In vitro sensitivity of *Colletotrichum acutatum* isolates from strawberry to tebuconazole, prochloraz, fludioxonil and thiophanate-methyl

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Abstract:

The objective of this study was to determine the *Colletotrichum acutatum* isolates sensitivity to several chemical compounds *in vitro* and to find their possibilities as a potential control agents for anthracnose disease on strawberry. *C. acutatum* J.H. Simmonds, a causing agent of anthracnose, is a very important pathogen of strawberry, which leads to devastating losses in its production. The pathogen is effectively controlled by the fungicides. Thus their application is necessary for achieving high yield and fruit quality. The sensitivity of 14 isolates of *C. acutatum*, collected from commercial strawberry plantations in Serbia, to four fungicides, was examined by an *in vitro* sensitivity assay. Based on the results of morphological, pathogenic and molecular characterization (up to the complex level), all 14 isolates were determined as *C. acutatum*. The commercial formulation of tebuconazole, fludioxonil, prochloraz and thiophanate-methyl were used for the sensitivity test. The mycelial growth assay method was used to investigate isolates sensitivity to fungicides. The tested isolates were very sensitive to prochloraz and fludioxonil, with mean EC_{50} values of 0.067±0.062 mg L^{-1} and 0.093±0.043 mg L^{-1}, respectively. Significantly higher mean EC_{50} values were observed for tebuconazole (1.473±0.878 mg L^{-1}) and thiophanate-methyl (1.718±1.592 mg L^{-1}). The toxicity of tested fungicides in the mycelial growth assay of *C. acutatum* isolates indicates the potential implementation of these fungicides in the protection programs against strawberry anthracnose disease.

Keywords: fungicide sensitivity; anthracnose; tebuconazole; prochloraz; fludioxonil; thiophanate-methyl

INTRODUCTION

The cultivated garden strawberry (*Fragaria ananassa* Duch.) is a very important berry crop with a major economic value worldwide. Turkey, Spain and Poland are the leading strawberry producers in Europe, with a total production of 415.150, 366.161 and 196.972 tons, respectively (Milivojević, 2018). According to the data obtained from the Statistical Office of the Republic of Serbia, strawberry was grown on over 6700 ha with a total production of 30.483 tons in Serbia in 2020.

There are many limiting factors responsible for the major losses in strawberry production. Strawberry anthracnose, caused by species from the *Colletotrichum* genus, is a very destructive and widespread disease of cultivated strawberries. *Colletotrichum* spp. contains an extremely large number of pathogens, and early studies reported *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *C. fragariae* A. N. Brooks and *C. acutatum* as the most common strawberry anthracnose pathogens (Freeman and Katan, 1997). Recent molecular methods for the identification of *Colletotrichum* species have demonstrated that these species are, in fact, "species complexes" composed of numerous diverse species (Damm et al., 2012). One of the most frequent species complex on strawberry, *C. acutatum* (teleomorph *Glomerella acutata* Guerber & J. C. Correll), infects other fruits (stone, pome and berry fruits),

grapes and vegetables (tomatoes, peppers), and many other crops (Howard et al., 1992; Bernstein et al., 1995; Smith, 2002). The pathogen is widespread in almost all areas of strawberry growing but primarily has tropical and subtropical distribution. All parts of the plant are affected, and the typical symptoms are flower blight, leaf spots, petiole lesions, crown rot, root rot and fruit rot (Haack et al., 2018). According to the findings of Ivanovic et al. (2007), fruit rot can cause over 80% strawberry yield losses in Serbia if conditions for disease development are favorable.

Effective control of *Colletotrichum* spp. can be achieved using a combination of chemical and non-chemical measures. The use of fungicides has major importance among other control methods, and successful protection highly depends on timely applications of multi- and single-site fungicides (Mertely et al., 2017a, b). Currently, there are no fungicides registered in the Serbian pesticide market for the strawberry anthracnose control, so chemical management of anthracnose is a challenge since farmers usually opt for the application of fungicides registered for other strawberry pathogens. Fungicides which are commonly used and highly effective for fruit rot disease control are azoxystrobin, pyraclostrobin and the combinations of fludioxonil+cyprodinil and boscalid+pyraclostrobin. Multi-site preventive fungicides, for example, captan, also provide sufficient control of *C. acutatum*, however, the weekly applications during the season are required since there is no curative effect (Mertely et al., 2017b). Generally, recent researchers suggested that *Colletotrichum* infection can be controlled by numerous fungicides: copper compounds, dithiocarbamates, phtalimides, triazoles and other chemicals like imazalil, prochloraz and fludioxonil (Cao et al., 2017; Gao et al., 2018; Baggio et al., 2018; He et al., 2019).

Differences in fungicide sensitivity among species from a single geographical location might be a result of fungicide selection pressure and variations, which are reflected in significant species heterogeneity within the complex itself. Therefore, knowing the fungicide sensitivity profile of species is important and necessary to create adequate protection programs and help farmers to apply only effective fungicides to overcome potential production problems (Dowling et al., 2020).

The objective of this study was to determine the *C. acutatum* isolates sensitivity to several chemical compounds *in vitro* and to find their possibilities as a potential control agents for anthracnose disease on strawberry.

MATERIALS AND METHODS

Fungal isolates

Colletotrichum acutatum researched in this study was isolated from anthracnose-symptomatic strawberry fruits, using a method given by Dhingra and Sinclair (1995). The samples were collected in 2019 from the strawberry production areas in the Rasina district in Serbia. Fruit fragments (1cm long) cut from the place between healthy and necrotic tissues were washed under running tap water for 10 minutes, surfaced sterilized with 5% sodium hypochlorite solution for 5 min, and then rinsed three times with sterile distilled water. The disinfested fragments were put on a blotting sheet for 20 minutes to dry. Thereafter, the fragments were moved to a Petri plate containing a combination of PDA and antibiotic media and incubated at 25°C for 10–14 days. Pathogen colonies with the typical morphological characteristics of *C. acutatum* were transferred to new dishes with a PDA medium. Based on the results of morphological, pathogenic and molecular characterization (up to the complex level), all 14 isolates were determined as *C. acutatum*.

Pathogenicity tests

Two representative isolates (C.a. 10 and C.a. 2) of *C. acutatum* were chosen for pathogenicity tests. Each isolate was tested on five fruits. The fruits were surface sterilized in 96% ethanol for 1 min, then rinsed in sterile distilled water and dried at 20°C. After that, the fruits were wounded with a sterile dissecting needle and inoculated with each isolate by applying fragments of the colony in the middle

of the injured fruits. Wounded but non-inoculated fruits were used as a negative control. The inoculated fruits were placed in a glass jar with a lid containing water-soaked paper wool and incubated at 25°C and a 12h photoperiod. Symptoms were evaluated 2–3 days after inoculation by a visual examination of necrotic surfaces. The experiment was repeated two times.

In vitro sensitivity to fungicides

Isolates' sensitivity to four selected fungicides was evaluated based on the mycelial growth assay method described by Zhang et al. (2012).

The commercial formulations of tebuconazole (Folicur 250 EW, 250 g a.i./L, Bayer, Germany), fludioxonil (Flux, 225 g a.i./L, Galenika-Fitofarmacija, Serbia), prochloraz (Mirage 45 EC, 450 g a.i./L, Adama Makhteshim Ltd., Israel) and thiophanate-methyl (Galofungin T, 450 g a.i./L, Galenika-Fitofarmacija, Serbia) were dispersed in sterile, distilled water to prepare stock solution series. Final concentrations of fungicides for the sensitivity test in PDA medium were: 0.1, 0.2, 0.5, 1, 5 and 10 mg a.i./l for tebuconazole, 0.02, 0.05, 0.1, 0.25 and 0.5 mg a.i./l for fludixonil, 0.01, 0.02, 0.1, 0.5, 1 and 5 mg a.i./l for prochloraz and 0.1, 1, 5, 50 and 500 mg a.i./l for thiophanate-methyl.

Mycelial plugs (10 mm in diameter) were cut from colony margins of 5-day-old cultures with a cork borer and transferred to Petri dishes with fungicide-amended PDA in three replications for each concentration. No fungicide was added to the control plates. The fungal colonies were incubated at 25°C for 10 days. When colonies reached 75% of the area in the control plate, colony radial growth (in mm) in all plates was measured. Measurements were performed in three directions in each Petri plate, and then the percent inhibition (PI) per each fungicide was calculated using the following formula:

$$(1)\ PI = \frac{a-b}{a} x100$$

where *a* was the colony diameter of control plates and *b* was the colony diameter of fungicide-amended plates.

The fungicide concentration that effectively inhibited radial fungal growth by 50% (EC_{50}) was determined for each isolate and fungicide. The probit analysis was used for the calculation of EC_{50} values. The resistance factor (RF) of each isolate was calculated by dividing the EC_{50} value of the isolate by the EC_{50} value of the most sensitive isolate in the study, where isolates were grouped as follows: sensitive (RF < 3), moderately resistant (RF = 4–20), resistant (RF = 20–100) and highly resistant (RF > 100) (Gouot, 1994; Dekker, 1995).

RESULTS AND DISCUSSION

Pathogenicity

Both tested isolates (C.a. 2 and C.a. 10) were able to infect their host. Inoculated strawberry fruits showed typical anthracnose symptoms and developed brown and necrotic lesions around the fruit wounds (Figure 1). *C. acutatum* was reisolated from the symptomatic fruit parts, according to Koch's postulates. No symptoms were observed on the negative control.

In vitro sensitivity of the studied isolates

The highest toxicity of tebuconazole was observed for isolate C.a. 10 (EC_{50}=0.791 mg L^{-1}) and the lowest for isolate C.a. 2 (EC_{50}=4.483 mg L^{-1}). The EC_{50} values for the remaining isolates were from 0.842 to 1.701 mg L^{-1}. RFs for tebuconazole for thirteen isolates were below 3, whereas C.a. 2 had RF of 5.7. The regression coefficients (*b*) for all tested isolates were below 1.64.

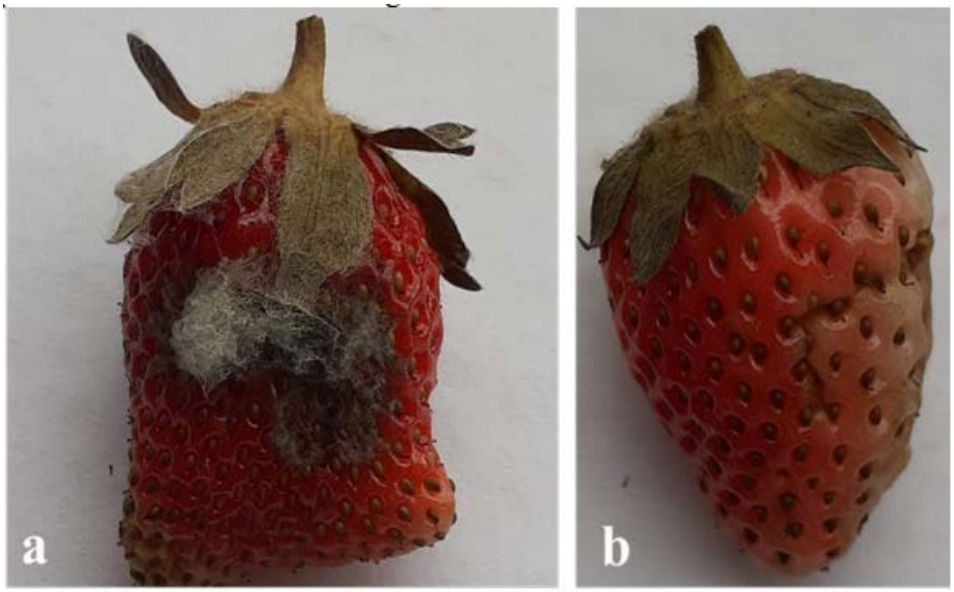

Figure 1.
The pathogenicity test of the selected C.a. 10 isolate; a strawberry fruit with an anthracnose symptom (a) and a negative control (b).

The studied isolates were very sensitive to prochloraz. Among 14 isolates of *C. acutatum*, the highest sensitivity was observed for isolate C.a. 16 (EC_{50}=0.020 mg L^{-1}), while C.a. 2 had the lowest sensitivity with the value of EC_{50}=0.272 mg L^{-1}. RFs for ten isolates were below 3, for one (C.a. 8), it was 3.1, while for three isolates (C.a. 2, C.a. 5, C.a. 7), RFs were between 4.2 and 13.6. The regression coefficients (*b*) were below 0.93.

All tested isolates expressed a relatively uniform response to fludioxonil (Figure 2), where EC_{50} values ranged from 0.030 to 0.184 mg L^{-1}. The highest toxicity of fludioxonil was observed for isolate C.a. 7 (EC_{50}=0.030 mg L^{-1}), while the lowest toxicity was determined for isolate C.a. 18 (EC_{50}=0.184 mg L^{-1}). RFs were below 3 for eight isolates, while the remaining six isolates had RFs between 3.8 and 6.1. The regression coefficient (*b*) ranged from 1.04 to 2.21.

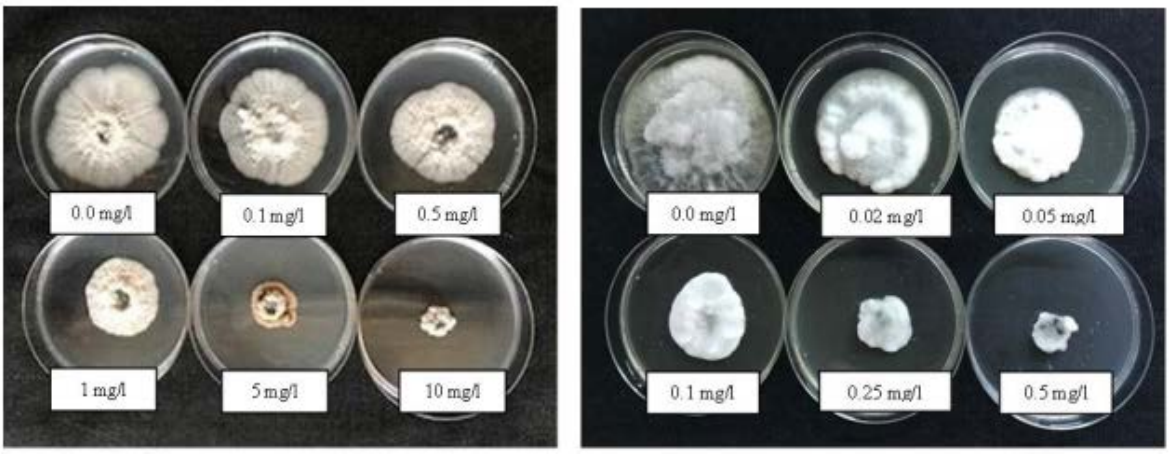

Figure 2.
Mycelial growth at concentrations of 0.0, 0.1, 0.5, 1, 5 and 10 mg L^{-1} for tebuconazole (left) and of 0.0, 0.02, 0.05, 0.1, 0.25 and 0.5 mg L^{-1} for fludioxonil (right).

Differences in the sensitivity of *C. acutatum* isolates to the examined fungicides are presented in Table 1 and Table 2.

Table 1. *In vitro* sensitivity of *C. acutatum* isolates to tebuconazole and prochloraz

	Tebuconazole			Prochloraz		
Isolate code	EC_{50} mg L^{-1}	*b*	RF	EC_{50} mg L^{-1}	*b*	RF
C.a. 15	0.842	1.14	1.1	0.031	0.71	1.6
C.a. 27	1.701	1.33	2.1	0.037	0.78	1.9
C.a. 16	1.023	1.30	1.3	0.020	0.74	1
C.a. 13	1.611	1.64	2	0.027	0.75	1.4
C.a. 18	0.990	1.24	1.2	0.058	0.61	2.9
C.a. 14	1.462	1.29	1.8	0.036	0.73	1.8
C.a. 2	4.483	1.01	5.7	0.272	0.75	13.6
C.a. 1	1.154	1.28	1.5	0.055	0.91	2.8
C.a. 8	1.501	1.26	1.9	0.061	0.82	3.1
C.a. 26	1.010	1.32	1.3	0.048	0.82	2.4
C.a. 4	1.310	1.42	1.6	0.053	0.93	2.7
C.a. 5	1.322	1.35	1.7	0.084	0.69	4.2
C.a. 10	0.791	1.15	1	0.035	0.85	1.8
C.a. 7	1.424	1.46	1.8	0.114	0.81	5.7

(b) – expressing the relative sensitivity of the isolates; (RF) – the resistance factor; it is calculated by dividing the EC_{50} value of the isolate by the EC_{50} value of the most sensitive isolate in the experiment.

The calculated EC_{50} for thiophanate-methyl ranged from 0.209 mg L^{-1} (C.a. 1) to 4.953 mg L^{-1} (C.a. 10). The EC_{50} for the other investigated isolates ranged from 0.367 mg L^{-1} to 3.878 mg L^{-1}. RFs for seven tested isolates were below 3, whereas for the other six, they were between 3.7 and 18.5, and for the remaining one (C.a. 10), it was 23.7. The regression coefficients (*b*) were below 0.90.

Numerous reports testify that *C. acutatum* affects a wide range of hosts (Damm et al., 2012). For farmers, the best management strategy in plant disease control depends on timed applications of preventive and systemic fungicides. This research examined *in vitro* sensitivity of *C. acutatum* isolates to four fungicides with different modes of action. Tebuconazole and prochloraz act by inhibiting C14-demethylation in the ergosterol biosynthesis (FRAC 3; G1), fludioxonil inhibits MAP/Histidine kinase in osmotic signal transduction (FRAC 12; E2), while thiophanate-methyl affects the inhibition of ß-tubulin assembly in mitosis (FRAC 1; B1).

Significant differences in the sensitivity of *Colletotrichum* to various fungicides are represented in several studies. Greer et al. (2011) state that *C. gloeosporioides* isolates are highly sensitive to benzimidazole fungicides, whereas isolates of *C. acutatum* are resistant to them. In this study, most of the isolates of C. acutatum were sensitive to the selected fungicides. The EC_{50} values were in the range from 0.030 to 0.184 mg L^{-1}, 0.020 to 0.272 mg L^{-1}, 0.791 to 4.483 mg L^{-1} and 0.209 to 4.953 mg L^{-1} for fludioxonil, prochloraz, tebuconazole and thiophanate-methyl, respectively. Therefore, prochloraz was the most toxic fungicide, with average EC_{50} values of 0.067±0.062 mg L^{-1}. Besides prochloraz, flu-

dioxonil was the fungicide which showed a strong inhibitory effect on mycelial growth, with a mean EC_{50} value of 0.093±0.043 mg L^{-1}. Comparing with other fungicides tested in this study, *C. acutatum* isolates showed less sensitivity to tebuconazole and thiophanate-methyl. Mean EC_{50} values were 1.473±0.878 mg L^{-1} for tebuconazole and 1.718±1.592 mg L^{-1} for thiophanate-methyl.

Table 2. *In vitro* sensitivity of *C. acutatum* isolates to fludioxonil and thiophanate-methyl

	Fludioxonil			Thiophanate-methyl		
Isolate code	EC_{50} mg L^{-1}	*b*	RF	EC_{50} mg L^{-1}	*b*	RF
C.a. 15	0.130	1.80	4.3	0.367	0.57	1.7
C.a. 27	0.085	1.16	2.8	0.387	0.58	1.8
C.a. 16	0.129	1.83	4.3	0.462	0.59	2.2
C.a. 13	0.123	1.98	4.1	0.374	0.57	1.8
C.a. 18	0.184	1.59	6.1	2.460	0.73	11.8
C.a. 14	0.079	1.04	2.6	0.328	0.57	1.6
C.a. 2	0.037	1.82	1.2	0.772	0.52	3.7
C.a. 1	0.084	1.88	2.8	0.209	0.54	1
C.a. 8	0.057	1.60	1.9	0.474	0.48	2.3
C.a. 26	0.134	1.94	4.5	3.066	0.59	14.7
C.a. 4	0.035	1.78	1.2	3.478	0.90	16.6
C.a. 5	0.076	1.90	2.5	3.878	0.79	18.5
C.a. 10	0.115	2.21	3.8	4.953	0.73	23.7
C.a. 7	0.030	1.67	1	2.840	0.81	13.6

(b) – expressing the relative sensitivity of the isolates; (RF) – the resistance factor; it is calculated by dividing the EC_{50} value of the isolate by the EC_{50} value of the most sensitive isolate in the experiment.

The results of this study for tested fungicides (except for thiophanate-methyl) are relatively similar to those reported by many other researchers for *in vitro* studies. Cao et al. (2017) determined the sensitivity of *Colletotrichum* species complexes to DMI fungicides, and the results showed that the mean EC_{50} value for prochloraz was 0.040 µg ml^{-1}. In the study conducted by Gao et al. (2018), where they investigated the sensitivity of 205 isolates of *C. acutatum*, EC_{50} values for fludioxonil were in the range from 0.011 to 0.080 µg ml^{-1} with a mean EC_{50} value of 0.031±0.057 µg ml^{-1}. According to the findings by He et al. (2019), the mycelial growth of different *Colletotrichum* species, including *C. acutatum*, was highly inhibited by tebuconazole, and EC_{50} values ranged from 0.36 to 0.48 mg L^{-1}. Baggio et al. (2018) reported that tested isolates of C. acutatum were insensitive to thiophanate-methyl, and EC_{50} values could not be determined. In their study, thiophanate-methyl was not able to inhibit the mycelial growth of *C. acutatum*, and the potential explanation they gave relates to the fact that the tested isolates were simply classified as belonging to the complex *C. acutatum* and were not determined further. In our study, isolates were also determined as a *C. acutatum* complex, but thiophanate-methyl had an inhibitory effect on tested isolates, which potentially indicates that these are different species within the complex compared to the previous research by Baggio et al. (2018). However, in our study, one isolate (C. a. 10) with RF value of 23.4 could potentially be characterized as resistant. However,

since we have done molecular identification up to the complex of *C. acutatum* (not further), we cannot claim with certainty that the resistance is present in the population.

The recent data show that *C. acutatum* species complex currently contains 41 species in total, which indicates the great variability in the sensitivity of *Colletotrichum* isolates to different fungicides. A potential solution for further investigations may be to use more precise molecular studies to distinguish the isolates, which may help explain the variations.

CONCLUSION

According to the obtained results, it can be concluded that tebuconazole, prochloraz, fludioxonil and thiophanate-methyl are effective against the mycelial growth of *C. acutatum*. The mycelium was highly inhibited by prochloraz and fludioxonil, while tebuconazole and thiophanate-methyl showed less inhibition in this study. Considering that the lack of registered plant protection products for controlling *C. acutatum* on strawberries in Serbia is an additional problem, these results indicate the potential integration of tested fungicides in the protection programs against strawberry anthracnose disease. Since this is preliminary research on fungicide sensitivity, further trials are needed to clarify the differences in the effects of these fungicides in the field conditions.

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Osetljivost izolata *Colletotrichum acutatum* sa jagode na tebukonazol, prohloraz, fludioksonil i tiofanat metil *in vitro*

Sažetak:

Cilj ovog istraživanja bio je da se ispita osetljivost izolata *Colletotrichum acutatum* na nekoliko različitih fungicida u uslovima *in vitro* i da se pronađe njihova potencijalna uloga u strategiji zaštite jagode od prouzrokovača antraknoze. *C. acutatum* J. H. Simmonds, prouzrokovač antraknoze, veoma je važan patogen jagode koji dovodi do ogromnih gubitaka u njenoj proizvodnji. Ovaj patogen se efikasno suzbija fungicidima, pa je njihova primena neophodna za postizanje visokog prinosa i kvaliteta plodova. U ovom istraživanju ispitivana je osetljivost 14 izolata *C. acutatum in vitro*, prikupljenih iz komercijalnih zasada jagode u Srbiji, na četiri fungicida. Svih 14 izolata determinisano je do nivoa kompleksa *C. acutatum* na osnovu morfoloških, patogenih i molekularnih karakteristika. Za test osetljivosti korišćene su komercijalne formulacije tebukonzola, fludioksonila, prohloraza i tiofanat-metila. Metoda praćenja porasta micelije na hranljivoj podlozi korišćena je za određivanje osetljivosti izolata na fungicide. Ispitivani izolati su bili veoma osetljivi na prohloraz i fludioksonil, sa srednjim EC_{50} vrednostima od 0,067±0,062 mg L^{-1} odnosno 0,093±0,043 mg L^{-1}. Značajno veće srednje EC_{50} vrednosti dobijene su kod tebukonazola (1,473±0.878 mg L^{-1}) i tiofanat-metila (1,718±1,592 mg L^{-1}). Toksičnost testiranih fungicida na porast micelije izolata *C. acutatum* ukazuje na mogućnost implementacije ovih fungicida u programe zaštite od prouzrokovača antraknoze na jagodi.

Ključne reči: osetljivost na fungicide; antraknoza; tebukonazol; prohloraz; fludioksonil; tiofanat-metil