Morphological and Molecular Identification of *Colletotrichum acutatum* from Tomato Fruit

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SUMMARY

Colletotrichum gloeosporioides, Colletotrichum acutatum, Colletotrichum coccodes, and *Colletotrichum dematium* are the four main species of *Colletotrichum* that cause tomato anthracnose. In Serbia, the occurrence of anthracnose on tomato fruit has been recorded during the last several years. Typical fruit symptoms include dark, sunken, and circular lesion with orange conidial masses. Pathogen isolates were obtained from a diseased tomato fruits, on PDA medium forming a white to gray colonies. The cultures developed black acervuli around the center of the colony. Conidia were hyaline, aseptate, and fusiform or rarely cylindrical. Appressoria were smooth, simple, clavate to ovate, and varied from light to dark brown. Pathogenicity tests with representative isolates were conducted on symptomless, detached tomato fruits. All tested isolates caused anthracnose lesions on tomato fruit after 7 days of incubation. Koch's postulates were fulfilled by reisolation from inoculated tomato fruits. PCR analysis (using species-specific primer pair, CaInt2/ITS4) of genomic DNA from tomato isolates resulted in an amplification product of 490 bp, specific for *C. acutatum*, further confirming the identity of the pathogen. Based on morphological and molecular characteristics, the isolates from tomato fruit were determined as *C. acutatum*.

Keywords: Anthracnose; Tomato; *Colletotrichum acutatum*; Identification

INTRODUCTION

The genus *Colletotrichum* (teleomorph *Glomerella*) contains an extremely diverse number of fungi including both plant pathogens and saprophytes. Plant pathogenic species are important worldwide, causing diseases commonly known as anthracnose of grasses, legumes, vegetables, fruits, and perennial tree crops. The disease can occur on leaves, stems, and fruits of host plant (Sutton, 1992). Anthracnose diseases appear in both developing and mature plant tissues. Two distinct types of diseases are: those affecting developing fruit in the field (preharvest) and those damaging mature fruit during storage (postharvest). The ability to cause latent or quiescent infections has grouped *Colletotrichum* among the most important postharvest pathogens (Bailey et al., 1992).

Anthracnose disease caused by several *Colletotrichum* spp. is a significant economic constraint on tomato (*Lycopersicon esculentum* Mill.) production worldwide. *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *Colletotrichum coccodes* (Wallr.) S.J. Hughes, and *Colletotrichum dematium* (Pers. ex Fr.) Grove are three main species of *Colletotrichum* that cause tomato anthracnose in the United States (Dillard, 1989; Byrne et al., 1997; Sanogo et al., 1997; LeBoeuf, 2007). In Bulgaria, *Colletotrichum acutatum* J.H. Simmonds has been reported as a causal agent of tomato fruit anthracnose (Jelev et al., 2008). The corky root of tomato caused by *Colletotrichum atramentarium* (Bert. et Br.) Taubenh., (synonym of *C. coccodes*) has also been found in Croatia (Panjan and Lušin, 1963).

In Serbia, the occurrence of anthracnose on tomato fruit was recorded in 2007 and 2008 (Živković et al., 2008). Typical symptoms include dark, sunken, and circular lesion that produces mucilaginous, orange conidial masses. Economic losses caused by the disease are mainly attributed to lower fruit quality and marketability.

Differentiation between *Colletotrichum* species based on host range or host of origin may not be a reliable criterion for fungi of this genus, since taxa such as *C. gloeosporioides, C. dematium, C. acutatum,* and others infect a broad range of host plants. Some taxa appear to be restricted to host families, genera or species within those families, or even cultivars, whereas others have more extensive host ranges (Freeman et al., 1998). Identification of *Colletotrichum* spp. is therefore a fundamental criterion in the development of more efficient control measures. Traditional identification and characterization of *Colletotrichum* species has relied primarily on differences in morphological features such as colony colour, size and shape of conidia and appressoria, growth rate, presence or absence of setae, and existence of the *Glomerella* teleomorph (Smith and Black, 1990; Gunnell and Gubler, 1992; Sutton, 1992). However, due to their morphological variability, the ample range of hosting crops, and wide variety of isolates, they are partially difficult to identify by traditional taxonomic methods, which must be complemented with molecular techniques (Whitelaw-Weckert et al., 2007). Construction of species-specific primers, especially from the ribosomal DNA internal transcribed spacer (ITS) region, has been proposed as the most efficient and reliable system for detection and differentiation of *Colletotrichum* spp. (Sreenivasaprasad et al., 1996; Freeman et al., 2000).

The objectives of the presented study were to substantiate the results obtained from previous investigations describing the symptoms, and identifying the species of *Colletotrichum* causing the anthracnose on tomato fruit using both classical and molecular techniques.

MATERIAL AND METHODS

Pathogen isolation and maintenance

Tomato fruit samples with typical anthracnose symptoms were collected from the markets during 2007 and 2008. Pieces of the diseased tissues were sterilized in 3% NaOCl for 3 min, followed by several rinses with sterile distilled water, and placed on water agar (WA) in Petri plates at 25°C for 5 days. Monoconidial cultures were produced for each isolate and maintained on potato dextrose agar (PDA) slants at 4°C. The reference isolates of *C. acutatum* (CBS 294.67) and *C. gloeosporioides* (CBS 516.97) were obtained from the Fungal Biodiversity Centre, Netherlands.

Pathogenicity test

Pathogenicity tests with representative isolates were conducted on symptomless, detached tomato fruits. The fruits were surface-sterilized with ethanol (70%), wounded with sterile needle, and inoculated with 20 μl of the conidial suspension (10^6 onidia/ml). Control fruit was inoculated with 20 μl of sterile distilled water. The fruits were then incubated in a plastic container at 25°C and >95% relative humidity, and examined for lesion development 7 days after inoculation. After 14 days, spores from diseased fruits were aseptically transferred and

subcultured onto PDA plates, which were incubated at 25°C in darkness. The resultant cultures were checked for colony and spore morphology to confirm Koch's postulates.

Morphological and cultural characteristics

The isolates were cultured on potato dextrose agar PDA in darkness at 25ºC. The appearance of the colonies, the occurrence of sectors, and the vegetative and reproductive structures were described after 10 days of incubation. The conidia were taken from actively growing colonies and suspended in sterile water. Length and width were measured for 100 conidia, and conidial shape was recorded using the light and scanning electronic microscopy. Appressoria were produced using a slide culture technique, in which 10 mm^2 squares of PDA were placed in an empty Petri plate. The edge of the agar was inoculated with spores taken from a sporulating culture, and a sterile cover slip was placed over the inoculated agar (Johnston and Jones, 1997). After 5 days, the shape and size of the 100 appressoria formed across the underside of the cover slip were examined microscopically. Morphological characteristics of conidia and appressoria of tomato isolates were compared with reference isolates of *C. acutatum* and *C. gloeosporioides*.

DNA extraction

Total genomic DNA was extracted from mycelium obtained from cultures grown on PDA for 7 days at 25°C. Aerial mycelium was removed from each culture using a sterile transfer needle and placed in a sterile 1.5-ml microcentrifuge tube containing 300 µl of extraction buffer (0.2 M Tris-HCl, 0.25 M NaCl, 25 mM EDTA, and 2% sodium dodecyl sulfate, pH 8.5). Tubes were uncapped and placed in a boiling water bath for 5 min and then cooled to 25°C; 200 µl of phenol that was equilibrated with extraction buffer (vol/vol), and 200 µl of chloroform were added. The tubes were vortexed for 4 min, and then centrifuged at 12.000 rpm for 5 min. The supernatant was pipetted to a new sterile 1.5 ml tube and 200 µl of chloroform was added; the mixture was vortexed for 30 s and then centrifuged at 12.000 rpm for 15 min. The supernatant was again transferred to a new 1.5 ml tube and 200 µl of isopropanol was added; the capped tube was inverted several times to adequately mix and precipitate DNA and then centrifuged at 12.000 rpm for 15 min. The supernatant was discarded, and the nucleic acid pellet was washed in 400 µl of 70% ethanol and centrifuged at 12.000 rpm for 5 min. Again, the supernatant was discarded, and the nucleic acid pellet was air-dried for 10 min, resuspended in 50 µl of low-TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 8.5), and gently agitated to dissolve the DNA. DNA was treated with Ribonuclease A for a final concentration of 10 µg of RNase/ml for 1 h at 37°C.

PCR amplification

Species-specific primers for *C. gloeosporioides* (CgInt; 5'-GGCCTCCCGCCTCCGGGCGG- 3'), and *C. acutatum* (CaInt2; 5'-GGCGCCGGCCCCGTCA-CGGGGG-3'), from the ITS1 region of the ribosomal DNA gene were used in combination with the conserved primer ITS4. PCR amplification was performed in a 25 µl reaction mixture containing 1.5 µl of DNA extract in low-TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0); 4 µl of 200 µM each of dATP, dCTP, dGTP, and dTTP; 2.5 µl of 10× *Taq* reaction buffer (500 mM KCl, 100 mM Tris-HCl pH 9.0, and 1% Triton X-100); 0.5 µl of 100 µM MgCl$_2$; 1.0 µl of 1 µM target primer; 1 µl of 1 µM ITS4 primer; 0.65 U *Taq* DNA polymerase, and 14.85 µl of sterile water. Amplifications were performed in Eppendorf Master Cycler programmed for the following cycling conditions: initial denaturation at 94°C for 5 min; 35 amplification cycles consisting of 1 min at 94°C, 2 min at 59°C, 1 min of extension at 72°C, and final extension at 72°C for 5 min. PCR products were separated using electrophoresis in 1% agarose gels in TBE buffer. Gels were stained in dilute ethidium bromide (0.2µg/ml), visualized by UV transilluminator, and photographed.

RESULTS

Disease symptoms

Fruit symptoms begin as small, dark, sunken lesions that have a water-soaked appearance, which increase in diameter and coalesce, leaving a large sunken soft area. Under favourable temperatures, lesions on ripe fruit become visible within 5 to 6 days after infection. Orange conidial masses may occur scattered or in concentric rings on the lesion (Figure 1a). Black acervuli are produced just beneath the skin of the infected fruit (Figure 1b).

Figure 1. Anthracnose symptoms on tomato fruit: (a) sunken necrotic lesion with orange conidial masses; (b) black acervuli on the infected fruit

Pathogenicity test

All tested isolates caused anthracnose lesions on tomato fruit after 7 days of incubation (Figure 2a). No lesions developed on fruit inoculated with water (Figure 2b). Koch's postulates were fulfilled by reisolation from inoculated tomato fruits. Spore shape, size, and colony morphology were identical for the original and recovered isolates.

Figure 2. Pathogenicity test: (a) necrotic lesion on tomato fruit inoculated with isolate PC-3; (b) control fruit inoculated with sterile water

Morphological and cultural characteristics

Colonies of tomato isolates were dense aerial, initially white or cream white, becoming gray and then turning dark gray, as the cultures aged on PDA (Figure 3a). Colony reverse was white to white gray. Bright orange spore masses were produced outward from the center of the colony. The cultures developed black acervuli around the centre of the colony (Figure 3b and 4d). No setae were observed. Dark structures, which resembled perithecia but did not produce asci, were often observed throughout. The cultures did not form sectors after 10 days of incubation.

Mycelia were branched, septate, and hyaline. Conidia were hyaline, aseptate, and fusiform or rarely cylindrical with obtuse apices and tapering basis (Figure 4a and 4b). Appressoria were observed on the underside of sterile covers slips arising from vegetative hyphae.

They were smooth, simple, clavate to ovate, and varied from light to dark brown (Figure 4c). Conidial and appressorial shape and size of tomato isolates, and reference isolates of *C. acutatum* and *C. gloeosporioides* are shown in Table1.
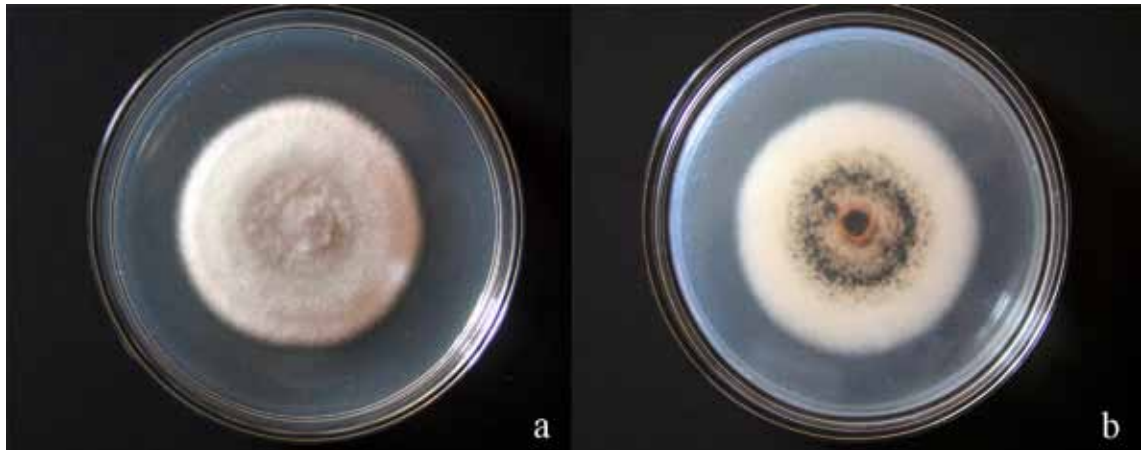

Figure 3. Cultural appearance of *C. acutatum*, isolate PC-3 on PDA: (a) top of culture; (b) bottom of culture
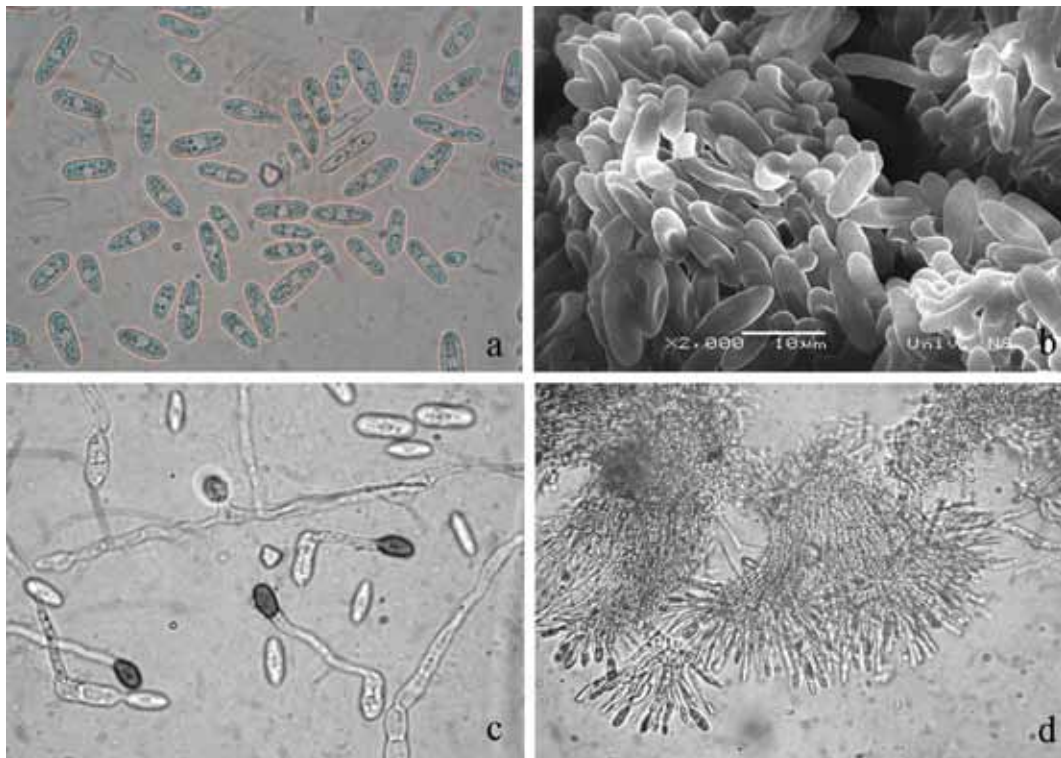

Figure 4. Morphological characteristics of *C. acutatum* from tomato: (a) conidia of isolate PC-4 (light microscope, x1000); (b) conidia of isolate PC-4 (scanning electronic microscope); (c) appressoria of isolate PC-6 (light microscope, x1000); (d) microscopic section of acervuli, isolate PC-1 (light microscope, x600)

Table 1. Morphology of conidium and appressorium of *Colletotrichum* isolates from tomato, and reference isolates of *C.acutatum* and *C. gloeosporioides*

Isolate	Conidium		Appressorium	
	ᵃ Shape	Size (μm) Length x Width min-(mean)-max	ᵇ Shape	Size (μm) Length x Width min-(mean)-max
PC-1	F, Ta	11.2-(14.3)-16.6 x 3.2-(3.6)-4.8	Cl	6.4-(7.2)- 9.6 x 4.8-(5.3)-6.4
PC-2	F, Ta	11.2-(13.9)-16.0 x 2.4-(3.7)-4.8	Cl	8.0-(8.5)-9.6 x 4.8-(5.5)-6.4
PC-3	F, Ta	11.2-(13.3)-15.2 x 2.4-(3.9)-4.8	Cl	6.4-(7.3)-9.6 x 5.6-(6.5)-7.2
PC-4	C, Ta	11.2-(13.7)-16.0 x 3.2-(3.7)-4.8	Ov	6.4-(7.3)-9.6 x 5.6-(6.2)-6.4
PC-5	F, Ta	12.8-(14.6)-17.6 x 2.4-(3.9)-4.8	Cl	8.0-(8.7)-10.4 x 5.6-(6.7)-7.2
PC-6	C, Ta	11.2-(13.7)-16.0 x 3.2-(3.9)-4.8	Ov	6.4-(7.2)-9.6 x 5.6-(6.2)-6.4
CBS 294.67	F, Ta	11.2-(13.2)-15.2 x 3.2-(3.9)-4.8	Cl	6.4-(8.1)-9.6 x 5.6-(5.8)-6.4
CBS 516.97	C, Ob	12.8-(17.9)-19.2 x 3.2-(3.7)-4.8	Ir, Ov	9.6-(12.3)-14.4 x 6.4-(7.9)-8.8

ᵃ Shape of conidium: F – fusiform; C – cylindrical; Ob – with obtuse ends; Ta – with tapering one end
ᵇ Shape of appressorium: Cl – clavate; Ir – irregular; Ov – ovate;

Molecular identification

The species-specific primer CaInt2 in conjunction with ITS4 primer amplified a 490 bp fragment from genomic DNA of tomato isolates, and the reference isolate of *C. acutatum* CBS 294.67, but not from DNA of *C. gloeosporioides* reference isolate (Figure 5a). In contrast, DNA of the tomato isolates, as well as the reference *C. acutatum*, were not amplified by the species-specific primer CgInt in conjunction with ITS4 primer, whereas a 450 bp fragment was amplified only from DNA of reference *C. gloeosporioides* CBS 516.97 (Figure 5b). No PCR products were produced with water controls in any of the reactions.
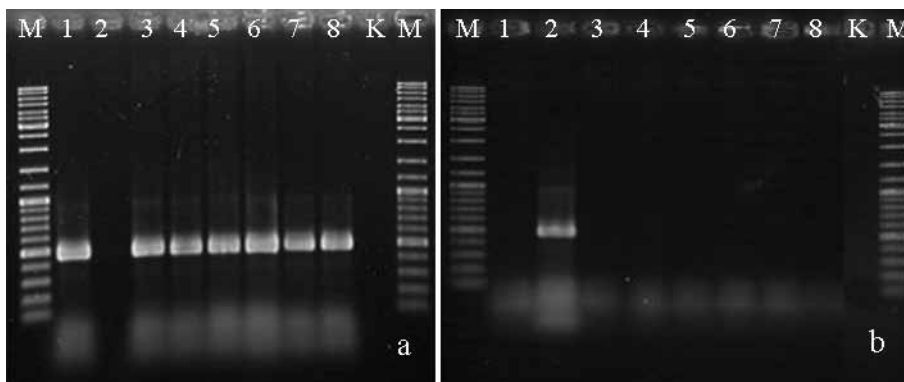

Figure 5. Amplification of specific DNA fragments from *Colletotrichum* isolates: (a) Primer pairs CaInt2/ITS4 specific for *C. acutatum*; (b) Primer pairs CgInt/ITS4 specific for *C. gloeosporioides*; reference isolate of *C. acutatum* CBS 294.67 (line 1); reference isolate of *C. gloeosporioides* CBS 516.97 (line 2); isolates PC-1,PC-2, PC-3, PC-4, PC-4, PC-5, PC-6 (lines 3 to 8); M = molecular size marker GeneRuler™ DNA Ladder Mix (100-10.000 bp); K = negative control (water)

DISSCUSION

Anthracnose caused by the fungi *C. acutatum* and *C. gloeosporioides* is an important disease in Serbia. Several host species can be affected, including sour cherry (Arsenijević, 1984; Ivanović and Ivanović, 1992), apple (Trkulja, 2003), strawberry (Ivanović et al., 2007), and pear fruit (Živković et al., 2009). Anthracnose on tomato fruit was observed during the last several years. The objective of this study was to identify the causing agent of tomato anthracnose using morphological and molecular analysis.

Morphological identification of tomato isolates was based on phenotypic traits such as colony appearance, and character of vegetative and reproductive structures. The colour of cultures may vary considerably within and between species of *C. acutatum* and *C. gloeosporioides*. Colonies of *C. gloeosporioides* were usually gray in appearance, while *C. acutatum* colonies had a chromogenic (pink) or nonchromogenic (white to gray) phenotype (Baxter et al., 1983; Freeman et al., 1998; Lardner et al., 1999; Forster and Adaskaveg, 1999). The results of cultures studies showed no distinct differences in characteristics among the tomato isolates. All isolates were nonchromogenic. The colours of colonies were white and white gray to gray. Tomato isolates were demonstrated to be pathogenic on wounded fruit, and were reisolated, fulfilling Koch's postulates.

Conidial size of *C. acutatum* was described variably as 8.3-14.4 × 2.5-4 μm (Simmonds, 1965), 8-16 × 2.5-4 μm (Dyko and Mordue, 1979), 12.3-14.7 × 4.6-5.3 μm (Smith and Black, 1990), and 12.5-20 × 3-5 μm (Gunnell and Gubler, 1992). Conidia of our isolates from tomato were compared with conidia of reference isolates of *C. acutatum*, and they were found to be similar in size. These results were almost fully consistent with description of Smith and Black (1990). In general, conidia of *C. acutatum* are elliptic-fusiform in shape; whereas conidia of *C. gloeosporioides* are cylindrical with obtuse ends (Dyko and Mordue, 1979; Baxter, et.al., 1983; Smith and Black, 1990). The conidial shape of most tomato isolates was fusiform, but conidia of PC-4 and PC-6 isolates were cylindrical with tapering end. The shape and size of appressoria have also been used for taxonomy of the genus *Colletotrichum*. Isolates from tomato fruits showed slightly smaller appressoria than reference isolate of *C. gloeosporioides*. These results correspond to description of Sutton (1992). The shape of appressoria of *C. acutatum* from tomato was clavate or ovate, and appressoria of *C. gloeosporioides* isolate CBS 516.97 were variable, irregular or ovate. Accordingly, appressoria of pathogens and reference isolates of *C. acutatum* and *C. gloeosporioides* differed in shape and size. Sanders and Korsten, (2003), and

Than et al., (2008) also found that appressorial morphology was unreliable in distinguishing *Colletotrichum* species. However, a definite identification of *Colletotrichum* species based on morphology is difficult because isolates have overlapping ranges of conidial and colony characteristics, and because the variation in morphology is accepted for isolates within a species (Sutton, 1992).

Based on morphological descriptions, many diseases reported before 1965 to be caused by *C. gloeosporioides* (or one of its synonyms) could have been caused by *C. acutatum* (Baxter, et al., 1983). *C. acutatum* represents a species that encompasses a wide range of morphological and genetic diversity. Characterization of *C. acutatum* has been enhanced by the use of molecular markers, which have identified genetically distinct and perhaps biologically discrete groups among morphologically similar isolates (Johnston and Jones 1997; Forster and Adaskaveg, 1999; Freeman et al., 2001).

Morphological characteristics of isolates from tomato fruit indicated that the causal agent could be *C. acutatum*, but culture morphology, and conidial and appressorial characteristics were insufficient to separate *C. acutatum* from *C. gloeosporioides*. However, PCR with primers specific for both species, followed by nucleotide sequencing of the amplicons, demonstrated that the causal agent of tomato anthracnose was *C. acutatum*. A PCR-amplified fragment of 490 bp was evident in all isolates from tomato fruits and *C. acutatum* CBS 294.67, but not in *C. gloeosporioides* CBS 516.97 isolate. *C. gloeosporioides* was not detected among the tomato isolates in this study. These results demonstrate that rDNA analysis is a reliable method for taxonomic species identification (Sreenivasaprasad, et al., 1996; Freeman, et al., 2000).

Anthracnose caused by *C. acutatum* is an emerging disease that may threaten the profitability of tomato and other crops in areas where it becomes established. Additional studies of the etiology and epidemiology of anthracnose are needed to define further the disease management strategies.

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Morfološka i molekularna identifikacija *Colletotrichum acutatum* sa ploda paradajza

REZIME

Četiri glavna prouzrokovača antraknoze paradajza iz roda *Colletotrichum* su: *Colletotrichum gloeosporioides, Colletotrichum acutatum, Colletotrichum coccodes* i *Colletotrichum dematium*. U Srbiji je tokom poslednjih godina zabeležena pojava antraknoze ploda paradajza. Tipični simptomi na plodu su tamne, ulegnute, kružne lezije sa masom narandžastih konidija. Sa obolelih plodova paradajza dobijeni su izolati patogena koji na KDA podlozi obrazuju bele do sivo obojene kolonije. Acervuli crne boje formiraju se u kulturi oko centra kolonije. Konidije su hialinske, neseptirane, fusiformne ili ređe cilindrične. Apresorije su glatke, jednostavne, okruglastog ili oblika izdužene palice, svetlo do tamno braon boje. Test patogenosti je obavljen sa reprezentativnim izolatima, na odabranim, zdravim plodovima. Svi ispitivani izolati prouzrokuju antraknozne lezije na plodu paradajza 7 dana nakon inokulacije. Kohovi postulati su zadovoljeni reizolacijama sa inokulisanih plodova paradajza. PCR analiza (korišćenjem para prajmera specifičnog za vrstu, Calnt2/ITS4) iz genoma DNA izolata sa paradajza rezultirala je amplifikacijom produkta od 490 bp, specifičnog za *C. acutatum*, što je potvrdilo identitet patogena. Na osnovu morfoloških i molekularnih karakteristika izolati sa ploda paradajza determinisani su kao *C. acutatum*.

Ključne reči: Antraknoza; paradajz; *Colletotrichum acutatum*; identifikacija