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IDENTIFICATION OF *COLLETOTRICHUM ACUTATUM* FROM NECTARINE FRUIT

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SUMMARY

Isolates of *Colletotrichum* spp. obtained from nectarine fruits with typical anthracnose symptoms in 2010 were identified using morphological and molecular methods. Pathogenicity test was conducted on symptomless, detached nectarine fruits. All tested isolates caused anthracnose lesions on nectarine fruits after 7 days of incubation. On PDA medium nectarine isolates forming dark green to dark gray mycelia. Growth rates of all isolates and reference strain of *C. acutatum* were lower at 25°C compared with reference strain of *C. gloeosporioides*. The conidia were hyaline, aseptate, and fusiform. Appressoria were dark brown, smooth, simple, and clavate to ovate. Using the primer set CaInt2/ITS4, the 490 bp DNA fragment was amplified from all nectarine isolates and reference strain of *C. acutatum* – CBS 294.67. Based on these results, the causal agent of anthracnose on nectarine fruits in Serbia was identified as *C. acutatum*.

Key words: anthracnose, nectarine, *Colletotrichum acutatum*, identification

INTRODUCTION

A large number of fungal pathogens attack the blossoms, foliage, fruits, branches, trunks and roots of peach (*Prunus persica*, L., Stokes) and nectarine (*P. persica*, variety *nectarine)*. Most of them occur preharvest in the orchard and determine the overall productivity and fruit quality, whereas other are postharvest pathogens that cause tremendous annual economic losses during storage (Adaskaveg et al., 2008).

Anthracnose disease caused by *Colletotrichum* spp. appears in both developing and mature plant tissues. The ability to cause latent or quiescent infections has grouped *Colletotrichum* among the most important postharvest pathogens (Bailey et al., 1992). In the United States and Brazil the pathogen causing anthracnose on peach and nectarine has been identified as *Colletotrichum acutatum* (Bernstein et al., 1995: Adaskaveg and Hartin, 1997; Kimati et al., 2005; Schnabel et al., 2006), and in Canada peach fruit may be infected with either *C. acutatum* or *C. gloeosporioides* early in development and remain symptomless until maturity (Zaitlin et al., 2000). In Serbia, anthracnose on nectarine fruits has been found during 2010 (Živković i sar., 2011).

Differentiation between *Colletotrichum* species based on host range or host of origin may not be a reliable criterion for fungi of this genus. 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). Classification of *Colletotrichum* spp. on the basis of morphological and cultural features (Cannon, 1998) failed to resolve relationships among several species, including *C. acutatum* and *C. gloeosporioides*, due to overlapping morphological characteristics. 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 methods, which have identified genetically distinct and perhaps biologically discrete groups among morphologically similar isolates (Johnston and Jones 1997; Forster and Adaskaveg, 1999). Lardner et al. (1999) suggested that *C. acutatum* J. H. Simmonds is a subspecific group within the broader *C. acutatum* complex, and Freeman et al. (2001) referred to this group as *C. acutatum* sensu Simmonds. Molecular analysis are now routinely used in conjunction with morphological characteristics to identify and characterize *Colletotrichum* spp. from various hosts (Guerber et al., 2003; Sreenivasaprasad et al., 2005; McKay et al., 2009).

The objective of the present study was identifying the causal agent of anthracnose disease on nectarine fruit using morphological and molecular techniques.

MATERIAL AND METHODS

Pathogen isolation and maintenance

Nectarine fruits with typical anthracnose lesions were collected from the markets. Symptomatic tissues were surface sterilized with 10% sodium hypochlorite solution for 2 min., and then rinsed several times with sterile distilled water before placing on potato dextrose agar (PDA) at 25°C. Monoconidial cultures were produced for each isolate and maintained on PDA slants at 4°C. The reference isolates of *C. acutatum* (CBS 294.67) and *C. gloeosporioides* (CBS 516.97) were obtained from Fungal Biodiversity Centre, Netherlands.

Pathogenicity test

Pathogenicity tests were conducted on symptomless, detached nectarine fruits. The fruits were

surface sterilized with ethanol (70%), wounded with sterile needle and inoculated with disk of PDA colonized with tested isolates. Non colonized PDA disk was used as negative control. 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 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 characterization

The isolates were cultured on PDA in darkness at 25°C for 7 days, and the diameter of mycelial growth was measured daily. The appearance of the colonies, the occurrence of sectors, and the vegetative and reproductive structures were described after 14 days 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 light microscopy. Appressoria were produced using a slide culture technique (Johnston and Jones, 1997). After 5 days, the shape and size of the 50 appressoria were examined microscopically.

DNA extraction and PCR amplification

Total genomic DNA was extracted from mycelium obtained from cultures grown on PDA for 7 days at 25°C. The 0.5 g of mycelium for each isolate was frozen in liquid nitrogen and ground in a sterile mortar. DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Extracted DNAs were preserved at -20°C.

Species-specific primers for *C. gloeosporioides* (CgInt: 5'- GGCCTCCCGCCTCCGGGCGG-3'), and *C. acutatum* (CaInt2: 5'- GGCGCCGGCCCCGTCACGGGGG-3') from the ITS1 region of the ribosomal DNA gene were used in combination with the conserved primer ITS4 (5'-TCCTCCGCTATTGATATGC-3'), (White et al., 1990). PCR amplification was performed in a 25 µl reaction mixture containing 1.5 µl of DNA extract in low-TE buffer; 4 µl of 200 µM each of dATP, dCTP, dGTP, and dTTP; 2.5 µl of 10× Taq reaction buffer; 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) and visualized by UV transilluminator.

RESULTS

Disease symptoms

The symptom begin as small, sunken lesion that have a water-soaked appearance, increase in diameter, and coalesce, leaving a large sunken soft area. The necrotic spots can expand and merge to cover the whole affected area. The color of the infected part darkens. Orange conidial masses may occur scattered or in concentric rings on the lesion (Figure 1a).

Pathogenicity test

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

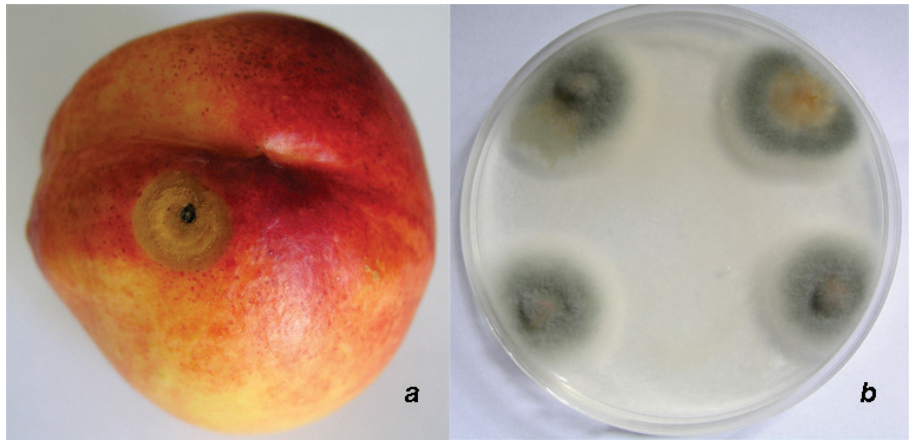

Figure 1. Anthracnose symptoms on nectarine fruit: a. Sunken necrotic lesion with orange conidial masses; b. Isolates from nectarine fruit on PDA.

Slika 1. Simptomi antraknoze na plodu nektarine: a. Ulegnuta nekrotična lezija sa narandžastom masom konidija; b. Izolati sa ploda nektarine na PDA.

Figure 2. Pathogenicity test: a. Necrotic lesion on nectarine fruit inoculated with isolate BC-1; b. Control fruit inoculated with sterile PDA disk.

Slika 2. Test patogenosti: a. Nekrotične lezije na plodu nektarine inokulisanim izolatom BC-1; b. Kontrolni plod inokulisan isečkom sterilne PDA podloge.

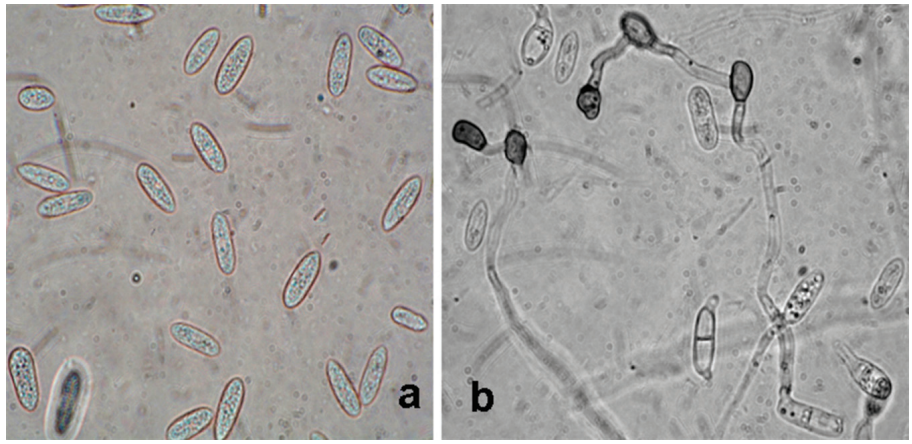

Figure 3. Morphological characteristics of nectarine isolates: a. Conidia of isolate BC-1 (x 400); b. Appressoria of isolate BC-1 (x400).

Slika 3. Morfološke karakteristike izolata sa nektarine: a. Konidije izolata BC-1 (x400); b. Apresorije izolata BC-1 (x400).

Table 1. Morphological characteristics and growth rate of isolates from nectarine fruits.
Tabela 1. Morfološke osobine i stopa porasta izolata sa plodova nektarine.

Isolate Izolat	Growth rate St.rasta (mm/day)	Conidia Konidije		Appressoria Apresorije	
		*shape oblik	size (µm) (length x width) veličina (µm) (dužina x širina)	**shape oblik	size (µm) (length x width) veličina (µm) (dužina x širina)
BC-1	9.2	F	12,8-17,6 x 2,4-4,8	Cl	6.4-9.6 x 5.6-6.2
BC-2	8.8	F	11.2-15.6 x 2.4-4.8	Cl	6.4-9.6 x 5.6-6.2
BC-3	8.7	F	11.2-16.2 x 3.2-4.8	Cl	8.0-9.2 x 5.6-7.2
BC-4	9.0	F	12.8-17.2 x 3.2-4.8	Ov	8.0-9.2 x 4.8-6.4
BC-5	8.9	F	11.2-15.6 x 2.4-4.8	Cl	6.4- 9.6 x 4.8-6.2
CBS 294.67	9.3	F	11.2-15.2 x 3.2-4.8	Cl	6.4-9.6 x 5.6-6.4
CBS 516.97	14.5	C	12.8-19.2 x 3.2-4.8	Ir, Ov	9.6-14.4 x 6.4-8.8

* Shape of conidium: F - fusiform; C - cylindrical;
** Shape of appressorium: Cl - clavate; Ir - irregular; Ov – ovate.

Morphological and cultural characterization

Colonies of nectarine isolates were dense aerial, initially dark green then turning dark gray, as the cultures aged on PDA (Figure 1b). Bright orange spore masses were produced outward from the center of the colony. The reverse of cultures was mostly greenish to dark grayish. The cultures developed black acervuli around the center of the colony. No setae were observed. Mycelia were branched, septate, and hyaline. Conidia were hyaline, aseptate, and fusiform (Figure 3a). Appressoria produced directly from conidia were dark brown, smooth, simple, clavate to ovate (Figure 3b). Conidial and appressorial shape and size, and growth rate of nectarine isolates are shown in Table 1.

Molecular identification

Using the primer set CaInt2/ITS4, the 490 bp DNA fragment was amplified from all nectarine isolates and reference strain of *C. acutatum* – CBS 294.67, but not from DNA of *C. gloeosporioides* (Figure 4a). In contrast, a primer par CgInt/ITS4 was amplified a 450 bp DNA fragment only from reference strain of *C. gloeosporioides* - CBS 516.97 (Figure 4b). No PCR products were produced with water controls in any of the reaction. Based on these results, the causal agent of anthracnose on nectarine fruits in Serbia was identified as *C. acutatum*.

DISSCUSION

Nectarine isolates of *C. acutatum* were demonstrated to be pathogenic on wounded fruits and were reisolated, fulfilling Koch's postulates.

Morphological identification of nectarine isolates based on phenotypic traits, such as colony appearance, growth rate, and characters of vegetative and reproductive structures. The color of cultures may vary considerably within and between species of *C. acutatum* and *C. gloeosporioides*. Colonies of *C. gloeosporioides* are 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 nectarine isolates. All isolates were nonchromogenic. The colors of colonies were dark green to dark gray. Growth rates of nectarine isolates and reference strain of *C. acutatum* were lower at 25°C compared with *C. gloeosporioides*. Maximum growth rates for *C. acutatum*

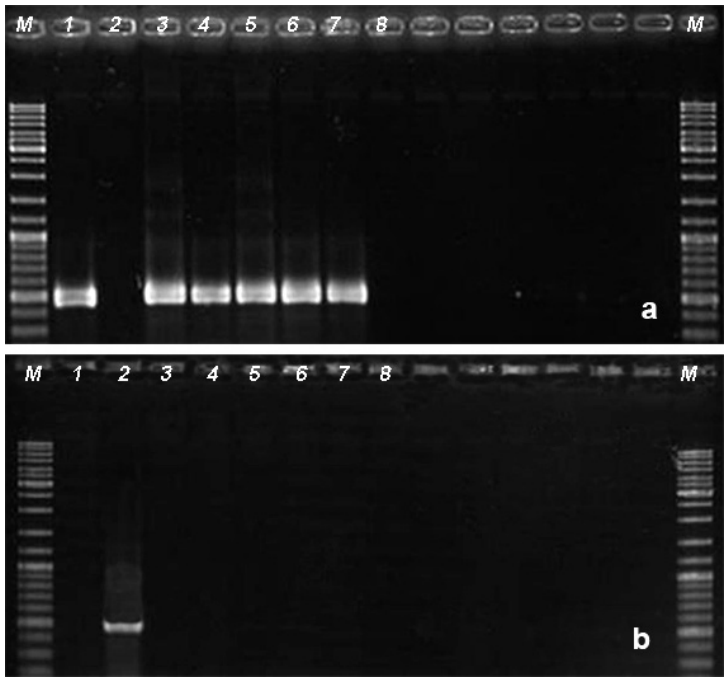

Figure 4. Amplification of specific DNA fragments from nectarine isolates: a. Primer pair CaInt2/ITS4 specific for *C. acutatum*; b. Primer pair CgInt/ITS4 specific for *C. gloeosporioides*; line 1 - reference strain of *C. acutatum* CBS 294.67; line 2 - reference strain of *C. gloeosporioides* CBS 516.97; lines 3-7 nectarine isolates; line 8 - negative control (water); M - marker GeneRuler™ DNA Ladder Mix (100-10.000 bp).

Slika 4. Amplifikacija DNA fragmenata *Colletotrichum* izolata sa nektarine: a. Par prajmera CaInt2/ITS4 specifičnih za *C. acutatum*; b. Par prajmera CgInt/ITS4 specifičnih za *C. gloeosporioides*; kolona 1 - referentni soj *C. acutatum* CBS 294.67; kolona 2 - referentni soj *C. gloeosporioides* CBS 516.97; kolone 3-7 izolati sa nektarine; kolona 8 - negativna kontrola (voda); M - marker GeneRuler™ DNA Ladder Mix (100-10.000 bp).

isolates were between 8.8 and 9.3 mm/day after 7 days, whereas reference strain of *C. gloeosporioides* had maximum growth rates of 14.5 mm/day. These results are consistent with other studies that used temperature relationships to distinguish *C. acutatum* from *C. gloeosporioides* (Sutton, 1992; Bernstein et al., 1995; Adaskaveg and Hartin, 1997).

Conidial size of *C. acutatum* was described variably as 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 nectarine fruits were compared with conidia of reference isolates of *C. acutatum*, and found to be similar size. The conidial shape of nectarine isolates was fusiform. In general, conidia of *C. acutatum* are 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). Shape and size of appressoria have also been used for taxonomy of the genus *Colletotrichum*. Isolates from nectarine fruits showed slightly smaller appressoria than reference strain of *C. gloeosporioides*. These results correspond to description of Sutton (1992). The shape of appressoria of *C. acutatum* from nectarine was clavate or ovate, and appressoria of *C. gloeosporioides* - CBS 516.97 were variable, irregular or ovate. Using these criteria, all of the nectarine isolates were distinct from the reference strain of *C. gloeosporioides*.

Morphological characteristics of isolates indicated that the causal agent of anthracnose could be *C. acutatum*, but PCR with primers specific for both species, demonstrated that the causal agent of nectarine anthracnose is *C. acutatum*. A PCR-amplified fragment of 490 bp was evident in all isolates from nectarine fruits and *C. acutatum* - CBS 294.67, but not in reference strain of *C. gloeosporioides* - CBS 516.97. *C. gloeosporioides* was not detected among the nectarine isolates in this study.

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IDENTIFIKACIJA *COLLETOTRICHUM ACUTATUM* SA PLODA NEKTARINE

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REZIME

Izolati *Colletotrichum* spp. dobijeni 2010. godine, sa antraknoznih plodova nektarine identifikovani su pomoću morfoloških i molekularnih metoda. Test patogenosti je obavljen sa reprezentativnim izolatima, na odabranim, zdravim plodovima. Svi ispitivani izolati prouzrokuju antraknozne lezije na plodu nektarine, 7 dana nakon inokulacije. Na PDA podlozi izolati formiraju tamno zelenu do tamno sivu miceliju. Stopa rasta izolata sa nektarine i referentnog soja *C. acutatum* je bila niža u odnosu na referentni soj *C. gloeosporioides*. Konidije su hialinske, neseptirane i fusiformne. Apresorije su tamno braon boje, glatke, jednostavne, okruglastog ili oblika izdužene palice. Korišćenjem para prajmera CaInt2/ITS4 iz genoma DNA izolata sa ploda nektarine i referentnog soja *C. acutatum* – CBS 294.67, amplifikovan je fragment veličine 490 bp. Na osnovu ovih rezultata, u Srbiji je kao prouzrokovač antrakoze plodova nektarine identifikovana vrsta *C. acutatum*.

Ključne reči: antraknoza, nektarina, *Colletotrichum acutatum,* identifikacija

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