EFFECT OF DIFENOCONAZOLE ON THE FORMATION OF OOSPORES BY PHYTOPHTHORA INFESTANS (MONT) DE BARY

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SUMMARY

Fungicide difenoconazole is applied for field treatment to protect potato and tomato against early blight, caused by the Alternaria fungi. It is practically important to estimate the impact of difenoconazole application against late blight pathogen Phytophthora infestans (Mont.) de Bary because of early and late blight often come together on potato. Effect of fungicide difenoconazole on the radial growth of colony of P. infestans and the formation of oospores in agar media and in detached leaves was investigated.

There was no significant influence of difenoconazole on the radial growth of P. infestans colony, small inhibition occurred only at high concentration of fungicide (100 mg/l). Difenoconazole inhibited the oospore formation in the medium with positive dependence on concentrations of fungicide and temperature increasing. Tested in leaves difenoconazol inhibited the oosporogenesis with positive dependence on concentrations of fungicide also.

The tiers of the plant were considered as source of oospores with different potential. In laboratory conditions (test on detached leaves) the higher production of oospores was detected in leaves from middle and low tiers rather than from top tiers.

Keywords: Phytophthora infestans, oosporogenesis, late blight of potato, resistance to fungicides, difenoconazole

INTRODUCTION

The oomycete Phytophthora infestans (Mont.) de Bary causes the serious potato and tomato disease known as late blight. The sexual dormant stage of P. infestans is a thick walled oospore which serves as the primary source of inoculum. Hybrid oospores from different genotypes increase the genotypic diversity of pathogenic population providing the quick adaptation to new releasing cultivars of potato and tomato as well as to fungicides. Production of oospores by P. infestans in field was reported in Russia (Smirnov et al., 1999), Norway (Hermansen et al., 2002), Sweden (Strömberg et al., 2001), the Netherlands (Kessel et al., 2002) and in other places. Oospores are viable for at least two years keeping in soil (Bodker et al., 2006) and able to infect the plants after overwintering (Ulanova et al., 2010; Lehtinen et al., 2002).

The oospore formation is inhibited with chemicals used for late blight protection of potato and tomato. The effect of fluazinam, dimethomorph, cytoxanil, metalaxyl, maneb, propamocarb was shown (Kessel et al., 2002). In our previous experiments in vitro we revealed the same effect for fungicide pencycuron, insecticide imidacloprid, and herbicide metribusin. They did not prevent the growth of P. infestans colonies but reduced the oospore formation (Mita et al., 2014). However number of other chemicals applied on potato do not prevent the growth of P. infestans mycelium directly and their effect on the formation of oospores is unknown. The object of this study was to investigate the suppression traits of systemic triazole fungicide difenoconazole that controls a broad spectrum of foliar, seed and soil-borne diseases caused by Ascomycetes, Basidiomycetes and Deuteromycetes (Dahmen and Staub, 1992; Kopacki and Wagner, 2006; Pobedinskaya et al., 2012). Difenoconazole is not recommended against late blight on potato or tomato for plants or seeds treatment and we have not found in literature any studies concerning activity of difenoconazole against Phytophthora infestans in vitro or in planta. Difenoconazole is widely used for treatment of potato and tomato plants against early blight (alternariosis). Because of early and late blight often come together on potato it is practically important to estimate the impact of difenoconazole application against late blight pathogen also.

It was already investigated that leaves of the same plant were variable in their resistance to P. infestans: apical leaves more resistant then basal leaves of the same plant at the same time (Visker et al., 2003). Basing on this concept we can suppose the differentiation of the tiers of the plant regarding the oospore production. Therefore, the study of the formation of oospores in leaves of different tiers of potato plant was performed.
MATERIALS AND METHODS

Five isolates of *P. infestans* with different mating types were isolated from potato leaves, collected in Moscow, Ryazan, and Leningrad regions. Each isolate was cultivated in three different Petri dishes to study the dependence of colony growth on concentration of fungicide. A block of colonized agar was placed onto the center of Petri dishes with hard oat medium of four gradual concentrations of fungicide: 0.1; 1.0; 10.0 and 100.0 mg/l. The medium without the fungicide served as a control. Two perpendicular diameters of each colony measured when diameter of control colony was 70-80% from radial size of Petri dish. After the measurements average diameter for each isolate was calculated.

The oospore formation was investigated on oatmeal agar (30 ml per dish) with different gradual concentration of difenoconazole 0.1; 1.0; 10.0; 100.0 mg/l and free medium as a control. Every two blocks of colonized agar with pairs of isolates of different mating types were placed onto the medium at 5 cm from each other. The incubation temperature was 18°C to provide the optimal growth condition for *P. infestans*. Additionally the extreme temperature conditions (25°C) for *P. infestans* growing and oospores formation was tested. Both experiments were processed during 20 days. After the incubation medium with oospores was resuspended in 30 ml of distilled water to investigate the samples under the microscope. Each sample was examined with 180 eyeshots (united probe of 3 Petri dish for each of 3 tested pairs of isolates were used). Concentration of oospores was calculated as number of units per 1 µl of the medium.

Cultivars Vektor belorussky and Briz were used to study the oospore formation in vivo. The three combinations of strains were set and tested on medium and only single pair of strains with different mating types, that produced maximal amount of oospores after crossing on the agar media, was examined on leaves. Leaves were collected from the middle tier of the plant. The primary leaves were weighted and scanned on the gridded paper plate with a camera to estimate their volume and size. Scaling leaves were placed on the sterile water (25 ml) in Petri dish keeping underside down. Then they were inoculated with single drop (10 µl) of zoosporangium mixture which was the washout of 7 days long separately incubated isolates of both mating types A1 and A2. Concentration of inoculums was 5-7 zoosporangium per eyeshot with optical magnification 80×. In 6 days after inoculation difenoconazole was added to water of all samples excepting control to achieve final concentration 10 and 100 mg/l. Each sample combined 3 potato leaves. After 20 days of incubation with 18°C each sample (3 leaves) was homogenized in grinder with 6 ml of distilled water to make a subsample for microscopy measurement. The average number of oospores (each sample was examined with 180 eyeshots) was calculated for 1 mm² of leaf tissue.

The primary leaves from different tires of potato plants were collected to estimate their potential for source of oospores. Three leaves were taken from the low tier (4 levels of basal leaves), three from the upper (4 apical levels) and three from the middle. Six cultivars were tested: early Sandrin, Zorachka, Uladar, Oziris, early maincrop Ilyinskiy, maincrop Yanka. Each primary leaf was inoculated with single drop of zoosporangium mixture of A1 and A2 mating types. Incubation and assessment were carried out following the methods describing before. Each cultivar was examined with 180 eyeshots from three united simple leaves subsamples.

Calculation of the confident interval of a mean (µ) was done as follows:

\[
\bar{X} - t \frac{s}{\sqrt{n}} \leq \mu \leq \bar{X} + t \frac{s}{\sqrt{n}}
\]

where \(s\) is the standard deviation, \(n\) is the number of observations, \(t\) constant of a t-test for the significance level 0.05.

The Student’s t-test was used to analyze differences between trials. All calculations were done in programs Excel 2007 and Origin 3.1 (t-test for two populations).

RESULTS

Neutral effect of difenoconazole on radial growth of *P. infestans* was registered. There was no significant influence of difenoconazole on the radial growth of *P. infestans* colony, very small inhibition occurred only at high concentration of fungicide (100 mg/l) (Fig. 1).

![Fig. 1. Growth rate of P. infestans colony in the medium with different concentrations of difenoconazole (+18°C). Error bars represent the confident interval of a mean for the significance level 0.05. Values followed by the same letter are not significantly different (P > 0.05).](image-url)
Suppression effect of difenoconazole on the oospore formation by *P. infestans* in the medium was found. The positive temperature dependence of this suppression effect was detected. Lower concentration of fungicide (0.1 mg/l) was sufficient to inhibit the oospore formation statistically at extreme for *P. infestans* growing meaning of temperature (25°C), comparing to higher concentrations (more than 10 mg/l) which triggered the same process at optimal conditions (18°C) (Fig. 2, Fig. 3).

Experiments with the separated potato leaves demonstrated suppression effect of difenoconazole on the oospore formation by *P. infestans* starting from concentration 10 mg/l (Fig. 4). The dependence of inhibition efficiency and concentration of fungicide was positive with maximum suppression of the oospore producing at 100 mg/l. We obtained the same rate of inhibition on both tested cultivars Briz and Vektar belorusky, which maturing and resistance to late blight were different. There were 14.0 and 31.5 oospores per mm² in the leaves of control plants (free of fungicide) for the cultivars Briz and Vektar belorusky, respectively. At concentration 10 mg/l there were 10.2 and 22.4 oospores per mm² in the leaves and at concentration 100 mg/l there were 5.2 and 12.8 oospores per mm² in the leaves for cultivars Briz and Vektar belorusky, respectively. Statistically evident was only differences between the oospore formation in the leaves at concentration 0 and 100 mg/l; 10 mg/l and 100 mg/l in the cultivar Vektar belorusky and between 0 and 100 mg/l in the cultivar Briz (Fig. 4).

The results of the tests of the infected leaves from the different tiers indicated their different potential as a source of oospores. In laboratory conditions the higher production of oospores was detected in the leaves from the middle and low tiers rather than from top. There was no difference between the rates of infection from the middle and low tiers for all the tested cultivars except Yanka (Fig. 5). The lowest concentrations of oospores were observed in the inoculated apical leaves. Therefore to examine the effect of difenoconazole we used the leaves from the middle tier.

There was significant variation of occurrence of oospores in the leaves among the cultivars. The maximum of oospores was formed in the leaves of the middle and low tiers of the cultivars Sandrin, Zorachka, Uladar, Oziris, and Ilyinskiy. Lower amount of oospores was observed in the cultivar Yanka. The occurrence of oospores between the top and low tiers was statistically different for all cultivars. The maximum of oospores from the top tiers was revealed in the leaves of cultivar Uladar. The decreasing of concentration in the top tier was observed in accordance with the following list of the cultivars: Uladar, Zorachka, Oziris, Yanka, Ilyinskiy, and Sandrin.

**DISCUSSION**

Difenoconazol widely used for the protection of potato and tomato. Concentration of difenoconazole in the liquid at the treatment of vegetative plants is 190-620 µg/ml. When injected into the plant concentration of the fungicide in the liquid decreases in several times. Difenoconazole is effective against *Alternaria* and other pathogens related to Ascomycetes fungi in very low concentration (Dahmen and Staub, 1992; Kopacki and Wagner, 2006; Pobedinskaya et al., 2012). Regarding this study low concentration of difenoconazole (less than 100 µg/ml) practically does not inhibit the mycelium growth of *P. infestans*. However, concentration of 10 µg/ml and above effectively
inhibited the formation of oospores in culture medium and in the leaves.

The inhibition of the oospore formation could be realized with following mechanism. Difenoconazole is a triazole fungicides, that acts by inhibition of C14-demethylase involving in ergosterol biosynthesis. Sterols are synthesized by many fungi, plants and other organisms to assimilate in cell membrane as structure blocks. Oomycetes are not able to synthesize their own sterols but they obtain the alien molecules of sterols from the host plant and modify them. Fungi from Phytophthora genus can survive and grow without any sources of sterols but the production of oospores is totally blocked (Elliott et al., 1966). Isofucosterol and \( \beta \)-sitosterol are precursors of sterols, when added to medium for oomycete colony they can stimulate the oospore formation (Knights, 1965). We speculate the targets of difenoconazole are sterol precursors. Using the oat-meal medium rich with \( \beta \)-sitosterol and isofucosterol (Knights, 1965) to investigate the effect of difenoconazole we observed the inhibition of the oospore formation instead of stimulation providing with precursors of sterol.

As it was shown before the leaves from the different tiers of the potato plants were various in resistance to late blight. The rate of resistance gradually increased up to the top of the plant but it did not depend on leaf freshness, age of the plant, and the potato cultivars (Visker et al., 2003). However there was no information about the oospore formation from the tiers. Our study revealed the gradient of the oospore formation from the tiers which should be taken into account during the sampling, processing the measurements and comparing the research data. The highest spore production was detected in the leaves of the middle and low tiers for most of the potato cultivars. The oospore formation in the top leaves was statistically less.

In the field the oospore formation in the mid- and bottom leaf levels usually relates to the following microclimate conditions: high humidity, low isolation, and reducing the day temperature change (Harrison, 1992). The techniques which deliver a fungicide directly into the middle and low tiers of the potato plant should be exploited to decrease the oospore formation. Among them the turbulent sprayers are suggested to be rather useful. Systemic fungicides are also recommended because of spreading through the plant and targeting the untreated loci. Effectiveness of systemic fungicide difenoconazol in this case was demonstrated here.

For the potato plants difenoconazole is traditionally applied against early blight, caused by Alternaria. Early blight and late blight in the field often occur at the same time. The application of difenoconazole controls the development of Alternaria effectively, and significantly reduces the number of produced oospores of P. infestans. This improves the phytosanitary situation on the field, prevents the increase of the soil inoculum and the appearance of highly aggressive and fungicide-resistant strains as a result of hybridization.

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REFERENCES


