PPO – SPECIAL REPORT NO 16 – 2014, 201-208

Effect of some pesticides on the *in vitro* oospore formation and mycelial growth of *Phytophthora infestans* (Mont.) de Bary

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SUMMARY
The effect of some pesticides, including fungicides (fludioxonil and difenoconazole), insecticides (thiamethoxam and imidacloprid), and herbicide (metribuzin), on the oospore formation and radial colony growth of *Phytophthora infestans* on oatmeal agar has been studied. Fludioxonil demonstrates a statistically significant inhibition of the radial colony growth; other pesticides had no any effect on this parameter. A statistically significant effect on the oospore formation has been shown for all pesticides tested, excepting thiamethoxam. The maximum inhibiting effect has been shown for fludioxonil and difenoconazole; however, low concentrations of difenoconazole (1 µg/ml) stimulate the oospore formation.

KEYWORDS
Phytophthora infestans, oospore formation, potato late blight, oomycete, fungicide resistance

INTRODUCTION

Late blight of potato, caused by the oomycete *Phytophthora infestans* (Mont) de Bary, is a common disease for almost all potato-growing regions of Europe. In the case of severe infection, yield losses can reach 30% or even more (Ivanyuk *et al.*, 2005).

P. infestans is able to form thick-walled sexual structures called oospores. Though single isolates are also able to form single oospores in infected tissues, the most intensive oospore formation occurs in the case of a contact between strains of different mating types. The formation of oospores in different tissues of potato and tomato plants was observed under field conditions in many countries, such as Russia (Smirnov and Elansky, 1999), Norway (Hermansen *et al.*, 2002), Sweden (Strömberg *et al.*, 2001), Netherlands (Kessel *et al.*, 2002), etc. Oospores, together with infected seed material and overwintered tubers, represent one of the main sources of primary inoculum. Oospores can survive in soil for several years and are able to infect plants during the whole of this period. The most intensive plant infection occurs within two seasons

after the harvesting of potato or even later. In Norway oospores survived in the soil for 31 months (Bødker *et al.*, 2006). In the Moscow region of Russia and in Finland, oospores overwintered and still kept the ability to infect plants (Bagirova *et al.*, 1998, Ulanova *et al.*, 2010, Lehtinen, 2002). Oospores are also able to survive in tomato fruits for a long time (Rubin *et al.*, 2001). Hybrid oospores, resulted from different crosses, improve the genetic diversity of a pathogen population and, therefore, accelerate the process of the pathogen adaptation to new potato cultivars and fungicides.

The basic method to control the late blight disease is a chemical protection of crops via the treatment of fields with fungicides. It is known that sub lethal concentrations of fungicides, used to control late blight, cause the *in vivo* and *in vitro* reduction of the intensity of the oospore formation and the viability of oospores (Kessel *et al.*, 2002). In our study we investigated the effect of pesticides, which are not registered for the use against late blight, on the oospore formation and mycelial growth of *P. infestans*.

MATERIALS AND METHODS

In this study the effect of some fungicides (Maxim and Skor), insecticides (Aktara and Tanrek), and herbicides (Zenkor) on the radial mycelium growth and oospore formation was examined (Table 1).

Table 1. *List of pesticides/active ingredients used in the study*

Pesticide	Active ingredient	Pesticide type
Skor	difenoconazole	Fungicide
Maxim	fludioxonil	Fungicide
Aktara	thiamethoxam	Insecticide
Tanrek	imidacloprid	Insecticide
Zenkor	metribuzin	Herbicide

In our studies we used *P. infestans* isolates, collected from infected potato leaves in the Moscow (4 isolates, 2012), Ryazan (5 isolates, 2012), and Leningrad (1 isolate, 2008) regions, and one isolate collected from a tomato fruit in 2007 in the Mariy El Republic (Table 2).

Table 2. *List of P. infestans isolates used in the study*

Isolate name	Mating type	Host plant, region of origin, and information about the use of pesticides on this field before the sample collection
08LK 3	A1	Potato leaves, Leningrad region, first late bligh foci, no any pesticide treatments.
07YTP40/1	A1	Tomato fruits, Mariy El Republic, Yoshkar-Ola, private plot, no any pesticide treatments.
12MGRK 19	A1	Potato leaves (cv. Sante), Moscow region, Odintsovo district, experimental field of the All-Russian Research Institute of Phytopathology, no any pesticide treatments.
12MGRK 25	A2	
12MGVK 15	A2	
12MGVK 55	A2	
12RKLS8	A1	Potato leaves (cv. Skarb), Ryazan region, commercial potato fields; the last fungicide treatment was applied 30 days before the sampling.
12RKLS15	A1	
12RKLS20	A1	
12RKLS47	A1	
12RKLYa15	A2	Potato leaves (cv. Yanka), Ryazan region, commercial potato fields; the last fungicide treatment was applied 30 days before the sampling.

The mating tests at different concentrations of fludioxonil and metribuzin were carried out using two pairs of isolates for the crossing; in the case of difenoconazole (18°C), thiamethoxam, and imidacloprid, such assessment was carried out using four pairs of isolates; finally, in the case of the same experiment with difenoconazole (25°C), six pairs of isolates were used (Table 3).
The assessment of the effect of the pesticide concentration on the radial colony growth was carried out using five *P. infestans* isolates. An agar block with the mycelium of each isolate was placed in the center of a Petri plate with oatmeal agar. All tests were made in three replications. The concentrations of active ingredients were the following: 0.1, 1, and 10 μg/ml for thiamethoxam and 1, 10, and 100 μg/ml for other substances. A radial colony growth was measured after 12-15 days of incubation, when the colony diameter in the control (pesticide-free) variant reached about 80% of the Petri plate diameter. For each variant, the results of measurements, obtained for all replications of the same concentration, were averaged.

Table 3. *Isolate pairs used for crossings*

Active ingredient used in the crossing experiments	Crossed pairs of isolates	
fludioxonil, metribuzin	12MGRK25 × 1MGRK19	12MGRK25 × 07YTP40/1
difenoconazole (18°C), imidacloprid, thiamethoxam	12MGRK25 × 12RKLS8 12MGRK25 × 12RKLS47	08LK3 × 12RKLYA15 08LK3 × 12MGVK15
difenoconazole (25°C)	12 RKLYA15 × 08LK3 12MGVK55 × 08LK3 12MGVK15 × 08LK3	12RKLS20 × 12MGRK25 12 RKLS15 × 12MGRK25 12RKLS47 × 1MGRK25

To study the effect of pesticides on the oospore formation, isolates of different mating types (see Table 3) were placed by pairs into Petri plates with oatmeal agar medium at a distance of 5 cm from each other. The medium was preliminary supplemented with the examined pesticides at the following concentrations: 0.1, 1, 10, and 100 µg/ml (difenoconazole); 0.1, 1, and 10 µg/ml (thiamethoxam); 1, 10, and 100 µg/ml (imidacloprid, fludioxonil, and metribuzin). Pesticide-free medium was used as the control variant. Each pair of isolates was planted in three repetitions (3 Petri plates) per each concentration variant, including the control. After the inoculation, Petri dishes were sealed with Parafilm. After 20-22 days of incubation at 18°C, the whole volume of medium from each Petri plate was re-suspended in 30 ml of distilled water. Three 30-µl samples were taken from each variant, placed onto an object-plate, covered with a cover glass, and then microscoped to detect oospores. For each Petri plate, 60 microscopic fields were examined; then the number of oospores per a microscopic field was recalculated to obtain their concentration per 1 mm^3 of medium. The results of measurements were averaged for each pesticide concentration.

RESULTS

Effect of the pesticide concentration in agar medium on the radial colony growth rate

The obtained results showed that difenoconazole, thiamethoxam, and imidacloprid did not have any statistically significant effect on the radial colony growth of *P. infestans* (Table 4). Metribuzin caused a minor delay in the colony growth during the first 5-7 days; however, the colony diameter became close to the control value to the 10^{th} day of incubation. Fludioxonil provided a statistically significant inhibition of the colony growth at the concentrations exceeding 10 µg/ml.

Table 4. *Effect of pesticide concentration in agar medium on the radial colony growth rate of P. infestans*

Active ingredient (AI)	Radial colony growth (mm) at different AI concentrations				
	Control (0 µg/ml)	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml
difenoconazole	82±7* (100%)	–**	76±9 (93%)	84±4 (102%)	81±6 (99%)
fludioxonil	82±6 (100%)	–	74±12 (90%)	56±10 (68%)	46±3 (56%)
thiamethoxam	82±6 (100%)	81±7 (99%)	82±6 (100%)	81±6 (99%)	–
imidacloprid	79±6 (100%)	–	76±9 (96%)	77±8 (97%)	76±5 (96%)
metribuzin	88±12 (100%)	–	85±12 (97%)	86±9 (98%)	80±5 (91%)

** A confidence interval for the significance level 0.05 is indicated after the ± symbol.*

*** The variant was not tested.*

Effect of the pesticide concentration in agar medium on the oospore formation

A statistically significant inhibition of the oospore formation was observed for all pesticides tested (Table 5). A weak effect was observed only in the case of thiamethoxam. A strong inhibition effect was observed in the case of imidacloprid, fludioxonil, and difenoconazole; in the

last case, the effect was higher at 25°C, i.e., at the temperature uncomfortable for *P. infestans*. A significant inhibition of the oospore formation was observed in the presence of imidacloprid, though it did not inhibit the radial colony growth even at a high concentration (100 µg/ml).

Table 5. *Effect of the pesticide concentration in agar medium on the oospore formation in P. infestans*

Active ingredient (AI)	Number of oospores per a mm3 at different AI concentrations				
	Control 0 µg/ml	0.1 µg/ml	1 µg/ml	10 µg/ml	100 µg/ml
thiamethoxam	79.6 ± 3.6* (100%)	79.8 ± 3.8 (100%)	79.1 ± 3.9 (100%)	71.4 ± 3.7 (90%)	–
imidacloprid	79.6 ± 3.6* (100%)	–**	70.0 ± 3.4 (88%)	66.0 ± 3.1 (83%)	35.8 ± 2.8 (45%)
fludioxonil	112.7 ± 6.9 (100%)	–	98.4 ± 8.6 (87%)	73.6 ± 5.4 (65%)	42.3 ± 3.7 (36%)
metribuzin	135.0 ± 9.5 (100%)	–	103.0 ± 9.8 (76%)	118.2 ± 9.3 (88%)	74.8 ± 8.1 (55%)
difenoconazole (18°C)	79.6 ± 3.6 (100%)	72.5 ± 3.6 (91%)	82.2 ± 3.7 (103%)	54.9 ± 2.8 (69%)	35.8 ± 2.3 (45%)
difenoconazole (25°C)	29.7 ± 2.3 (100%)	14.1 ± 1.4 (47%)	22.6 ± 1.8 (76%)	12.0 ± 1.3 (40%)	10.8 ± 1.3 (36%)

** A confidence interval for the significance level 0.05 is indicated after the ± symbol.*
*** The variant was not tested.*

DISCUSSION

The study of the effect of pesticides, which were not registered for the use against the late blight, on the radial colony growth of *P. infestans* showed the expected absence of any statistically significant growth inhibition. However, it was found that fludioxonil inhibits the mycelial growth of *P. infestans* at concentrations exceeding 10 µg/ml. An allowed fludioxonil concentration in a working solution is 10 (or even more) times higher than the levels tested (see Table 6) and is able to significantly influence on the *P. infestans* development in infected seed tubers, killing zoospores and zoosporangia on their surface. Probably this fact can explain a delay in the late blight development, observed in the case of the planting of seed tubers treated with the Maxim (AI - fludioxonil) preparation (A.V. Filippov, personal communication; Anisimov *et al.*, 2009).

As we have already mentioned earlier, all tested pesticides inhibited the oospore formation process. The tested pesticide concentrations were lower or, in the case of imidacloprid and thiamethoxam, about equal to concentrations allowed for the use in a working solution (Table 6). In our experiments, the level of inhibition of the oospore formation increased as the dosage of a preparation increased; therefore, one could suppose an increased effect in the case of a contact of the pathogen with a more concentrated working solution. A weak effect observed for thiamethoxam is possibly explained by a low value of the maximum experimental concentration (10 µg/ml) comparing to those of other preparations (100 µg/ml).

The inhibition of the oospore formation was earlier shown for fungicides used for late blight control. Kessel *et al.* (2002) studied more than 10 commercial fungicides recommended for late blight control. All these preparations taken in sub lethal concentrations inhibited the oospore formation to a greater or lesser extent; this effect was observed on both nutrient medium and potato leaves. Hanson and Shattock (1998) demonstrated an inhibiting effect of metalaxyl on the oospore formation by pairs of isolates, in which one or both parental isolates were sensitive to this fungicide; a decreased oospore formation was also observed for the crossing of two resistant isolates. Taking into account our results and results obtained by other researchers, we can conclude that almost all pesticides applied on potato are able to inhibit the oospore formation to a greater or lesser extent. A weak effect was revealed in the case of such fungicides as mancozeb and chlorothalonil (Kessel *et al.*, 2002) and the insecticide thiamethoxam (our studies).

Table 6. *Concentrations of active ingredients used in the study and the recommended concentrations of the same ingredients in their working solutions*

Active ingredient (AI)	AI concentration used in the study, µg/ml	Recommended AI concentration in the working solution*, µg/ml
difenoconazole	0.1, 1, 10, 100	188-625
fludioxonil	1, 10, 100	1000
thiamethoxam	0.1, 1, 10	37-75
imidacloprid	1, 10, 100	50-100
metribuzin	1, 10, 100	1630-4900

** According to the State Catalogue of pesticides and agrochemicals permitted for the use on the territory of Russian Federation.*

In our experiments, difenoconazole, taken at the concentration equal to 1 µg/ml, was able to stimulate the oospore formation under optimal growth conditions (18°C) that was observed for all pairs of the isolates tested. The stimulating effect of low fungicide concentrations on the oospore formation was also described by Groves and Ristaino (2000), who studied the process of the oospore formation in a monoculture. Authors supposed that fungicide preparations are able to imitate the action of hormones and to influence on the mating type system. Probably the same reason explains the stimulating effect of difenoconazole on the oospore formation revealed in our study.

Difenoconazole represents a systemic pesticide able to penetrate into a plant. The difenoconazole concentration in plant tissues is lower than on the surface of a plant; it can be below the inhibiting level and close to the level optimal for the stimulation of the oospore formation. Therefore, in the case of a severe late blight infection, the treatment of potato plants with difenoconazole, especially at the minimum allowed dosage, is able to stimulate the oospore formation rather than to inhibit this process.

The obtained results show that pesticides applied on potato plants are able to inhibit the oospore formation even in the case when they do not possess any direct inhibiting effect on the pathogen growth. This improves a general phytosanitary situation on the fields and prevents the appearance of highly aggressive and fungicide resistant *P. infestans* strains caused by the hybridization process.

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