Colletotrichum gloeosporioides causing anthracnose on pomegranate in Turkey

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Abstract

During spring 2017, an anthracnose-like foliar disease was observed in the pomegranate orchards in the region. Disease incidence (%) ranged from 15 to 22. The objectives of this study were to characterise this fungal pathogen, based on morphology, molecular characteristics and pathogenicity. Foliar symptoms progressed light to dark brown, concentric, circular, distinct spots with yellowish halos. Also, salmon coloured spore masses were observed on pomegranate cv. Hicaz leaves in Kozan district. Cultural and morphological examinations and DNA sequence data revealed that the fungus associated with symptomatic leaves was *Colletotrichum gloeosporioides*. Pathogenicity testing was performed by inoculation of healthy leaves with a spore suspension of *C. gloeosporioides*. This confirmed *C. gloeosporioides* as causal agent of anthracnose symptoms on pomegranate in Turkey.

Keywords *C. gloeosporioides* · Pomegranate · Pathogenicity · PCR

Pomegranate (*Punica granatum*) is a commercially important fruit both for domestic consumption and as an export commodity in tropical and subtropical countries. In Turkey, total pomegranate production (tonnes) between 2011 and 2016 increased by 114% (TUIK 2018). The Mediterranean Region, including provinces Hatay, Adana, Mersin and Antalya, the main pomegranate growing areas of the region, accounts for approximately 52.7% of the total pomegranate production in Turkey. Pomegranate production is also popular in other parts of the country. At present, the known fungal disease agents of pomegranate are *Alternaria* spp., *Botrytis cinerea*, *Aspergillus niger*, *Colletotrichum gloeosporioides*, *Coniella* spp., *Nematospora* spp., *Pilidiella granati*, *Pestalotiopsis versicolor*, *Syncephalastrum racemosum*, *Penicillium* spp. and *Rhizopus* spp. (Palou et al. 2013; Kanetis et al. 2015; Munhuweyi et al. 2016). Among the various fungal diseases, anthracnose caused by *Colletotrichum gloeosporioides* is one of the most serious diseases of pomegranate worldwide (Munhuweyi et al. 2016). All stages of pomegranate tree

development are susceptible to the disease. Latent infection may occur invisibly in healthy plants causing extensive crop losses due to anthracnose disease related symptoms (Munoz et al. 2009). Primary and secondary sources of inoculum are from infected leaves and windborne conidia, respectively (Munhuweyi et al. 2016).

In the spring of 2017, an anthracnose-like leaf spot symptoms were observed in the pomegranate orchards in the Mediterranean Region of Turkey. Disease incidence (%) ranged from 15 to 22. The objectives of this study were to isolate, identify and characterise this fungal pathogen, based on cultural morphology, molecular characteristics and pathogenicity. Foliar symptoms progressed light to dark brown, concentric, circular, distinct spots with yellowish halos (Fig. 1). Also, salmon coloured spore masses were observed on pomegranate cv. Hicaz leaves in Kozan district (geographical coordinates: N 37°30'35.870" E 035°52'13.727") of Adana province in the region. The necrotic areas with irregular shapes were 1–3 cm in size. No symptoms were observed on the stems and fruits of pomegranate trees.

For fungal isolation, the epidermal tissues (approximately 5 mm) of leaves affected were collected and cut into pieces, surface-sterilised by dipping in a 1% sodium hypochlorite (NaOCl) solution for 2 min, rinsed twice in sterile distilled water, and blotted dry on sterile filter papers. Small pieces of

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Fig. 1 Typical leaf anthracnose symptoms on the upper side of pomegranate leaves caused by *Colletotrichum gloeosporioides*

disinfested tissues were plated five pieces/plate on the surface of potato dextrose agar (PDA) amended with $100 \mu\text{g ml}^{-1}$ streptomycin sulphate (Sigma Aldrich, St. Louis, MO) to inhibit bacterial growth in 90 mm-diam petri dishes. The plates were incubated for 7–10 days at 25°C in the dark. The morphological characteristics of the fungal structures such as acervuli and conidia originating from the leaves were carefully scraped using a sterile needle and examined under an upright microscope (Nikon Eclipse Ni-U, Japan) equipped with a digital camera (Nikon DS-Ri2, Japan) for measurements and photographs.

Colony pigmentation after 15 days of incubation on Czapek-dox Agar and PDA (Fig. 2) ranged from creamy, white to pale olivaceous grey with slightly raised aerial mycelium to a dense cottony mycelium with abundant small, dark-based acervuli with deep orange conidial ooze and scattered setae (Fig. 3). The conidial shape was oval to cylindrical, hyaline, aseptate, unicellular with blunt round ends. The average length and width of the conidia were 15.5 to 18.6 and 4.6 to 6.7 μm , respectively (Fig. 4), fitting descriptions published by Cannon et al. (2008) and Weir et al. (2012). Average growth rate for the isolates was 7.3 mm per day on PDA and Czapek Dox Agar at $25 \pm 0.5^\circ\text{C}$. Pure cultures of five *Colletotrichum*-like isolates were obtained by single-spore isolation. In order to obtain pure cultures, hyphal tips from the margin of each emerging fungal colony were subcultured on fresh PDA. Based on these morphological characters, the fungus was temporarily identified as *Colletotrichum gloeosporioides*. Isolate PoCg1 was selected as a representative strain for further tests. The pure culture of the *C. gloeosporioides* has been deposited in the Microbial

Fig. 2 Morphological characteristics of *Colletotrichum gloeosporioides* PoCg1 after 15 days incubation on Czapek-dox Agar (a) and Potato Dextrose Agar (b) at 25°C

Culture Collection of Plant Health Clinic, Mustafa Kemal University (BİSAK), Hatay, Turkey, with accession number BİSAK-PoCg66.

To confirm the identity, PCR was performed using *Colletotrichum gloeosporioides* species-specific primers (Carbone and Kohn 1999; Templeton et al. 1992). The total genomic DNA was extracted by using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions from five-day-old fungal mycelium grown from single hyphal tips on PDA at 28°C . Fragments of GAPDH (GDF1- GDR1), ACTIN (ACT512F-ACT783R), CHS (CHS79F-CHS345R) genomic regions of *C. gloeosporioides* isolate PoCg1 was amplified and sequenced in both directions. From the amplification of ACTIN, GAPDH and CHS gene regions, a 220, 227, 243-bp PCR product, respectively, was obtained for the fungal isolate and the resulting sequences were submitted to the NCBI GenBank. BLASTn analysis of these sequences had a 100% homology to *Colletotrichum gloeosporioides* strains. Gene sequences matched with ACTIN, GAPDH and CHS sequences (GenBank accession nos. MG387956, KX620243, KY440785, respectively), confirming *C. gloeosporioides* as the causal organism. Phylogenetic tree (Fig. 5) based on alignment of nucleotide sequences of partial ACT gene and GAPDH gene was constructed using MEGA version 7.0 (Kumar et al. 2016). The DNA sequences of *C. gloeosporioides* were deposited into GenBank under Accession Nos. MF522261,

Fig. 3 Abundant bright orange conidial ooze (conidiomata) showing through mycelium from agar surface (a), acervuli (b) and setae (c) of *Colletotrichum gloeosporioides* isolate PoCg1 on PDA medium

Fig. 4 Conidial growth after 10 day incubation from single hyphal tips of *Colletotrichum gloeosporioides* on PDA. Bars, 10 μ m

MG775282 and MG775281 for ACTIN, GAPDH and CHS genes, respectively.

A pathogenicity test was carried out by spraying on surface-sterilised and wounded pomegranate (cv. Hicaz) leaves each with 10 μ l of a suspension of conidia (4×10^6 conidia ml^{-1}) obtained from a single spore culture. Leaves sprayed with sterile distilled water served as controls. Both

inoculated and control leaves were covered with polythene bags. Fifteen seedlings of twenty-month-old pomegranate were kept separately in a growth chamber at 25 °C, 85% relative humidity, and 12-h photoperiod. No symptoms developed on the leaves sprayed with sterile water. Dark-grey spot symptoms reproduced after 9–12 days were similar to those initially observed on the trees. The fungus was successfully

Fig. 5 Phylogenetic relationship between *Colletotrichum gloeosporioides* and some reference isolates of *Colletotrichum* species retrieved from GenBank based on alignment of nucleotide sequences of partial ACT gene and GAPDH gene. The tree was constructed by the UPGMA method using MEGA version 7.0

reisolated from symptomatic leaves on PDA and exhibited the same morphological characteristics that were observed upon the initial isolation, thus fulfilling Koch's postulates.

Morphological characters, pathogenicity and the BLAST analysis of the nuclear gene region sequences against the NCBI database as described above revealed that the isolate we obtained in this study could be identified as *Colletotrichum gloeosporioides*. This pathogen has been reported on *Citrus reticulata*, *Ficus carica*, *Hedera helix*, *Persea americana*, *Persicaria perfoliata* and *Rhododendron ponticum* in Turkey (Farr and Rossman 2018). Although *C. gloeosporioides* on pomegranate has been previously reported from China, USA, Greece, India, Myanmar, Puerto Rico, and Virgin Islands (Farr and Rossman 2018), to the best of our knowledge, this is the first report of anthracnose on pomegranate in the Mediterranean Region of Turkey. Further studies are required to reliably assess the potential threat posed by this pathogen for commercial pomegranate production and to better define the host range of *C. gloeosporioides* in Turkey. The present study provides basic information for epidemiologic studies and developing disease management strategies. Also, it should search for effective bio-control agents and fungicides with acceptable residue levels for use in commercial pomegranate production in Turkey.

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