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Sensitivity of *Phytophthora infestans* (Mont.) de Bary Isolates to Fluazinam, Fosetyl-Al and Propamocarb-hydrochloride

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SUMMARY

A survey of *in vitro* sensitivity of twelve isolates of the *Phytophthora infestans* to the fluazinam, fosetyl-Al and propamocarb-hydrochloride was conducted. The isolates were isolated from infeceted potato leaves collected from eight different localities in Serbia during 2005-2007. All *P. infestans* isolates were sensitive to tested fungicides. The obtained values of resistance factor were in the range from 1.0 to 2.8. The EC_{50} values of fluazinam were from 0.14 to 0.27 mg l^{-1}, fosetyl-Al from 30.2 to 85.8 mg l^{-1}, propamocarb-hydrochloride between 12.1 and 31.1 mg l^{-1}, respectively.

Keywords: *Phytophthora infestans*; Sensitivity; *in vitro*; Fluazinam; Fosetyl-Al; Propamocarb-hydrochloride

INTRODUCTION

Potato late blight, caused by *Phytophthora infestans* (Mont.) de Bary, is the economically most important potato disease in Serbia. The asexual life cycle of *P. infestans* is short; and sporulating foliar lesions develop three to seven days after successful infection under conducive conditions resulting in a polycyclic epidemic (Stein and Kirk, 2002).

In recent years, the severity of late blight increased in many parts of the world. This is frequently associated with migrations that have introduced new, more aggressive, populations of the pathogen (Fry and Goodwin, 1997). The late blight epidemic in 1999 was devastating in Serbia. The new genotypes of *P. infestans* are being detected in the countries from which a large quantity of potato seed is imported to Serbia for its own potato production, in particular from Holland, Germany and Hungary. Ivanović (2005) reported that the A2 mating type occurs in Serbia. The appearance of more aggressive strains of *P. infestans*, containing both A1 and A2 mating types makes this problem even more complicated.

Control of *P. infestans* in Serbia relies on intensive use of fungicides often without any appropriate program. Discovery and development of the first systemic fungicides from phenylamides class was a significant improvement and one of the most important contributions to agrochemical industry. However, the intensive use of metalaxyl led to the rapid selection of metalaxyl-resistant strains of *P. infestans* in Europe within one year from its introduction (Parra and Ristaino, 2001).

Twenty one active substances besides phenylamid fungicides are registered in Serbia for control of potato late blight (copper sulfate, copper oxide, copper hydroxide, copper oxysulfate, copper oxychloride, mancozeb, propineb, chlorothalonil, metriram, cineb, fluazinam, cymoxanil, metalaxyl, famoxadone, dimethomorph, propamocarb hydrochloride, fluopicolide, mandipropamid, cyazofamid, azoxystrobin and zoxamide) (Janjić and Elezović, 2010). However, according to FRAC(a) (2011) report, *P. infestans* developed resistance to the phenylamide fungicides quite rapidly but not at all to dimethomorph, iprovalicarb, fluazinam, cymoxanil, azoxystrobin and fenamidone (QoI fungicides), cyazofamid (QiI fungicide), propamocarb and organotins. Therefore, FRAC(a) (2011) re-classified *P. infestans* as a high risk pathogen for the RNA polymerase target only, and as a medium risk pathogen for all other modes of action.

Knowledge of the sensitivity of *P. infestans* isolates to fungicides with different mode of action, is very important for the development of late blight management strategies in Serbia.

The objective of this study was to test the sensitivity of *P. infestans* isolates collected in Serbia over the period 2005-2007, to the fungicides fluazinam, fosetyl-Al and propamocarb-hydrochloride.

MATERIAL AND METHODS

Samples collection and isolation

Isolates were collected from the major potato growing regions in Serbia during 2005 and 2007 growing season. The host variety on which late blight was detected, plot size, fungicides used, and late blight incidence and severity were also recorded. *P. infestans* was isolated from the infected potato leaves collected from eight different locations according to the methods described by Mukalazi et al. (2001) and Zhu et al. (2008). Fragments of infected leaf tissue were placed under a thin, surface disinfected slice of potato tuber of Desiree variety and incubated at 18°C for seven days to allow mycelial growth through the potato slice. Mycelia was picked from the surface of the slice using a sterile needle, and then put on rye B medium (200 g rye, 20 g glucose, 17 g agar, 1 l deionized water) amended with rifampicin 20 μg ml^{-1}, ampicillin 200 μg ml^{-1}, and nystatin 100 μg ml^{-1}, and incubated at 18°C in darkness for 7 days. After two transfers of hyphal tips on media containing antibiotics, twelve pure isolates were obtained (Table 1). The identity of isolates of *P. infestans* was confirmed by polymerase chain reaction (PCR) using species-specific primers (Tooley, 1998) and their morphological traits according to Erwin and Ribeiro (2005). The isolates were kept on potato dextrose agar (PDA) at 5°C in the Culture Collection of the Institute of Pesticides and Environmental Protection, Belgrade.

Table 1. *Phytophthora infestans* isolates and their origin

Code of isolate	Location	Year of isolation
DO7	Dobanovci	2005
GU6	Guča	2005
KS3	Kosjerić	2005
KS2	Kosjerić	2005
KT1	Kotraža	2006
KT2	Kotraža	2006
KV1	Kraljevo	2006
KV5	Kraljevo	2006
P11	Prijepolje	2007
P12	Prijepolje	2007
PR1	Prilike	2007
VK1	V. Kamenica	2007

Fungicides

Commercial formulations of fungicides were used as active ingredients (a.i.) respectively: fluazinam provided by Syngenta Agro Service, Serbia, (Shirlan 500-SC, 500 g l^{-1}), fosetyl-Al (Aliette 80-WP, 800 g kg^{-1}, Bayer CropScience), and propamocarb-hydrochloride (Previcur 607-SL, 722 g l^{-1}, Bayer CropScience). Each fungicide was diluted into a set of stock solutions with sterile distilled water. Freshly-made stock solutions were prepared to give specific concentrations of active ingredient in ml l^{-1}. Volumes of stock solution were added to molten (50°C) sterile culture media prior to pouring, thereby, producing active ingredient concentrations ranging from 0.01 to 100.0 mg l^{-1} (Locher and Lorenz, 1991).

Fungicide sensitivity and EC_{50} assays

P. infestans isolates grown on rye B medium amended with the fungicides: fluazinam, fosetyl-Al, and propamocarb-hydrochloride, were used for sensitivity tests. Based on the preliminarily obtained results, the selected concentrations of fluazinam for further study was: 0.01, 0.1, 1.0 and 10.0 mg l^{-1}; fosetyl-Al: 0.1, 1.0, 10.0 and 100.0 mg l^{-1}; propamocarb-hydrochloride 0.1, 1.0, 10.0 and 100.0 mg l^{-1}. Control plates were not amended with fungicides. Tests for each isolate were replicated three times per each concentration of each fungicide. Mycelial plugs (10 mm in diameter) were removed from the margins of colonies grown on rye B medium, placed upside down on the fungicide-amended and fungicide-free rye B medium in Petri dishes, and incubated at 18°C. After 10 days, the colony diameter of each isolate was measured in two directions (minus the diameter of the inoculation plug) and the percent inhibition (PI) values of each of the fungicide rates were calculated using the formula given below:

percent inhibition = (a – b) / a x 100

where a = the colony diameter of the control plate and b = the colony diameter of the fungicide-amended plate

PI values were subjected to regression analysis against the logarithmic values of the fungicide rates. The EC_{50} (fungicide concentration which inhibits mycelial growth by 50%) was determined for each isolate and data on fungicide concentration and relative inhibition were analysed using probit analysis, according to Finney (1971).

The resistance factor (RF) was expressed as the ratio of the EC_{50} and the lowest EC_{50} of the isolates tested (Gouot, 1994). The level of resistance factor (RF) was expressed according to following scale (Gouot, 1994):

- RF < 3 – sensitive isolates;
- RF = 3 – 20 > – moderately resistant isolates;
- RF = 100 > – highly resistant isolates.

RESULTS

Sensitivity of *P. infestans* isolates to the fungicides tested are shown in Tables 2, 3 and 4. Among twelve *P. infestans* isolates tested for sensitivity to fluazinam, the PR1 isolate showed greatest sensitivity. The EC_{50} value of this isolate was 0.14 mg l^{-1}. The DO7, KT2 and KV1 isolates had the highest EC_{50} value (0.27 mg l^{-1}). The EC_{50} values of the remaining tested isolates were between 0.15 and 0.24 mg l^{-1} (Table 2).

Table 2. Sensitivity of isolates of *P. infestans* to fluazinam

Code of isolate	Fluazinam				
	EC_{50} (mg l-1)		b		RF
	Value	Range*	Value	Range*	
DO7	0.27	0.09-0.58	0.46	0.40-0.52	1.9
GU6	0.23	0.06-0.54	0.41	0.35-0.47	1.8
KS3	0.22	0.08-0.44	0.51	0.44-0.58	1.6
KS2	0.15	0.03-0.39	0.38	0.32-0.44	1.1
KT1	0.19	0.07-0.38	0.55	0.48-0.62	1.4
KT2	0.27	0.09-0.56	0.47	0.35-0.51	1.8
KV1	0.27	0.07-0.64	0.39	0.33-0.46	1.9
KV5	0.19	0.07-0.41	0.50	0.43-0.56	1.4
P11	0.17	0.06-0.36	0.52	0.45-0.59	1.2
P12	0.18	0.04-0.44	0.41	0.34-0.47	1.3
PR1	0.14	0.03-0.35	0.40	0.34-0.47	1.0
VK1	0.24	0.07-0.53	0.44	0.37-0.50	1.7

EC_{50} – Fungicide concentration which inhibits mycelial growth by 50%; RF – The resistance factor was expressed as the ratio of the EC_{50} and the lowest EC_{50} for the isolates tested; b – Regression coefficient; *95% confidence interval (P=0.05)

Table 3. Sensitivity of isolates of P. *infestans* to fosetyl-Al

Code of isolate	Fosetyl-Al				RF
	EC_{50} (mg l-1)		b		
	Value	Range*	Value	Range*	
DO7	64.3	30.8-185.6	0.52	0.45-0.59	2.2
GU6	85.8	38.6-283.8	0.50	0.43-0.57	2.8
KS3	49.9	23.8-144.8	0.50	0.43-0.56	1.6
KS2	39.9	18.6-120.9	0.45	0.39-0.52	1.3
KT1	59.1	24.4-232.7	0.41	0.35-0.48	1.9
KT2	32.9	14.7-106.6	0.41	0.35-0.48	1.1
KV1	79.5	34.8-278.0	0.48	0.41-0.54	2.6
KV5	57.7	32.0-127.6	0.66	0.59-0.74	1.9
P11	50.2	25.0-133.8	0.53	0.46-0.60	1.7
P12	30.2	14.2-86.9	0.44	0.38-0.52	1.0
PR1	46.8	22.8-128.7	0.51	0.44-0.57	1.5
VK1	36.6	20.8-75.7	0.64	0.57-0.71	1.2

EC_{50} – Fungicide concentration which inhibits mycelial growth by 50%; RF – The resistance factor was expressed as the ratio of the EC_{50} and the lowest EC_{50} for the isolates tested; b – Regression coefficient; *95% confidence interval (P=0.05)

Table 4. Sensitivity of isolates of P. *infestans* to propamocarb-hydrochloride

Code of isolate	Propamocarb-hydrochloride				RF
	EC_{50} (mg l-1)		b		
	Value	Range*	Value	Range*	
DO7	22.0	13.5-40.29	0.69	0.61-0.76	1.8
GU6	12.1	6.88-23.87	0.55	0.49-0.61	1.0
KS3	21.7	13.47-38.28	0.72	0.65-0.80	1.8
KS2	18.6	12.0-30.91	0.78	0.71-0.86	1.5
KT1	27.3	16.49-50.69	0.70	0.62-0.77	2.6
KT2	16.2	10.11-28.12	0.70	0.63-0.78	1.3
KV1	19.1	11.52-35.04	0.66	0.59-0.73	1.6
KV5	25.9	13.60-60.56	0.52	0.45-0.58	2.1
P11	22.2	13.61-40.33	0.70	0.62-0.77	1.8
P12	30.9	17.32-65.40	0.60	0.53-0.67	2.5
PR1	31.1	16.01-75.67	0.52	0.45-0.59	2.6
VK1	26.5	14.79-56.06	0.58	0.52-0.65	2.2

EC_{50} – Fungicide concentration which inhibits mycelial growth by 50%; RF – The resistance factor was expressed as the ratio of the EC_{50} and the lowest EC_{50} for the isolates tested; b – Regression coefficient; *95% confidence interval (P=0.05)

The calculated EC_{50} values for inhibition of hyphal growth ranged from 30.2 to 85.8 mg l^{-1} for fosetyl-Al. This fungicide was the most toxic for the mycelium of isolates P12 and KT2 (30.2 and 32.9 mg l^{-1}) (Table 3). The highest EC_{50} values were determined in isolates KV1 (79.5 mg l^{-1}) and GU6 (85.8 mg l^{-1}) (Table 3).

Propamocarb-hydrochloride exhibited greater toxicity for the isolates GU6 (EC_{50} =12.1 mg l^{-1}) and KT2 (EC_{50}=16.2 mg l^{-1}) then for the rest of the isolates. Among the tested isolates the PR1 isolate showed the lowest susceptibility to dimethomorph (EC_{50}=31.1 mg l^{-1}). The EC_{50} values of the other investigated isolates were between 18.6 and 30.9 mg l^{-1} (Table 4).

DISCUSSION

All tested isolates of *P. infestans* were highly sensitive to fluazinam. Fluazinam is the only commercially available product from the class of pyridinamines for

the control of potato late blight in Serbia and worldwide. The biological mode of action is uncoupling of oxidative-phosphorilation in both true fungi and pseudofungi (Stein, 2002). The obtained EC_{50} values were in a very narrow range (0.14 to 0.27 mg l^{-1}), and were similar to the values established by Matheron and Porchas (2000) for *Phytophthora parasitica* isolates (0.1 mg l^{-1}) originating from the United States. All isolates were sensitive to fluazinam (RF=1.0-1.9) according to criteria established by Gouot (1994). FRAC(b) (2011) also classified fluazinam as fungicide with low risk of resistance.

As a member of carbamate fungicides, propamocarb-hydrochloride is highly specific for fungi from order Peronosporales. The biological mode of action has not been elucidated but disruption of cellular membrane and/or function was observed. The biological activity of propamocarb-hydrochloride is relatively low compared to other semi-systemic late blight fungicides in the terms of rate comparisons, and large amounts have to be applied for comparable activity (Stein, 2002). Widest range of EC_{50} values of tested isolates was observed in tests conducted with this carbamate fungicde; they varied from 12.1 mg l^{-1} (GU6) to 31.1 mg l^{-1} (PR1). The obtained values of RF indicate that all isolates were sensitive to propamocarb-hydrochloride. The results were similar to those of Hu et al. (2007) for *Phytophthora nicotianae* (EC_{50} = 2.2-90.1 mg l^{-1}). FRAC(b) (2011) states that propamocarb-hydrochloride, in terms of resistance, is a low to medium risk fungicide. However, as propamocarb-hydrochloride since its introduction to the Serbian market was combined with contact or systemic fungicides (mancozeb, chlorothalonil, fosetyl-Al and fenamidone), the intensity of selection pressure of *P. infestans* was relatively lower.

Fosetyl-Al showed the lowest toxicity among all tested fungicides (30.2 to 85.8 mg l^{-1}). EC_{50} values obtained in our experiments were similar to the values obtained by the Farih et al. (1981).

However, the values of RF indicate that most isolates obtained from infected potato leaves in Serbia are sensitive to fosetyl-Al. Although fosetyl-Al and its breakdown product, phosphorous acid are not always more active against *P. infestans in vitro*, they are much more selective for this pathogen *in vivo* (Erwin and Ribeiro, 2005). Fosetyl-Al cause disturbance at several metabolic sites in the mycelial phase of the life-cycle, and inhibit sporulation at low concentrations, without affecting mycelial growth. Even at high concentrations (1-10 mM), fosetyl-Al is fungistatic rather than fungitoxic (Erwin and Ribeiro, 2005). The probability of high levels of resistance emergence in the field is reduced by the multi-site action of fosetyl-Al in the matebolism of *P. infestans* (Erwin and Ribeiro, 2005; FRAC(b), 2011)

Management of potato blight will continue to rely on chemical control. Low-risk fungicides (fluazinam, fosetyl-Al and propamocarb-hydrochloride) with different modes of action will be needed in problematic fields. Our study suggests that continuous monitoring of *P. infestans* field populations with respect to fungicide resistance is very important for the development of late blight management strategies.

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Osetljivost izolata *Phytophthora infestans* (Mont.) de Bary na fluazinam, fosetil-Al i propamokarb-hidrohlorid

REZIME

Ispitivana je osetljivost 12 izolata *Phytophthora infestans* na fluazinam, fosetil-Al i propamokarb-hidrohlorid u *in vitro* uslovima. Izolati su dobijeni iz zaraženih listova krompira sakupljenih tokom 2005-2007 godine sa osam različitih lokaliteta iz Srbije. Svi ispitivani izolati su bili osetljivi na testirane fungicide. Dobijene vrednosti faktora rezistentnosti bile su u intervalu od 1.0 do 2.8. Vrednosti EC_{50} za fluazinam su bile od 0.14 do 0.27 mg l^{-1}, fosetil-Al od 30.2 do 85.8 mg l^{-1} i propamokarb-hidrohlorid od 12.1 do 31.1 mg l^{-1}.

Ključne reči: *Phytophthora infestans*; osetljivost; *in vitro*; fluazinam; fosetil-Al; propamokarb-hidrohlorid