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First report of *Colletotrichum nigrum* causing tomato anthracnose in Serbia

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Abstract: During the survey of open tomato fields in Vojvodina (Serbia), characteristic anthracnose disease symptoms, including black, circular, sunken, and watery lesions, were observed in about 20% of fruits in September 2018. Subsequent pathogen isolations formed white aerial mycelia and numerous spherical, black conidiomata on the Potato Dextrose Agar. Ten representative isolates produced hyaline, smooth-walled, cylindrical, and aseptate conidia. The presence of initially brownish necrotic lesions on the tomato, which later expanded into large rotted areas, ultimately leading to complete fruit rot, confirmed the pathogenicity of the tested isolates. Molecular identification was performed using Bayesian analysis of concatenated ITS, TUB2, CHS-1, and ACT loci. Based on the combination of the morphological and molecular features, the fungus *Colletotrichum nigrum* was identified as the causal pathogen. As this is the first report on this fungal pathogen on tomatoes in Serbia, it highlights the importance of early and accurate detection for effective disease prevention, thus reducing crop damage and market losses.

Keywords: *Solanum lycopersicum*; identification; fruit rot

Tomato (*Solanum lycopersicum* L.) is the second most important vegetable at the global level and is currently cultivated on an area of 5.16 mil. ha with a production of 189.13 mil. t (FAO 2021). In Serbia, 7 800 ha are presently under this valuable crop (SYRS 2023), which can be potentially threatened by anthracnose. Globally, for this destructive disease, multiple fungal pathogens belonging to the genus *Colletotrichum* have been identified, including *C. gloeosporioides*, *C. acutatum*, *C. coccodes*, *C. dematium*, and *C. nigrum* (Liu et al. 2011; Rivera et al. 2016; Belov et al. 2018; Manova et al. 2022; Sopialena et al. 2022). Thus far, only *C. acutatum* has been reported as the causal agent of tomato anthracnose in Serbia (Živković et al. 2010). Therefore, this study aimed to determine and characterize the causal agent of the observed *Colletotrichum*-like symptoms in tomato in Serbia.

MATERIAL AND METHODS

Sampling and isolation. In September 2018, fruit anthracnose, characterized by black, circular, sunken, watery lesions, was noticed on about 20%

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of fruits during the survey of tomato fields covering a 144 ha area situated in the Bačka Palanka locality (Bačka is a part of the Vojvodina Province in northern Serbia) (Figure 1A). A total of 20 diseased tomato fruits were collected for further laboratory testing. Isolation was performed by taking small pieces from the zone between the infected and healthy tissues (surface sterilized in 75% alcohol for 5 min and rinsed with sterilized water) and placing them onto a potato dextrose agar (PDA) medium for incubation at 25 °C for seven days. Fungi was subsequently purified by transferring single spores or hyphal tips onto the new PDA. Finally, ten representative isolates were stored at 4 °C until required for further analyses.

Morphological characterization. For this purpose, isolates were grown on sour synthetic agar (SSA) at 25 °C and the culture characteristics (colony colour, texture), conidiomata and conidial morphology (length and width) were assessed after 10 days. Conidiomata and conidial size (length and width) were computed from 100 measurements per isolate using a microscope (BTC).

Pathogenicity. Pathogenicity was assessed on healthy/symptomless, detached, mature tomato fruits (cultivar Novosadski Jabučar) by placing a 7 day old culture of mycelium plugs (5 mm) in a wound made by a sterile needle. Fruits were disinfected with 70% ethanol for 5 min, washed in distilled water, and dried before being artificially inoculated. The inoculated fruits were kept at 25 °C under high humidity (70–80%) for 12 days. The reisolations were performed on PDA to confirm Koch's postulates.

Molecular identification. Total DNA was extracted from isolates grown on PDA for eight days using a genomic DNA isolation kit (DNeasy Plant Mini Kit, Qiagen). The identification was performed based on the sequences of four gene regions (Liu et al. 2022) — the internal transcribed spacers (ITS), beta-tubulin (TUB2), chitin synthase (CHS-1), and actin (ACT) — which were amplified with ITS1/

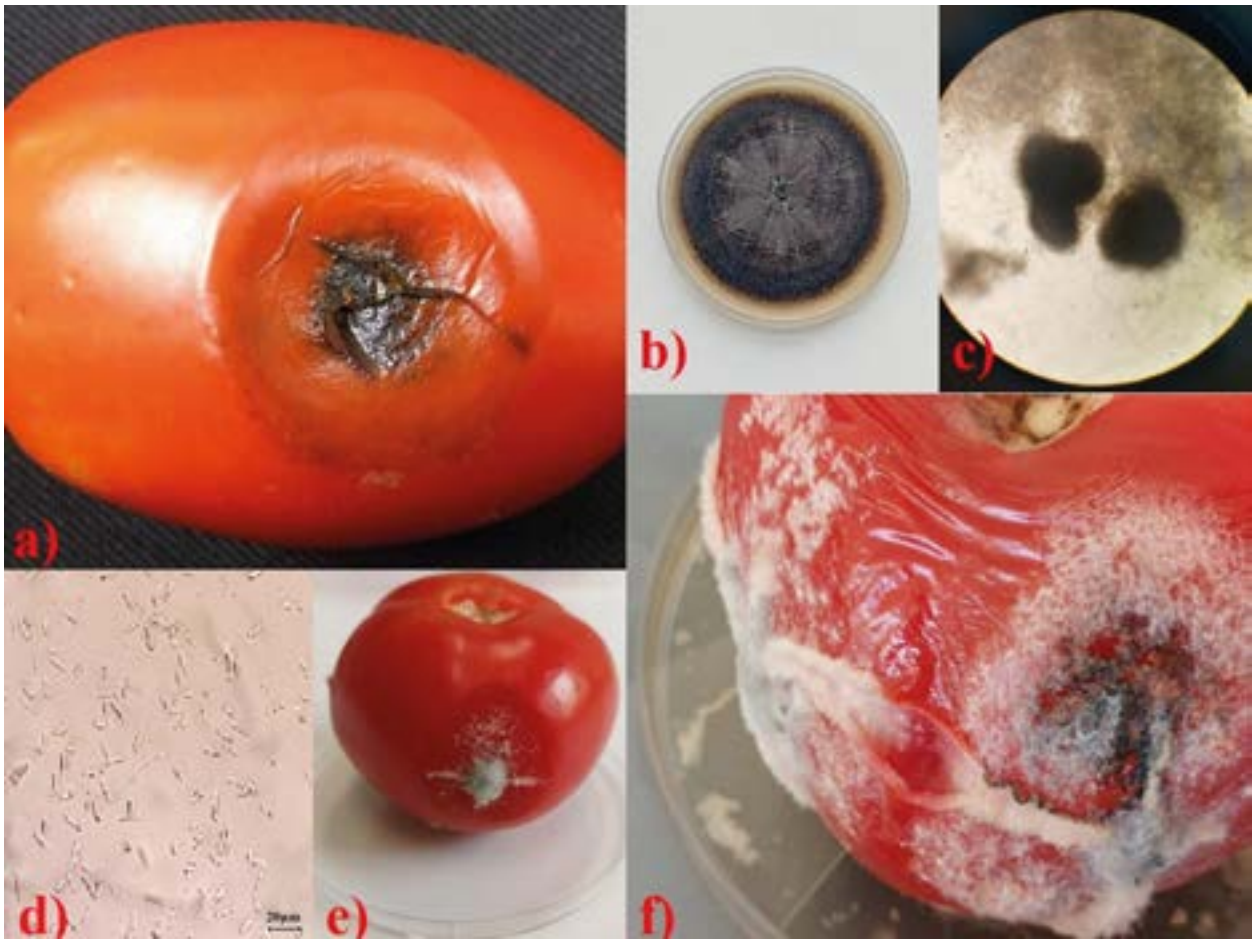

Figure 1. *Colletotrichum nigrum*: (A) symptom of naturally infected tomato fruit; (B) culture on SSA; (C) conidiomata; (D) conidia; (E), (F) inoculated tomato fruits

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ITS4 primers (White et al. 1990) (the annealing temperature was adjusted to 58 °C), T1/Bt2b primers (Glass & Donaldson 1995; O'Donnell & Cigelnik 1997), CHS-79F/CHS-345R primers (Carbone & Kohn, 1999) (the annealing temperature was altered to 58 °C) and ACT-512F/ACT-783R (Carbone & Kohn 1999) (the protocol was modified by setting the temperature to 59 °C), respectively. All amplifications were performed in a 25 µL volume containing 5 ng DNA, 1 × PCR buffer (20 mM Tris.HCl pH 8.4, 50 mM KCl), 1 µM of each primer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP and 1 UTaq DNA polymerase (Fermentas) in a Mastercycler Nexus GSX1 (Eppendorf). Amplicons were sent for sequencing in an automated DNA sequencer (ABI PRISM 3 700, Macrogen Inc.).

The obtained sequences were edited and assembled using FinchTV (version 1.4.0) and were aligned with MAFFT (version 7.520). After concatenating, these sequences were used for phylogenetic analyses comparing Serbian tomato isolates with other reference *Colletotrichum* sequences retracted from NCBI reported in *Solanum* spp. plants. Maximum-likelihood (ML) was performed using PAUP (Phylogenetic Analysis Using Parsimony) software (version 4.0a169) (Swofford 2003), and Bayesian analysis was conducted in MrBayes (version 3.2.7a) (Ronquist & Huelsenbeck 2003) using the concatenated ITS, TUB2, CHS-1 and ACT data. The heuristic search option with 100 random sequence additions was adopted for PAUP, with tree bisection and reconstruction as the branch-swapping algorithm. Alignment gaps were treated as missing data. The robustness of the obtained trees was evaluated through 10 000 bootstrap replications. The Bayesian information criterion (BIC) indicated the best-fitting nucleotide substitution model using the software jModeltest (version 2.1.10) (Darriba et al. 2012). Accordingly, a Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities. The analyses of two MCMC chains were run from random trees for 1 000 000 generations and were sampled at 100 generation intervals. The first 25 trees were discarded as burn-in samples, and the standard deviation of the split frequencies was checked at the end of each run until its value declined below 0.01. The convergence of the MCMC chains and their stationarity were checked using Tracer (version 1.5), and the phylogenetic tree was visualized using FigTree (version 1.4).

RESULTS AND DISCUSSION

All collected tomato fruits showed *Colletotrichum*-like symptoms, white to gray mycelia with profuse and numerous spherical; black conidiomata were consistently isolated on PDA. On SSA, all isolates produced white circular mycelia, which became grey-black (Figure 1B). Acervular conidiomata ranging from 150 to 350 µm in diameter were observed after 10 days (Figure 1C). Conidia were hyaline, smooth-walled, cylindrical and aseptate, measuring 12.6–23.1 × 2.1–4.2 µm in size (Figure 1D). Their morphological characteristics corresponded to *Colletotrichum* sp. (Liu et al. 2013; Pal & Testen 2021). Pathogenicity test performed on tomato fruits resulted in brownish necrotic lesions around the inoculation sites five days after inoculation (Figure 1E), which expanded into large rotted areas, ultimately leading to complete fruit rot 12 days after inoculation (Figure 1F). The reisolates were examined for colony and spore morphology, confirming their identity as the original isolates and thus fulfilling Koch's postulates.

A BLAST search with each individual, ITS, TUB2, CHS-1, and ACT sequences indicated that all tomato isolates corresponded 100% to the referent strain *Colletotrichum nigrum* CBS 127562. Sequences of the three selected tomato isolates — R1PA, R2PA/1 and R3PA/2 — were deposited in the NCBI database under the accession numbers PP152324—152326 (ITS), PP171497—PP171499 (TUB2), PP171500—PP171502 (CHS-1) and PP171494—496 (ACT).

The Bayesian information criterion (BIC) employed in jModelTest based on the 2421 bp length of the ITS, TUB2, CHS-1, and ACT concatenated sequences recognized a general time reversible model with gamma distribution (GTR + G) as the most appropriate substitution model. Tree topologies yielded by the maximum-likelihood and Bayesian analyses were congruent; therefore, one overlapped phylogenetic tree for every phylogenetic analysis was shown with both bootstrap values (Figure 2). The phylogenetic tree revealed a high bootstrap value for the clade of *C. nigrum* isolates, grouping three selected Serbian tomato isolates (R1PA, R2PA/1, and R3PA/2) with reference *C. nigrum* strain CBS 127562 (Figure 2).

Pathogen *C. nigrum* has been reported on tomatoes in New Zealand, the USA, China, and Russia (Liu et al. 2013; Rivera et al. 2016; Yarmeeva et al. 2023). However, it can also be a significant pathogen on

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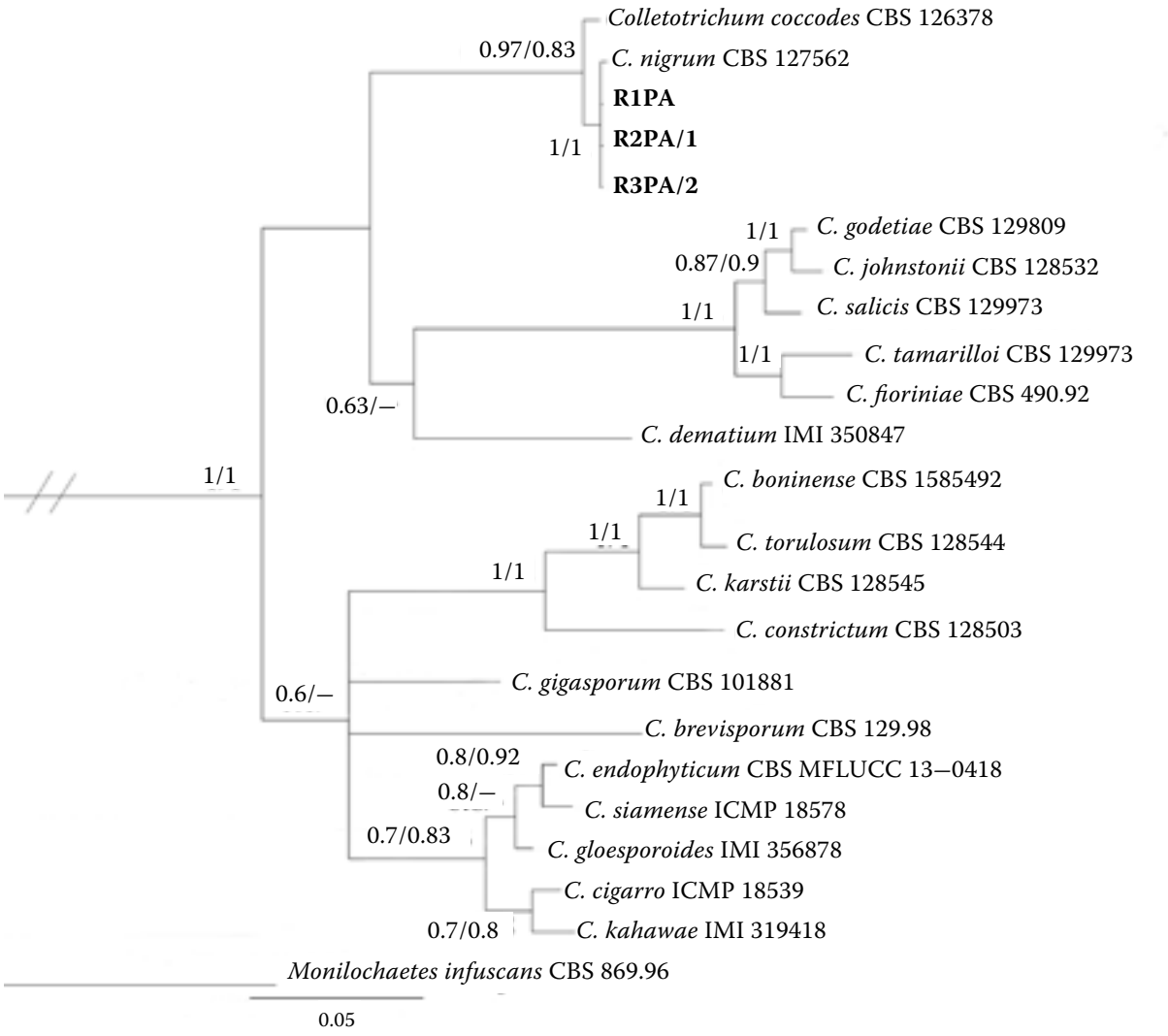

Figure 2. The consensus phylogenetic tree based on the Bayesian and Maximum-likelihood analyses of the combined ITS, TUB2, CHS-1 and ACT gene sequences of *Colletotrichum nigrum* from Serbia and reference isolates from the GenBank

The isolates from this study are marked in bold. Bootstrap support values (expressed as percentages of 1 000 replications) are given at the nodes (only the Bayesian/Maximum-likelihood bootstrap values exceeding 60 are shown)

Capsicum annum, *Cichorium intybus*, *Fragaria* sp., *Helianthus tuberosus*, and *Salvia gregii* (Liu et al. 2013; Jayawardena et al. 2021). In Serbia, *C. nigrum* was previously described only on *H. tuberosus* (Liu et al. 2013). Thus this is the first report of its presence on tomatoes in Serbia, enhancing our knowledge of its geographical distribution. As a significant threat to tomato production, *C. nigrum* can reduce yield and quality, causing economic losses both in fields and post-harvest, as it decreases the market value of tomatoes (Rivera et al. 2016). Its wide host range necessitates specific control strategies, highlighting its substantial economic impact on commercial agriculture. Since *C. nigrum* survives on tomato roots and in infested soils, crop rotation with non-host crops and elimination of wild hosts is recommended to reduce the inoculum levels in the soil.

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