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# *In Vitro* and *In Vivo* Toxicity of Several Fungicides and Timorex Gold Biofungicide to *Pythuim aphanidermatum*

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**SUMMARY**

A survey of *in vitro* and *in vivo* sensitivity of *Pythuim aphanidermatum* to several commercial fungicides and a biofungicide was undertaken. An isolate of *P. aphanidermatum* pathogenic to pepper was collected from a naturally infested greenhouse soil from Smederevska Palanka, Serbia. The *P. aphanidermatum* isolate was sensitive to all tested products. The obtained $EC_{50}$ values were as follows: 10.21 mg/l for propamocarb-hydrochloride, 302.65 mg/l for fosetyl-Al, 11.18 mg/l for mancozeb, 1.27 mg/l for mefenoxam, 0.05 mg/l for azoxystrobin, and 175.33 mg/l for tea tree oil. Under greenhouse conditions, fosetyl-Al was the most efficient fungicide among the tested substances (97.5%). The biofungicide tea tree oil (Timorex Gold) (35.0%) exhibited the lowest efficacy among the tested materials, but it was still significantly better than the untreated control plot. The efficacies of propamocarb-hydrochloride (Previcur 607 SL), mancozeb (Mankogal 80 WP), azoxystrobin (Quadris) and mefenoxam (Ridomil gold 480 SL), were 72.5%, 77.5%, 57.5% and 75.0%, respectively.

**Keywords:** *Pythium aphanidermatum*; Sensitivity; *In vitro*; *In vivo*; Fungicides; Biofungicide

## INTRODUCTION

Serbia has 19.600 hectares of pepper fields with a slight tendency of decline at an average rate of 0.3% per year. The average yield of peppers is 7.2 tons per hectare, which is three times less than the European average (Privredna komora Srbije, 2013). Adverse environmental conditions, such as high temperatures in July and August with low relative humidity and minimum amount of precipitation during that period of vegetation affect pepper yields. However, besides abiotic factors and inadequate growing technology, pepper production is also threatened by many pathogens. One of the most important, which threatens plants at the earliest stages, are species of the genus *Pythium*. There are over 200 species of that genus that have been recognized as pathogens of more than 270 plant species (Dick, 1990). These pathogens cause seed and seedling diseases in bedding plants and greenhouse-grown transplants (Abbasi and Lazarovits, 2006). The roots

of mature plants may also be attacked. *Pythium* species that cause damping off diseases occur primarily in cold and wet soils where young seedlings of directly seeded crops may be killed before or soon after their emergence. Considering the fact that young plant tissue is very sensitive to pathogens, it is necessary to apply some control measures (substrate disinfection, fungicide treatment, etc.). Methyl bromide has been widely used for soil disinfection, but it is planned to be withdrawn by the year 2015 (in developing countries) because of its harmful impact on human health and the environment (Watson et al., 1992). In developed countries the ban on methyl bromide has been in force since 2005. Chemical treatment of soil is the most common means of controlling *Pythium* sp. on pepper. Fungicides commonly used to control *Pythium* sp. on pepper include propamocarb-hydrochloride, fosetyl-Al, metalaxyl and azoxystrobin (Tomlin, 2009; Janjić and Elezović, 2010). However, these fungicides sometimes do not show satisfactory efficacy because of the pathogen's ability to survive in the environment for a long time and spread by way of various agricultural practices.

Numerous research studies have focused on finding alternative ways to control damping off in pepper. A significant amount of research work is currently focused on the use of beneficial microorganisms and essential oils in the control of *Pythium sp.* (Moulin et al., 1996; Mao et al., 1997; Lewis and Larkin, 1998; Chatterton et al., 2004).

Tea tree oil is an essential oil obtained by steam distillation of the Australian plant *Melaleuca alternifolia* (Maiden and Betche) Cheel. It contains over 100 components, mostly monoterpenes, sesquiterpenes and their alcohols (Brophy et al., 1989). The main active components of tea tree oil are terpinen-4-ol (42%), a-terpineol (3%) and 1.8 cineole (2%) (Hart et al., 2000). The oil is an effective antiseptic, fungicide and bactericide (Carson et al., 2006). A new formulation, Timorex Gold, containing 23.8% of tea tree oil, is effective against a broad spectrum of pathogens of various vegetables, herbs, field crops, fruit trees and grapevines, while causing no phytotoxic effects (Reuveni et al., 2006). The mode of action of tea tree oil is not clearly understood, but it acts as a protector against a wide range of fungi by inhibiting spore germination and mycelial growth. In yeast cells and isolated mitochondria, α-pinene and β-pinene destroy cellular integrity, inhibit respiration and ion transport processes and increase membrane permeability. Timorex Gold has been tested as a biofungicide against a wide range of phytopathogens but tests against *Pythium* species have been scarce (Reuveni et al., 2006).

The objective of this study was to evaluate *in vitro* sensitivity of a *Pythium* sp. isolate originating in Serbia to several commercial fungicides and tea tree oil, and their biological efficacy in controlling damping off disease of pepper in a greenhouse.

## MATERIAL AND METHODS

### *Pythium aphanidermatum* isolate and preparation of inoculum

*P. aphanidermatum* pathogenic to pepper was isolated from a naturally infested greenhouse soil originating from Smederevska Palanka, Serbia. The identity of the isolate was confirmed by polymerase chain reaction (PCR) using universal primers (White et al., 1990) and their morphological traits according to Waterhouse and Waterston (1966).

Infested wheat seed (hard red winter wheat) was used as an inoculum source. A mixture of 25 ml of deionized water and 20 g of wheat seed was allowed to soak for 24 h in each of two 250-ml flasks with the isolate. The flasks were then autoclaved twice on two consecutive days. Each flask was inoculated with five 5-mm disks from a 2-day-old culture grown on PDA (potato dextrose agar) media. The flasks were incubated for 2 to 4 weeks in the dark at 25°C and shaken periodically to ensure uniform growth of inoculum (Chellemi et al., 2000).

### Crop protection products

Commercial formulations of fungicides were used as active ingredients (a.i.), respectively: propamocarb-hydrochloride, provided by Bayer Crop Science, Serbia, (Previcur 607-SL, 722 g/l), fosetyl-Al (Aliette 80-WP, 800 g/kg, Bayer Crop Science), mancozeb (Dithane DG neotec, WG, 750 g/l, Dow AgroScience), azoxystrobin (Quadris, 250 g/l, SC, Syngenta Agro), mefenoxam (Ridomol Gold 480 SL, 480 g/l, Syngenta Agro), and tea tree oil (Timorex Gold, 23.8%, EC, Stockton Agrimor).

A set of stock solutions for each fungicide was made using sterile distilled water. Freshly-made stock solutions were prepared to give specific concentrations of each active ingredient in ml/l. Volumes of stock solution were added to molten (50°C) PDA prior to pouring, thereby producing active ingredient concentrations ranging from 0.5 to 1000.0 mg/l (Locher and Lorenz, 1991).

### *In vitro* tests

The *P. aphanidermatum* isolate grown on PDA medium amended with the fungicides: propamocarb-hydrochloride, fosetyl-Al, mancozeb, azoxystrobin, metalaxyl, and the biofungicide based on tea tree oil, was used for sensitivity tests (Table 1). Based on preliminary results, the following concentrations were selected for further study: 3.9, 7.81, 15.6, and 31.2 mg/l of propamocarb-hydrochloride; 250, 350, 500 and 700 mg/l of fosetyl-Al; 6.25, 12.5, 25 and 50 mg/l of mancozeb; 0.006, 0.0125, 0.025, 0.05 and 0.1 mg/l of azoxystrobin; 0.5, 1, 5, 10 and 100 mg/l of mefenoxam; and 62.5, 125, 25, 500 and 1000 mg/l of tea tree oil. Control plates were not amended with fungicides. Tests for each isolate were replicated three times per each concentration of each fungicide. Mycelial plugs of 2 days old culture (10 mm in diameter) were removed from the margins of colonies grown on PDA medium, placed upside down on the fungicide-amended and fungicide-free PDA media in Petri dishes, and incubated at 20°C. After 2 days, colony diameter of each isolate was measured in two directions (minus the diameter of inoculation plug) and the percent inhibition (PI) of each fungicide rate was calculated using the formula below:

$$\text{percent inhibition} = [(a - b) / a] \times 100$$

where a = colony diameter of control plate and b = colony diameter of fungicide-amended plate. The 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).

### Greenhouse assays

Pots (10 cm in diameter and 5 cm in height) were filled with 250 ml sterile growth substrate (Floragard@, Germany) and each planted with 10 pre-germinated seeds of pepper (cv. California Wonder). The pots were held for 15 days at 25°C (24-27°C) in dim light to ensure slight etiolation of the seedlings (4000-6000 lux with a 15 h photoperiod). The pots were regularly watered to ensure good development of seedlings (EPPO, 2004).

On the 15th day, the pots were drenched with water (to give 100% of water-holding capacity of the sterile substrate). Sixty ml of inoculum (mixtures of inoculated wheat and sterilized substrate) was added to each pot around plant collars. It is recommended that the humidity of this soil should be at 70-80% of its water-holding capacity. After the addition of inoculum, the pots were placed at 19-21°C under moderate light (6000-8000 lux with a 15 h photoperiod). Seedlings inoculated with the isolate and watered with 60 ml of sterile distilled water served as the positive control. The tested products were applied by drenches immediately after inoculation at the dosage specified for the intended use (Table 2). The dosage is expressed as a concentration (%) and a volume of drench per unit area, and the appropriate calculated dose of product was applied per pot in 60-100 ml water (according to moisture condition of the soil in pots at the time of treatment). The experimental design was a complete randomized block with five replicates per treatment and one pot with 10 seedlings per replicate. The experiment was conducted twice (EPPO, 2004).

The seedlings were scored as either healthy or infected 7 days after fungicide application. Symptoms ranged from brown necrotic lesions at the collar to collapse and death (EPPO, 2004).

The efficacy was evaluated using Abbott's formula. The data were analyzed separately for each trial using ANOVA and the means were separated by Duncan's multiple range test.

## RESULTS

### *In vitro* tests

Sensitivity of the *P. aphanidermatum* isolate to the tested fungicides and tea tree oil is shown in Table 1. Among all tested products, azoxystrobin exhibited the greatest toxicity. The tested *Pythium* sp. isolate was capable to grow well at 0.006 mg/l azoxystrobin concentration, but it was severely inhibited at 0.0125 mg/l and higher concentrations (Figure 1). The calculated $EC_{50}$ value for inhibition of hyphal growth was 0.05 mg/l. The *P. aphanidermatum* isolate also showed a high susceptibility to metalaxyl ($EC_{50}$=1.27 mg/l). Its hyphal growth was severely inhibited at 1.0 mg/l or higher concentrations (Figure 2).

Fosetyl-Al exhibited the lowest toxicity to the *P. aphanidermatum* isolate ($EC_{50}$=302.65 mg/l). The tested isolate was capable of growing at 250 mg/l, but was severely inhibited at 500 mg/l or higher concentrations (Figure 3). Propamocarb-hydrochloride and mancozeb showed similar toxicity (Figure 4 and Figure 5). Their $EC_{50}$ values were 10.21 and 11.18 mg/l, respectively.

**Table 1.** *In vitro* sensitivity of *P. aphanidermatum* to fungicides and tea tree oil

| Fungicide | $EC_{50}$ (mg/l) | | b | |
|---|---|---|---|---|
| | Value | Range* | Value | Range* |
| Propamocarb--hydrochloride | 10.21 | 8.62-12.01 | 1.88 | 1.67-2.09 |
| Fosetyl-Al | 302.65 | 260.73-337.15 | 3.07 | 2.65-3.49 |
| Mancozeb | 11.18 | 9.46-12.9 | 2.29 | 2.06-2.52 |
| Azoxystrobin | 0.05 | 0.04-0.06 | 1.33 | 1.18-1.48 |
| Mefenoxam | 1.27 | 0.64-2.1 | 0.56 | 0.48-0.64 |
| Tea tree oil | 175.33 | 132.46-201.57 | 2.29 | 2.06-2.51 |

$EC_{50}$ - Fungicide concentration which inhibits mycelial growth by 50%; *95% confidence interval (P=0.05).

The tested *P. aphanidermatum* isolate demonstrated an ability to tolerate tee tree oil at higher concentrations than all other fungicides except fosetyl-Al. The isolate was capable to grow at 125 mg/l, but it was severely inhibited at 500 mg/l or above (Figure 6). The obtained $EC_{50}$ value was 175.33 mg/l.

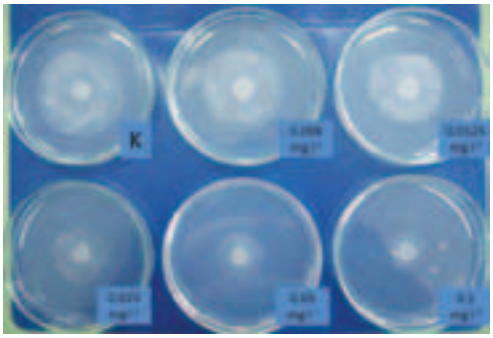


**Figure 1.** Growth of *P. aphanidermatum* on PDA amended with azoxystrobin

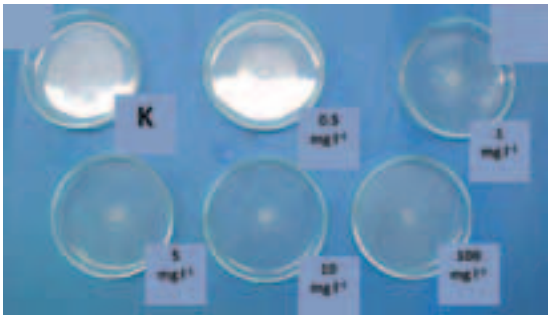


**Figure 2.** Growth of *P. aphanidermatum* on PDA amended with metalaxyl

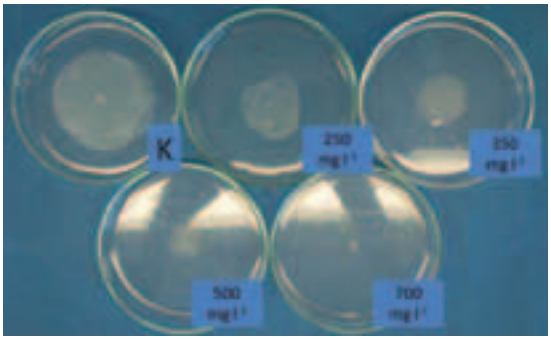


**Figure 3.** Growth of *P. aphanidermatum* on PDA amended with fosetyl-Al

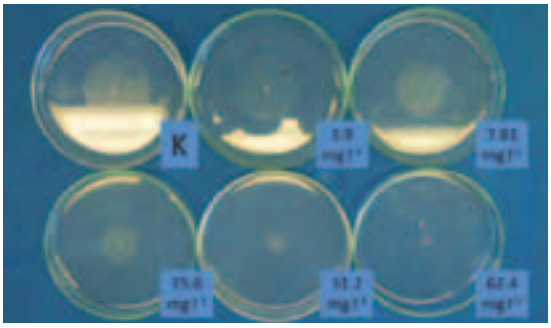


**Figure 4.** Growth of *P. aphanidermatum* on PDA amended with propamocarb-hydrochloride

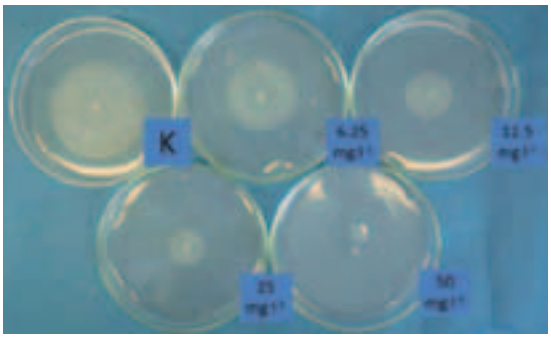


**Figure 5.** Growth of *P. aphanidermatum* on PDA amended with mancozeb

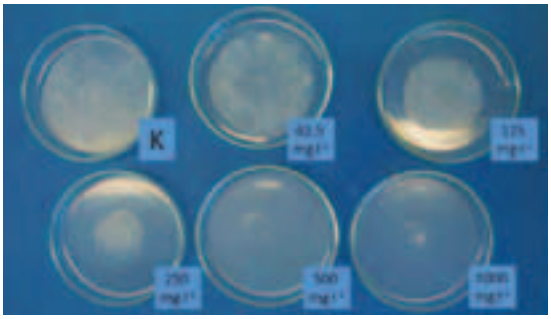


**Figure 6.** Growth of *P. aphanidermatum* on PDA amended with tea tree oil

### Greenhouse assays

Table 2 summarizes data for the average number of diseased pepper plants and efficacy of the tested fungicides and tea tree oil (Timorex Gold). All pepper plants were diseased (Ms=10.0) in inoculated and untreated control plots. Among the tested conventional fungicides and the biofungicide, Aliette flash (97.5%) showed the highest efficacy. There was a statistically significant difference in the efficacy between Aliette flash and all other tested products. The biofungicide Timorex Gold (35.0%) exhibited the lowest efficacy among all tested materials. The efficacy of the fungicides Previcur 607 SL, Mankogal 80 WP, Quadris and Ridomil gold 480 WP was 72.5%, 77.5%, 57.5% and 75.0%, respectively.

**Table 2.** *P. aphanidermatum* – Average number of diseased pepper plants and fungicides and biofungicide efficacy

| Treatment | Rate (%) | Ms[1] | Sd[2] | Efficacy (%) |
|---|---|---|---|---|
| Previcur 607 SL | 0.15 | 2.8 b* | 1.7 | 72.5 |
| Aliette flash | 0.5 | 0.3 a | 0.5 | 97.5 |
| Mankogal 80 WP | 0.25 | 2.3 b | 0.5 | 77.5 |
| Quadris | 0.075 | 4.3 b | 2.2 | 57.5 |
| Ridomil gold 480 SL | 0.03 | 2.5 b | 1.3 | 75.0 |
| Timorex Gold | 1.0 | 6.5 c | 1.3 | 35.0 |
| Control | – | 10.0 d | 0.0 | – |

[1]Average number of diseased plants; *Mean values in columns followed by different letters are significantly ($p<0.05$) different according to Duncan's test; [2] Standard deviation

## DISCUSSION

Data in the present study showed that the used *P. aphanidermatum* isolate was sensitive to the tested fungicides. However, relevant $EC_{50}$ values differed depending on fungicide. Fosetyl-Al showed the lowest toxicity of all tested products (302.65 mg/l). The $EC_{50}$ values recorded in our experiments were similar to those reported by Fenn and Coffey (1984). They found that mycelia of four *Pythium* species were inhibited when corn meal agar (CMA) was amended with phosphorous acid at concentrations of 276 and 552 mg/l. Although fosetyl-Al and its breakdown product phosphorous acid are not always more active against *P. aphanidermatum in vitro*, they are much more selective for this pathogen *in vivo* (Erwin and Ribeiro, 2005). In our trials we also noticed that commercial products based on fosetyl-Al (Aliette) exhibited the greatest efficacy in controlling *P. aphanidermatum* in greenhouse assays, compared to the other tested materials. The ability of phosphonates to alter the pathogen's metabolism in such a way that a more rapid and more effective defense response can be mounted by the host is one of the explanations (Erwin and Ribeiro, 2005). Also, these alterations involve a reduction in the amount of suppressor molecules present on the pathogen surface, or an increase in the amount of elicitor molecules exposed to receptors on the host cells, or both. These changes are not inhibitory to the pathogen *in vitro*, but lethal in the presence of an active host defense system (Erwin and Ribeiro, 2005; Abbasi and Lazarovits, 2006).

In our study, azoxystrobin exhibited the greatest toxicity to the used isolate of *P. aphanidermatum* ($EC_{50}$=0.05 mg/l). However, Wheeler et al. (2005) reported that *Pythium* species isolated from diseased peanut pods were much more susceptible to mefenoxam than to azoxystrobin. The $EC_{50}$ values for mefenoxam and azoxystrobin obtained in that study ranged from 0.001 to 0.270 mg/l, and from 1 to 103 mg/l, respectively. The fact that some fungi species, even within the same genus, use an alternative respiratory pathway that can interfere with the activity of azoxystrobin in a petri dish assay is a possible explanation. Wheeler et al. (2005) added that these results could not be compared directly to assays in which salicylhydroxamic acid is used to inhibit *Pythium* from using an alternative respiratory pathway. The efficacy of the conventional fungicide Quadris (azoxystrobin) was insufficient in greenhouse tests (57.5%).

As a member of the carbamate group of fungicides, propamocarb-hydrochloride is highly specific to fungi from the class Oomycetes. The biological mode of action has not been elucidated but a disruption of cellular membrane has been observed. The biological activity of propamocarb-hydrochloride is relatively low compared to the other semi-systemic fungicides in rate comparisons, and large amounts must be applied for comparable activity (Stein, 2002). Some earlier *in vitro* tests had indicated that the $EC_{50}$ of propamocarb-hydrochloride ranged from 0.5 to 10 mg/l for *P. aphanidermatum*, *P. splendens*, *P. irregulare*, *P. ultimum*, and *P. arrhenomanes* (Papavizas et al., 1978; Moorman and Kim, 2004). In the present study, the tested *P. aphanidermatum* isolate also exhibited similar sensitivity to propamocarb-hydrochloride in *in vitro* trial ($EC_{50}$=10.0 mg/l). However, Moorman and Kim (2004) obtained *P. ultimum* and *P. irregulare* isolates which exhibited resistance to propamocarb-hydrochloride in geranium seedlings ($EC_{50}\geq$1000.0 mg/l). The authors also emphasized that sensitivity of the tested isolates to propamocarb-hydrochloride *in vitro* was not a good predictor of *in vivo* sensitivity.

For mancozeb, the $EC_{50}$ was 11.18 mg/l. Similar values of $EC_{50}$ for mancozeb have been reported for Central African isolates of *P. aphanidermatum* (Suleiman, 2011). As a fungicide with multi-site contact activity (acts by disrupting lipid metabolism), mancozeb is generally considered as a low-resistance-risk fungicide with permanent good efficacy (FRAC, 2013).

The biofungicide Timorex Gold showed a much lower fungitoxic effect against the pathogen than the other fungicides tested in our study ($EC_{50}$=175.33 mg/l). The biological efficiency of tea tree oil in the greehhouse trial was also very low (35.0%). Since the biofungicide Timorex Gold has never been tested or applied against pathogens of pepper in Serbia before, the results of our sensitivity tests and the biological efficiency against *P. aphanidermatum* isolates are initial findings.

*P. aphanidermatum* is an important pathogen of pepper, particularly in its early stages of growth. An understanding of the sensitivity of *P. aphanidermatum* to commercial fungicides and some alternative natural compounds is important for damping off management in fields where pathogenic *Pythium* spp. are present. Our study also suggested that continuous monitoring of fungicide resistance in *Pythium* spp. field populations is very important for development of damping off management strategies.

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# *In vitro* i *in vivo* toksičnost fungicida i biofungicida za *Pythuim aphanidermatum*

**REZIME**

Ispitivana je osetljivost *Pythuim aphanidermatum* u *in vitro* i *in vivo* uslovima na odabrane fungicide i biofungicid. Izolat *P. aphanidermatum* je izolovan iz zemljišta iz staklenika gde je prethodno uočeno poleganje biljaka paprike (Smederevska Palanka, Srbija). Izolat *P. aphanidermatum* je ispoljio osetljivost na sve testirane preparate. Dobijene su sledeće $EC_{50}$ vrednosti: propamokarb-hidrohlorid 10,21 mg/l, fosetil-Al 302,65 mg/l, mankozeb 11,18 mg/l, mefenoksam 1,27 mg/l, azoksistrobin 0,05 mg/l i ulje čajnog drveta 175,33 mg/l. U uslovima staklenika, fosetil-Al (Aliette flash) je ispoljio najveću efikasnost (97,5%) u odnosu na sve ispitivane preparate. Biofungicid na bazi ulja čajnog drveta (Timorex Gold) je ispoljio najmanju efikasnost (35,0%) u odnosu na druge ispitivane fungicide ali je intenzitet zaraze statistički značajno bio manji u poređenju sa inokulisanom i netretiranom kontrolom. Efikasnost preparata na bazi propamokarb-hidrohlorida (Previcur 607 SL) bila je 72,5%, mankozeba (Mankogal 80 WP) 77,5%, azoksistrobina (Quadris) 57,5% i mefonoksama (Ridomil gold 480 SL) 75,0%.

**Ključne reči:** *Pythium aphanidermatum*, osetljivost, *in vitro*, *in vivo*, fungicidi, biofungicid