, Козловский, 1992/.

Aggressiveness of Belarusian *P. infestans* isolates is shown to vary considerably. However, the differences in aggressiveness between strains from different geographic regions were insignificant ($p = 0.36$, χ^2 – test). Of all isolates analyzed 29.6% were weakly aggressive, 29.3% were highly aggressive and 41.1% showed intermediate aggressiveness level (Table 2). It is necessary to mention that different approaches and protocols for CAI calculation have established in different research laboratories worldwide. So, according to Spielman et al. (1992), overall aggressiveness consists of 3 components: IF, SC and LP. In the Netherlands IF, SC, LP and MGR (maximal curve growth rate) serve as aggressiveness parameters /Flier, Turkensteen, 1999/. As a result, ranking of strains according to their overall aggressiveness is, most likely, rather subjective. Therefore, the majority of *P. infestans* investigators deal with individual aggressive components of the pathogen /Carlisle et al., 2002; Suassuna et al., 2004/.

Correlation analysis of aggressiveness components have shown that strains with high rate of penetration into plant tissue, as a rule, caused more lesions of larger size (negative correlation between IF and IP, IF and LP, LA and LP) (Table 4). In addition, the quicker the isolate penetrates into plant tissue, the faster sporulation forms (correlation between LP and IP). SC, however, does not depend on other aggressiveness components. Big sporulation area can yield traces of sporangia and inversely, small zones covered with mycelium can give an abundant sporulation. It denotes requirement to check all the components when analyzing *P. infestans* aggressiveness.

Table 4. The correlation between aggressiveness parameters of *P. infestans* isolates

Aggressiveness components	LA	SC	IP	LP	CAI
Infection Frequency (IF)	0.36* $p = 0.10$	0.13 $p = 0.38$	–0.57 $p<0.05$	–0.73 $p<0.05$	0.67 $p<0.05$
Lesion Area (LA)		0.16 $p = 0.28$	–0.63 $p<0.05$	–0.42 $p<0.05$	0.75 $p<0.05$
Sporulation Capacity (SC)			-0.27 $p = 0.57$	–0.26 $p = 0.64$	0.59 $p<0.05$
Incubation Period (IP)				0.80 $p<0.05$	–0.82 $p<0.05$
Latent Period (LP)					–0.77 $p<0.05$

* "p" shows significance level of Spearman correlation coefficient

Strains from different geographic regions of the country are similar in their aggressive components, except LP and IP (Table 5). Consequently, isolates from Grodno region had significantly lower LP than other strains. The most prolonged LP and IP was shown for Brest and Gomel isolates.

Table 5. Aggressiveness factors (means of ranked values) of *P. infestans* isolates collected in different regions of Belarus (ANOVA, Duncan range test)

Aggressiveness components	Average values for strains from different regions of Belarus						Significance level (p)	Significance of differences between groups of strains
	Brest	Gomel	Grodna	Minsk	Mahiliou	Vitebsk		
Infection frequency	4.70	4.30	5.00	4.78	5.00	4.75	0.60	–
Lesion area	1.63	2.04	1.78	2.33	2.28	2.24	0.48	–
Sporulation capacity	3.61	3.28	3.77	2.53	3.01	3.53	0.48	–
Incubation period	4.67^{a*}	4.40^{a}	3.60^{b}	4.08^{ab}	4.22^{ab}	4.18^{ab}	0.06	–
Latent period	4.73^{b}	4.77^{b}	3.60^{a}	4.47^{b}	4.26^{ab}	4.38^{b}	0.08	–
Composite aggressiveness index	3.02	3.07	3.84	3.29	3.51	3.54	0.69	–

* "a" and "b" indices show statistically significant differences between groups

Unlike strain originating region, fungicide application affects significantly some aggressiveness components of *P. infestans*. Strains collected from unsprayed potato plants showed higher aggressiveness regarding all its factors when compared to isolates sampled from conventional fields though these differences were significant only for SC, IP and CAI (Table 6). So, fungicides suppress *P. infestans* strains, considerably lowering their aggressiveness on potato. Nevertheless, as mentioned above, phenylamides serve as selective agent for the increase of insensitive strains' proportion in population. In that case there is every reason to suppose that resistant strains are less aggressive than sensitive ones. Comparative analysis of aggressive components of isolates with different levels of metalaxyl sensitivity has not supported this hypothesis (Table 7). Moreover, six strains collected in conventional potato fields after several fungicide sprays proved to be very aggressive. It indicates that even against a background of severe fungicide pressure field populations of pathogen can contain highly aggressive strains. Results obtained have revealed similar competitive ability and aggressiveness of metalaxyl sensitive and resistant strains. These forms co-exist in heterogeneous pathogen populations, conditioning their high level of adaptation to environmental conditions. In the case of fungicide pressure resistant strains receive an advantage, sensitive and intermediate forms are predominant in unsprayed potato fields. Plenty of strains with intermediate sensitivity result from high adaptability of "new" pathogen populations, their improved ecological flexibility.

Table 6. Aggressiveness factors (means of ranked values) of *P. infestans* isolates collected in home gardens and conventional fields

Aggressiveness components	Average values for strains		Significance level (p)	Significance of differences between groups of strains
	Home gardens	Conventional fields		
Infection frequency	4.88	4.67	0.09	–
Lesion area	2.68	1.95	0.12	–
Sporulation capacity	4.39	3.07	0.02	+
Incubation period	3.83	4.34	0.02	+
Latent period	4.15	4.53	0.19	–
Composite aggressiveness index	4.25	3.14	0.02	+

It is noteworthy that the majority of potato plantings in Belarus (about 90%) constitute home gardens, where fungicide applications are rather rare. So, these fields serve as a reserve for phenylamide sensitive *P. infestans* isolates. Selective action of phenylamides results in appearance and rapid accumulation of insensitive forms with compatible level of aggressiveness.

Table 7. Aggressiveness factors (means of ranked values) of *P. infestans* isolates with different levels of metalaxyl sensitivity

Aggressiveness components	Average values for strains with different sensitivity to metalaxyl			Significance level (p)	Significance of differences between groups of strains
	Sensitive	Intermediate	Resistant		
Infection frequency	4.80	4.95	4.43	0.10	–
Lesion area	2.14	2.30	1.82	0.17	–
Sporulation capacity	3.34	3.37	3.18	0.89	–
Incubation period	4.56	4.07	4.38	0.14	–
Latent period	4.64	4.24	4.65	0.14	–
Composite aggressiveness index	3.36	3.65	2.99	0.10	–

Conclusions

1. It has been demonstrated in this work that most part (about 90%) of the tested strains possess intermediate and high resistance to metalaxyl. It sets conditions for more careful phenylamide application for late blight control in Belarus.

2. Strains sampled from unsprayed potato fields were found to be more sensitive and aggressive than strains from conventional fields with application of fungicides, including phenylamides. The difference in aggressiveness between strains with different metalaxyl sensitivity levels cannot be confirmed statistically. It conditions similar competitive ability of sensitive and resistant strains of pathogen and their co-existence in field populations.

3. Diversity of *P. infestans* and microevolution processes going on in pathogen populations under the influence of chemical substances should be seriously taken into account when planning effective late blight control strategies. If high insensitivity level is determined, phenylamide application should be reduced. In this case it would be more effective to use contact fungicides according to their application recommendations.

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