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ANTHRACNOSE — A NEW STRAWBERRY DISEASE IN SERBIA AND ITS CONTROL BY FUNGICIDES

ABSTRACT: Anthracnose is a destructive disease of strawberry fruits in warm and continental climate. During 2004, in the vicinity of Valjevo, there were severe losses in two strawberry plantations due to fruit anthracnose. Two fungal isolates, GG-6A and GG-JUP were recovered from strawberry stolons, and fruits showing severe anthracnose symptoms. Based on morphological and pathological characteristics, and PCR analyses with specific primers of reference species, isolate GG-6A was identified as *Colletotrichum gloeosporioides*, and GG-JUP isolate as *C. acutatum.* This is the first identification of *C. acutatum* in strawberry in Serbia.

In order to control strawberry anthracnose, five fungicides and their combinations were applied four times during the flowering. The best fruit protection was achieved by fungicides Metiram + piraclostrobin (Cabrio top), Captan FL and Fludioksinil + ciprodinil (Swich). Less effective were Benomil (Benlate) and Krezoksim-metil (Stroby).

Pathogen is transmitted by planting material, so phytosanitary measures are extremely important in preventing the disease.

KEY WORDS: anthracnose, *Colletotrichum acutatum*, planting material, strawberry disease

INTRODUCTION

The strawberry (*Fragaria x ananassa* Duch.) production in Serbia, both in the field and under the plastic, is increasing. Deficiency of certified planting material is frequent, so the import is necessary. However, with importation of the planting material there are possibilities of introducing new strawberry diseases.

In two new strawberry plantations founded by the imported planting material, in the vicinity of Valjevo, a new disease was registered in 2004. The first visible symptoms of fruit rotting were in the maturity, and yield was reduced over 80%. In 2005 the appearance of the disease was mild, and in 2006 it was weak.

Anthracnose diseases of strawberry are caused by three fungal pathogens: *Colletotrichum acutatum* J.H. Simmonds, *C. fragariae* Brooks and *C. gloeosporioides* (Penz.) Penz & Sacc. (teleomorph *Glomerella cingulata* (Stoneham.) Schrenk & Spaulding). All three species incite diseases which cannot be distinguished in the field by symptoms alone (Peres et al., 2005). *C. fragariae* is most often associated with anthracnose crown rot of strawberry grown in hot, humid areas, such as the southern United States. *C. gloeosporioides* usually cause petiole and stolon lesions and crown rot on a strawberry, but may also produce fruit symptoms (Smith, 1998).

Anthracnose, caused by *C. acutatum* is responsible for the major losses in strawberry production worldwide. Fruit rot and flower blight are common symptoms in fruiting fields (Howard et al., 1992), whereas lesions on stolons, petioles and leaves are particularly harmful in plant nurseries (Freeman & Katan, 1997). *C. acutatum* was first described as a separate species by Simmonds (Simmonds, 1968). The teleomorph of the fungi was recently described as *Glomerella acutata* Guerber & Correll (Guerber & Correll, 2001).

None of the three mentioned anthracnose diseases have been registered on strawberry in Serbia even though *gloeosporioides* has been present for many years as the pathogen of sour cherry, apple and other plants (Ivanović & Ivanović, 2005).

In order to study the causal agent of strawberry disease in new plantations, in the vicinity of Valjevo, and to optimize the control strategy for the disease, this research has been carried out.

MATERIAL AND METHODS

Pathgen isolation and maintenance. Strawberry stolons and fruits from naturally infected cv. Favet were collected in January and May in 2005 respectively. Fruit lesions were clear, frequently coalesced and sporulated quickly, but stolon lesions were atypical, unclear. Both fruit and stolon lesions were cut in small sections, their surface was sterilised in 70% ethanol, cultured on a laboratory prepared potato dextrose agar (PDA), and kept under laboratory ambient conditions until colony was developed. Mycelial fragments were cut from the edges of the developed colonies, and transferred to a new PDA to get a pure culture. The cultures were stored on PDA slants at 10°C and transferred every 4 weeks. Two isolates, GG-6A and GG-JUP recovered from stolons and fruits, respectively, were chosen for further research. Reference isolates of *C. acutatum* (TUT 137A) and *C. gloeosporioides* (AVO 37 4B), used in this study, were acquired from V. Trkulja, Banja Luka, Republic of Srpska.

Colony growthrate assessment and spore dimensions. The effect of PDA, carrot agar (CA), and oatmeal agar (OM) media on the colony growth of two isolates GG-6A and GG-JUP, and reference isolates (TUT 137A and AVO 37 4B) were studied under ambient laboratory conditions. The diameter of colonies was measured after 9 and 15 days. Morphology of the colonies, the

occurrence of sectors, and the vegetative and reproductive structures were described after 14 days of incubation.

For conidial measurements, isolates were cultured under continuous fluorescent light for 3 days at room temperature on PDA, to promote sporulation. The spores were suspended in sterile water using a sterile needle. The length and width of too condition were measured, using measuring program IM 1000, and conidial shape was recorded at x400 magnification (10 x ocular, 40x objective) using bright field microscope (Leica DMLS).

Pathogenicity test. Strawberry fruits were inoculated with previously mentioned four isolates. Spores were produced on PDA media. Cultures were flooded with sterile distilled water and filtered through four layers of cheesecloth to remove mycelia. Conidia concentration was determined using a haemocytometer, and adjusted with sterile distilled water to 4×10^5 conidia per ml. Strawberry fruits of cv. Favet were, just before the ripening, inoculated by injecting 20 µl of conidial suspension. Control fruits were inoculated with 20 µm of sterile distilled water. Inoculated fruits were held on the top of the metal screen in plastic containers at 25 ± 2°C. In other to maintain RH, hot water was added to the bottom of each container which was then sealed tightly. Lesion diameter was measured for four days, after inoculation. The test was repeated two times.

Nucleic acid extraction and PCR. Nucleic acid extraction was performed following the protocol described by Day and Shattock (1997). Extracted nucleic acids were resuspended in TE buffer and maintained at –20°C. PCR was performed using ITS4 universal primer in pair with, in separated reactions, primer CgInt specific for *C. gloeosporioides*, and CaInt2 specific for *C. acutatum.* PCR was performed in 25 ml total volume composed of: 1X PCR master mix (Fermentas, Lithuania) (0,625 U Taq polymerase, 2 mM $MgCl_2$, 0,2 mM of each dNTP, 1 ml of each primer (20 mM) and 1 ml of extracted DNA. PCR conditions were 35 cycles: 94°C 1 min (denaturation), 59°C 2 min (annealing) and 72°C 2 min (extension). Visualization of PCR products was performed in 1% agarose gel, stained with ethidium bromide, under UV light.

Control of strawberry fruits anthracnose in naturally infected plantation. Field trials for strawberry anthracnose control were conducted during 2005 and 2006 in naturally infected plantation in the vicinity of Valjevo. Five fungicides, single or in a combination of two, were supplied by market companies in Serbia. The plot for each treatment consisted of double rows, 50 m long (100 plants per each row planted in zigzag position). All trials were arranged in a completely randomised block design with three replications. Fungicide treatments, doses and application timing are listed in **Table 1**. Fungicide treatment was applied on 7 days schedule from the beginning of flowering till the beginning of ripening (April 23 till May 16). Disease incidence was measured at each of the four harvests by collecting the fruits with anthracnose, their measuring and comparing with the weight of diseased fruits in untreated control plot.

Tab. 1 — Treatments, doses and application timing of fungicides applied to control strawberry anthracnose

Fungicide — Generic name	Fungicide chemical name	Doses/ha	Data of application for all fungicides (in 2005)
Kaptan FL	Captan F	2,5 l	April 23; April 30; May 7, and May 16.
Benfungin	Benomil	1,0 kg	
Stroby DF	Krezoksim-metil	0,20 kg	
Cabrio top	Mertira + piraklostrobin	2,0 kg	
Swich 62, 5WG	Fludiksinil + Ciprodinil	0,80 kg	

RESULTS

Diseases symptoms. The most visible symptoms of anthracnose on strawberry were on fruits at maturation. At the beginning infected fruit form of brown, circular, sunken, initially water-soaked lesion (**Figure** 1). Under optimal temperature (25°C) and humidity they rapidly increased, giving lesion of 1 to 2 cm in diameter within 3 to 4 days (**Figure** 2). With warm weather the disease spreads fast causing fruit rotting. The symptoms on vegetative parts of plants, stolons, leaves during the vegetation were unlikely to be seen.

Fig. 1 — Water-soaked lesion on strawberry fruit caused by *C. acutatum*

Colony and spore morphology. Uniform colony growth of all investigated isolates was on the PDA and OM. The colonies filled Petri dishes, Ø 90 mm, after 15 days. Three isolates (GG-6A, GG-JUP and TUT 137 A) had significantly less growth on CA than on PDA and OM (**Table** 2). Only reference AVO 37 4B isolate (*C. gloeosporioides*) had the same colony diameter in all three media. The colonies of GG-6A and GG-JUP isolates were effuse, first

Fig. 2 — Typical anthracnose lesion on ripen strawberry fruit

white later becoming orange, then turning into greenish grey as the cultures aged and later became covered with pink to salmon conidial masses on PDA. Light orange spore masses were formed around the centre of the colony. Older cultures developed black acervuli in the centre of the colony.

Tab. 2 — Effect of different media on colony growth of *Colletotrichum* isolated from strawberry and two reference species *C. acutatum* and *C. gloeosporioides*

Fungi	**Media**	**Diameter of colony (mm) after days**	
		9	**15**
GG-6A	PDA	73.3 a	90.0 a
	CA	62.7 b	83.3 b
	OM	56.7 c	90.0 a
GG-JUP	PDA	67.3 a	90.0 a
	CA	34.3 b	45.0 b
	OM	63.3 a	90.0 a
TUT-137A	PDA	72.0 a	90.0 a
	CA	28.0 c	37.0 b
	OM	63.3 b	90.0 a
AVO 37 4B	PDA	90.0 a	90.0 a
	CA	86.3 b	90.0 a
	OM	85.7 c	90.0 a

The same alphabet are not statistically different by Duncan test (P = 0.05)

Colony reverse was brownish orange to black. There were no differences in the colony morphology among the mentioned isolates, nor among the two reference isolates of *Colletotrichum.*

Conidia were hyaline, unicellular, and cylindrical with obtuse apices and tapering base. Conidia of GG-JUP isolate are usually ellipsoid and fusiform at least at one end (**Figure** 3), while conidia of isolate GG-6A are typically having both end rounded. Setae are not produced in the culture but are present in diseased strawberry fruits. Pigmented appressoria are produced after the germination of the conidia and vary in shape and size. There was no registered sexual stage formation under the laboratory conditions.

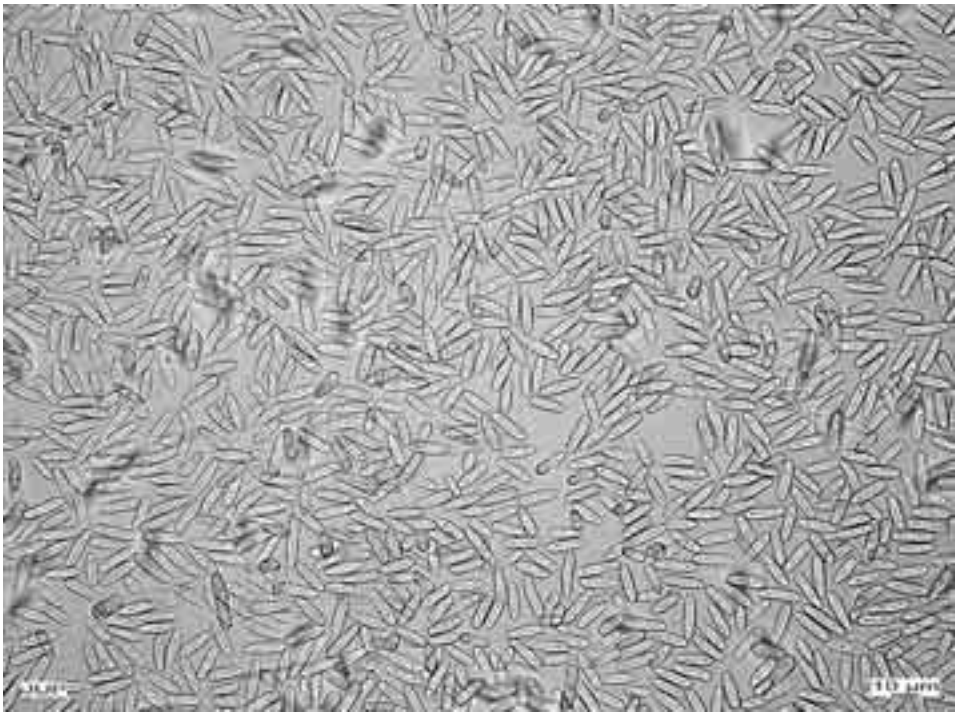

Fig. 3 — Conidial morphology of *C. acutatum*, isolate GG-JUP

Conidial size has been given in **Table 3**. Average conidial length of TUT 137A reference isolate is statistically shorter than the conidial length of other three isolates. However, GG-JUP and the referent isolate TUT 137A have statistically more similar conidia than GG-6A and AVO 37 4B. Concerning the ratio of conidial length:width, GG-JUP and TUT 137 A did not differ statistically, but GG-6A and AVO 37 4B did (**Table** 4).

Tab. 3 — Size of conidia of two isolates of *Colletotrichum* isolated from strawberry, and two reference isolates *C. acutatum* (TUT 137A), and *C. gloeosporioides* (AVO 37 4B).

Isolate	Length of conidia (µm)			Width of conidia (µm)		
	Min.	Average	Max.	Min.	Average	Max.
GG-6A	12,63	15,31 ± 0,49 a	22,03	4,98	5,66 ± 0,08 a	6,53
GG-JUP	12,96	15,49 ± 0,38 a	19,37	3,77	4,57 ± 0,08 c	5,22
TUT 137A	12,59	14,08 ± 0,39 b	17,49	3,53	4,32 ± 0,09 c	5,01
AVO 37 4B	12,57	15,07 ± 0,35 a	17,24	4,68	5,78 ± 0,17 a	7,82

The same alphabet are not statistically different by Duncan test (P = 0.05)

Tab. 4 — Ratio of conidia length/width of two isolates of *Colletotrichum* isolated from strawberry, and two reference isolates *C. acutatum* (TUT 137A), and *C. gloeosporioides* (AVO 37 4B).

Isolate	Min.	Average	Max.
GG-6A	2,56	2,98 ± 0,08 b	3,83
GG-JUP	2,76	3,42 ± 0,12 a	5,14
TUT 137A	2,78	3,27 ± 0,96 a	4,62
AVO 37 4B	1,63	2,65 ± 0,09 c	3,59

The same alphabet are not statistically different by Duncan test (P = 0.05)

Pathogenicity test on strawberry fruit. Both reference and our isolates caused lesions on artificially inoculated strawberry fruits. More pathogenic isolates were GG-JUP and TUT 137A, compared to the isolates GG-6A and AVO 37 4B (**Table** 5). Fruits challenged with distilled water did not develop lesions. On inoculated fruits, symptoms first appeared as whitish, water soaked lesions up to 3 mm in diameter. As lesions developed, they turned a light tan to dark brown and eventually became sunken and black within 2 to 3 days. After several days lesions may be covered with salmon-coloured spore masses. Infected fruits dried down to form hard, shrivelled mummies.

Tab. 5 — Pathogenicity of two isolates of *Colletotrichum* and two referent isolates of *C. acutatum* and *C. gloeosporioides* to strawberry fruits.

Isolates	Appearance of lesions	Diameter of lesion	Acervuli presence
GG-6A	Rounded cowered with sparse mycelia	13	No present
GG-JUP	Big, sunken, brownish, cowered with whitish mycelia	20	Present, creamy
TUT 137A	Big, sunken, brownish, cowered with whitish mycelia	20	Very present
AVO 37 4B	Smaller spot cowered with whitish mycelia	12	Not present

Molecular identification. Using CgInt primer specific for species *C. gloeosporioides*, in pair with ITS4 primer, expected length amplicons were obtained with isolates GG-6A which were, based on the conidia size, identified as *C. gloeosporioides.* Using CaInt2 primer specific for species *C. acutatum*, in pair with ITS4 primer, expected length amplicons were obtained with isolates GG-JUP which were, based on the conidia size, identified as *C. acutatum.*

Identification of fungal isolates was beside morphological characteristics, confirmed by using molecular technique PCR with primers specific for fungal species *C. gloeosporioides* and *C. acutatum.*

Control of anthracnose in strawberry fruits. Results obtained in this investigation showed that strawberry anthracnose can be controlled with fungicides application. The best fruit protection was achieved by fungicides Metiram + piraclostrobin, Captan FL and Fludioksinil + ciprodinil. Krezoksim-metil and Benomil did not protect strawberry fruit from anthracnose attack (**Table** 6).

Tab. 6 — Effect of the fungicides on anthracnose incidence in strawberry in 2005.

Date of harvesting	Weight of affected strawberry fruits (g)/treatment — (two-rows 50 m)					
	Market name of fungicides					
	Kaptan FL	Benomil	Stroby DF	Cabrio top	Swich	Control
May 21	20	20	300	0,0	0,0	1500
May 23	30	800	230	0,0	55	1250
May 26	150	500	700	150	300	1250
June 01	100	600	350	50	150	2750
Total (g)	**300**	**1920**	**1580**	**200**	**505**	**6750**

DISCUSSION

Our first objective was to identify the species of *Colletotrichum* causing strawberry anthracnose disease in our country. Morphological characteristics indicated that the causal agent of fruit rotting, isolate GG-JUP, could be *C. acutatum*. This statement was confirmed by fungus isolation from the infected fruits, pathogenicity tests, as well as molecular tests.

The isolates recovered from stolons, according to the morphological and molecular characteristics could be determined as *C. gloeosporioides*. Since this is the first finding of the pathogen on strawberry, further researches are needed to collect more isolates in order to study their morphological, pathological and molecular characteristics.

Other researches have shown that the characterisation of fungi isolated from strawberry plants, affected by anthracnose is complex. Lewis Ivey et al. (2004) found that the characteristics such as the colony morphology, the conidial shape, the presence or absence of setae and sclerotia, and the appressorium shape and size could be used for differentiation of the genus *Colletotrichum.* Morphological features, however, are highly variable among the isolates and often subject to interpretation. Concerning the same characteristics we did not find full consistency as well.

Since the anthracnose was first registered in 2004 in the plantations founded by the imported planting material, there are possibilities that the pathogen was introduced by the planting material from abroad.

Transmission of the pathogen by planting material is reported by Legard (2000) in the US. Investigation of Eastburn and Gubler (1990) suggested that the fungus is transmitted in infested soil attached to strawberry

crowns. Petioles of foliage harbour inoculum for fruit and flower for several *Colletotrichum* spp. (T i m m e r and B r o w n, 2000).

In OEPP/EPPO Bulletin (2004), it was emphasized that the infected planting material of strawberry is the main mean of introduction, but symptoms of anthracnose are unlikely to be seen on this material as the fungus is usually inactive in living vegetative tissues.

Several studies have pointed out that *C. acutatum* can develop quiescent infection on strawberry plants (H o w a r d et al., 1992). Production of secondary conidia and appressoria of *C. acutatum* on symptomless strawberry leaves, under a range of environmental conditions suggests that these processes also occur under field conditions and contribute to inoculum availability during the growing season (L e a n d r o et al. 2003).

Anthracnose is a serious disease of strawberry in Northern hemisphere. Three species of the *Colletotrichum* are responsible for strawberry anthracnose. *C. gloeosporioides* and *C. fragariae* usually cause petiole and stolon lesions and crown rot on strawberry but may also produce fruit symptoms (S m i t h, 1998). *C. acutatum* is predominantly on flowers and fruits inducing rotting, and causing the most yield reduction worldwide.

In other to develop recommendations for the management of the disease, we assessed the efficacy of several fungicides in reducing disease incidence. The best fruit protection was achieved by fungicides metiram + piraclostrobin, captan FL and fludioksinil + ciprodinil. Less effective were Benomil and Krezoksim-metil. Benomil was not good in anthracnose control in Ohio (E l l i s, 2004), nor was it in our trials in sour sherry anthracnose control (I v a n o v i ć & I v a n o v i ć, 1992). Various fungicides were assessed for their ability to control strawberry anthracnose caused by *C. acutatum* in Israel (F r e e m a n et al., 1997), and anthracnose of immature bell peppers in Ohio (M e l a n i e et al., 2004). In Florida, strawberry anthracnose management is based on the use of Captan or thiram by regular weekly application (M e r t e l y & P e r e s, 2005).

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АНТРАКНОЗА, НОВА БОЛЕСТ ЈАГОДЕ У СРБИЈИ И ЊЕНА КОНТРОЛА ФУНГИЦИДИМА

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Резиме

Антракноза је деструктивна болест плодова јагоде у топлим и континенталним климатским условима. Током 2004. године, на 2 плантаже јагода у близини Ваљева, било је великих губитака проузрокованих антракнозом. Два изолата гљива ГГ-6А и ГГ-ЈУП су изолована из столона јагоде, и плодова са израженим симптомима антракнозе. На основу морфолошких и патолошких карактеристика, и ПЦР анализе са специфичним прајмерима за референтне врсте, изолат ГГ-6А је идентификован као *Colletotrichum gloeosporioides*, а изолат ГГ-ЈУП као *C. acutatum*. Ово је први налаз *C. acutatum* на јагоди у Србији.

У циљу контроле антракнозе јагоде пет фунгицида и њихових комбинација су примењени 4 пута током цветања. Најбоља заштита плодова јагоде је постигнута применом фунгицида Metiram + piraclostrobin (Cabrio top), Captan FL и Fludioksinil + ciprodinil (Swich). Мање ефективни су били Benomil (Benlate) и Krezoksim-metil (Stroby).

Патоген се преноси садним материјалом па су фитосанитарне мере веома важне у превенцији болести.