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Pomegranate dieback and fruit rot caused by *Coniella granati* recorded in Croatia

Odumiranje šipka i trulež plodova uzrokovani patogenom *Coniella granati* u Hrvatskoj

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ABSTRACT

Pomegranate (*Punica granatum*) is a native shrub and a fruit crop cultivated in regions with warmer climate. In Croatia, pomegranates are grown all along the coast, but with only a few commercial plantations. In 2022, yellowing of leaves, twig blight, tree dieback and fruit rot were observed on cultivars Hicaz and Wonderful in several pomegranate orchards in Neretva Valley. The symptoms resembled those described to be caused by the fungus *Coniella granati*, a potentially serious pathogen of pomegranate. Samples of twigs, fruits and trunk base fragments were collected from three locations: Metković, Opuzen and Buk Vlaka. The same fungus was regularly isolated from all symptomatic plant parts. Based on cultural, morphological and molecular analyses, the fungus was identified as *Coniella granati*. Pathogenicity test on pomegranate fruits cv. Wonderful reproduced the same symptoms of fruit rot as seen in the field. To our knowledge, this is the first record of *Coniella granati* on pomegranate in Croatia.

Keywords: *Punica granatum*, identification, crown rot, twig blight

SAŽETAK

Šipak (*Punica granatum*) je samonikli grm i voćna kultura koja se uzgaja u područjima s toplijom klimom. U Hrvatskoj se šipak uzgaja duž cijele obale, ali komercijalnih nasada je malo. Tijekom 2022. u dolini Neretve, u nekoliko voćnjaka na kultivarima Hicaz i Wonderful uočeno je žućenje listova, sušenje grana, odumiranje stabala i trulež plodova šipka. Simptomi su odgovarali opisu simptoma koje uzrokuje gljiva *Coniella granati*, potencijalno štetni patogen šipka. Uzorci plodova, grana i dijelova podnožja debla su uzeti na tri lokacije: Metković, Opuzen i Buk Vlaka. Morfološki ista gljiva izolirana je iz svih simptomatičnih dijelova. Na temelju izgleda kolonije, morfološke i molekularne analize, svi izolati su potvrđeni kao vrsta *Coniella granati*. Simptomi truleži plodova jednaki kao zabilježeni u voćnjacima razvili su se nakon umjetnih zaraza plodova šipka (cv. Wonderful). Prema našim saznanjima, ovo je prvi nalaz vrste *Coniella granati* na šipku u Hrvatskoj.

Ključne riječi: *Punica granatum*, identifikacija, trulež korijenova vrata, sušenje grana

INTRODUCTION

Pomegranate (*Punica granatum*) is a native shrub and a fruit crop cultivated in regions with warmer climate. The largest producers of pomegranates are China, India, Iran, USA and Turkey (Jain and Desai, 2018). In Croatia, pomegranates are grown all along the coast, but with only a few commercial plantations. The centre of pomegranate cultivation in Croatia is Neretva Valley, Dubrovnik - Neretva County. Pest and diseases of pomegranate in Croatia are relatively poorly investigated. Fungal species *Elsinoë punica*, *Penicillium* spp., *Aspergillus* spp. and *Alternaria* spp. were recorded as pomegranate fruit pathogens (Miličević et al., 2017). In 2017, symptoms of pomegranate dieback were observed in public areas and private gardens in Dalmatia. The fungus *Cytospora punicae* (Ascomycota, Fungi) was detected in samples collected (Piškurić, 2019; Ivandić, 2020). This pathogen is known to cause wood canker and twig dieback in pomegranates (Peduto et al., 2014; Triki et al., 2015; Samouel and Kanetis, 2015; Palou and Vincent, 2019).

In 2022, severe symptoms obviously caused by an unknown disease were observed in several pomegranate orchards in Neretva Valley. Tree dieback and visible crown necrosis resembled typical Pytophthora crown and root rot. However, yellowing of leaves, twig blight and unusual fruit rot also developed extensively, leading to poor production and high yield losses. The symptoms were the most prominent on cvs. Hicaz and Wonderful. Similar symptoms have been described in the literature to be caused by the fungus *Coniella granati* (Sacc.) Petr. & Syd. (Uysal et al., 2018; Jabnoun-Khiareddine et al., 2018; Tekiner et al., 2020). *Coniella granati* is a serious pomegranate pathogen, emerging rapidly in pomegranate-growing areas of the world (Linaldeddu et al., 2020; EFSA, 2023). So far, it has been confirmed in Cyprus (1957), Turkey (1973), Greece (2008), Spain (2010), California (2010), Israel (2011), Iran (2012), China (2014), Italy (2016), Tunisia (2018) and Hungary (2020) (EFSA, 2023). Yield losses are reported to reach up to 50% (EFSA, 2023). Warm Mediterranean climate with wet periods seems conductive to the development of the pathogen (Michailides et al., 2010; Palou et al., 2013; Thomidis, 2015; Munhuweyi et al., 2016; Uysal et al., 2018). Different chemical and biological control options are investigated (Uysal et al., 2018; Tekiner et al., 2020; Yang et al., 2021).

The aim of this study was to identify and characterize the pathogen associated with pomegranate dieback and fruit rot observed in 2022 in Croatia.

MATERIALS AND METHODS

Visual inspections and sample collection

Visual inspections were carried out in three pomegranate orchards in Neretva Valley, in September 2022. Samples were collected from symptomatic trees showing fruit rot, twig dieback and general decline. Sampling was carried out in three locations: Metković, Opuzen and Buk Vlaka. Twenty-eight samples were collected: 15 samples of rotten fruit, five samples of symptomatic twigs, four samples of inner bark from the base of the trunk and four soil samples. Trunk base fragments were taken from declining trees by removing the outer bark tissue and cutting the inner wood fragments with visible necrotic tissue. Four soil samples were taken for the eventual detection of *Phytophthora* species. Approximately five cm of topsoil was removed around declining trees, and about 100 g of soil and fine roots were collected from a depth of 15–20 cm. Each sample was placed in a separate bag. The material sampled was delivered and analysed in the Laboratory for Mycology, Centre for Plant Protection – CAAF in Zagreb.

Isolation

Symptomatic pomegranate fruits, twigs and trunk base wood fragments were washed under the tap water for several minutes. Parts of symptomatic and visually healthy fruit tissue were cut into smaller pieces, approximately 5 mm in diameter, surface-sterilized in 70% ethanol for two minutes, rinsed three times in sterile distilled water and dried in laminar flow. Cut fragments were inoculated on potato dextrose agar (PDA; Biolife Italiana S.r.l., Italy) and carrot piece agar (CPA; 50 g of cut carrot root pieces and

15 g of agar; Select Agar, Sigma-Aldrich) in Petri dishes. Petri dishes were incubated in darkness at 25 °C for five to seven days. To obtain axenic cultures, hyphal tips of emerging fungal colonies were sub-cultured on fresh PDA and CPA medium. Isolates were incubated at 22 °C and 12 h/12 h photoperiod.

Randomly selected trunk base wood fragments were also plated on P_5ARP medium (Erwin and Ribeiro, 2005), to check for the presence of *Phytophthora* species. Plates with P_5ARP were placed in a growth chamber at 15 °C for three to five days in darkness. The development of colonies was examined visually and under the microscope. As the eventual *Phytophthora*-resembling colonies did not emerge, no further isolation was performed.

Soil samples were analysed for the presence of *Phytophthora* species using the bait method (Themann and Werres, 1998). Freshly picked rhododendron leaves were used as a bait. Baiting on rhododendron leaves was carried out in a growth chamber, in darkness at 15 °C. After five to ten days, baited rhododendron leaf pieces, approximately 3 x 3 mm in size, were plated on P_5ARP media. As no *Phytophthora* colonies developed, no further isolation was performed.

Identification of isolates

Out of 24 pomegranate plant samples, 23 yielded similar fungus of PDA and CPA. As all colonies sporulated abundantly on both media, only PDA was used for morphological identification. Fourteen isolates, designated as Cg HR1 to Cg HR 14 were selected for examination of colony appearance, morphology of pycnidia, and morphology and measurement of conidia. Dimensions and morphology of 100 randomly selected conidia were measured in all isolates. Olympus BX53 optical microscope was used for examination. Measurements were performed using Olympus CellSens Dimension® software. Descriptions of *Coniella granati* by Alvarez et al. (2016), Uysal et al. (2018) and Jabnoun-Khiareddine et al. (2018) were used for the identification.

To confirm the identification of *C. granati* based on morphological characteristics, PCR amplification and the subsequent sequencing of ribosomal (ITS) gene region was performed on four selected isolates (Cg HR1, Cg HR2, Cg HR3 and Cg HR4). Before DNA extraction, mycelium grown on PDA was harvested from 7-day-old cultures with a sterile scalpel, stored in 2 ml tubes and ground into powder using liquid nitrogen. Nucleic acids extraction was performed using the Dneasy® Plant Mini Kit (Qiagen Inc., USA) according to the manufacturer's instructions. Primer pair ITS1 and ITS4 (White et al., 1990) was used for amplification. PCR reaction was performed in a total volume of 25 µl, containing 12.5 µl of EmeraldAmp® Max PCR Master Mix 2x (Takara), 5.5 µl of water, 1 µl of 10nM primers each and 5 µl of the extracted DNA. Reactions were performed by initial denaturation at 95 °C for 2 minutes, followed by 30 cycles of denaturation at 95 °C for 20 seconds, annealing at 55 °C for 25 seconds, extension at 72 °C for 50 seconds, with a final extension at 72 °C for 10 minutes. Electrophoresis was performed in 1% agarose gel in 1xTAE buffer (AccuGENE®, Lonza, Switzerland) for 45 minutes at 110 V. Results were visualized using UVIdoc HD2® gel documentation system. PCR products were purified by GenElute® PCR Clean-Up Kit (Sigma-Aldrich, USA) and sequenced at Macrogen Europe. Sequences were trimmed using Geneious Prime (Biomatters Inc., USA) and compared with the GenBank sequences using standard nucleotide BLAST.

Pathogenicity test

To confirm the pathogenicity of the identified fungus *Coniella granati*, pathogenicity test was performed on pomegranate fruits, cv. Wonderful. Nine fruits were surface-sterilized by immersion in 90% ethanol for 5 minutes, washed three times in sterile water and dried on sterilized blot paper. Fruits were wounded with a cork borer to a depth of 3 mm and 6 mm in diameter. Mycelial plugs from 10-day-old cultures on PDA were made with a cork borer of the same dimensions, and were inoculated into each wound. Isolates Cg HR1 and Cg HR4 were selected for the test. Each fruit was inoculated twice, on two opposite positions. Totally nine fruits were inoculated, with 18 inoculation points. Pure PDA plugs were used as control. Inoculated fruits were placed separately in

plastic boxes on moist filter blot, and incubated for 20 days in the growth chamber (RK-2, Kambič, Slovenia) at 70% humidity at 25 °C. The appearance of symptoms was assessed on a daily basis. As necrosis appeared after several days and progressed rapidly, re-isolation was carried out from cut fragments of necrotic fruit tissue after 10 days, as described previously. The fruits were left in the chamber, and the appearance of pycnidia was recorded after 20 days of incubation.

RESULTS AND DISCUSSION

Visual inspections in pomegranate orchards in Metković, Opuzen and Buk Vlaka revealed similar symptoms. Yellowing of leaves, dieback of twigs and branches, and rotten or mummified fruits covered with an orange-brown coating were observed in all inspected sites (Figure 1). Black fungal fruiting bodies were visible on the surface of infected fruits. After bark removal, necrotic areas were observed on the trunk base of declining trees. About one-third of the trees and about half of the fruits were symptomatic in all three orchards inspected. Almost identical symptoms caused by *C. granati* have been described in China (Chen et al., 2014), Tunisia (Jabnoun-Khiareddine et al., 2018), Turkey (Uysal et al., 2018), India (Mahadevakumar et al., 2019), Italy (Linandeddu et al., 2020) and Hungary (Szendrei et al., 2022).

A fungus identified as *Coniella granati* was isolated from 23 out of 24 symptomatic plant samples (fruits, twigs and trunk base wood). The presence of *Phytophthora* species was not confirmed in the soil samples or crown tissues. All isolates obtained in this study developed white to yellowish compact mycelium on PDA (Figure 2). Irregular concentric rings of pycnidia developed within 15 days of incubation. On CPA, the mycelium was less compact and whitish, with pycnidia regularly within one week of incubation. The effects of different culture media on the

Figure 1. Natural infection observed on twig and fruit of pomegranate cv. Wonderful

appearance, mycelial growth and pycnidia production of *Coniella granati* were in accordance with the previous descriptions of Jabnoun-Khiareddine et al. (2018) and Uysal et al. (2018).

Pycnidia of 14 examined isolates on PDA after 15 days of incubation were spherical, ostiolate and solitary, dark brown to black in color and 105 – 190 µm in diameter. The morphology of pycnidia was consistent with the descriptions of other authors (Jabnoun-Khiareddine et al. 2018; Uysal et al. 2018; Yang et al. 2021; Szendrei et al. 2022). Conidia were ellipsoid with truncated base, unicellular and hyaline, 8.38 – 16.46 µm x 2.8 – 4 µm in size. Morphological characters of all isolates were in line with the descriptions of *C. granati*, and the species isolated from pomegranate samples were morphologically identified as *Coniella granati* (Sacc.) Petr. & Syd. ITS sequences of Cg HR1, Cg HR2, Cg HR3 and Cg HR4 showed 100% similarity with the sequence of *C. granati* KX507098 and 99.80% with the sequence MT6112233 (Linaldeddu et al. 2020). The sequence of Cg HR1 isolate was deposited in GenBank database under the accession number OQ525804.

In pathogenicity tests, all pomegranate fruits of cv. Wonderful inoculated with *C. granati* developed typical symptoms of fruit rot within five days. Control fruits remained symptomless. Brown to black lesions appeared around the inoculation site and rapidly spread into the fruit flesh and seeds. Infected tissue turned brown, soft and juicy and finally rotted completely within 20 days. The results are in agreement with the symptoms reported on cv. Gabi (Jabnoun-Khiareddine et al. 2018), cv. Hicaz (Uysal et al. 2018), and observations by Tekiner et al. (2020). Within 20 days' post-inoculation, dark brown to black pycnidia developed on the surface of inoculated fruits. *Coniella granati* was consistently re-isolated from the infected tissue.

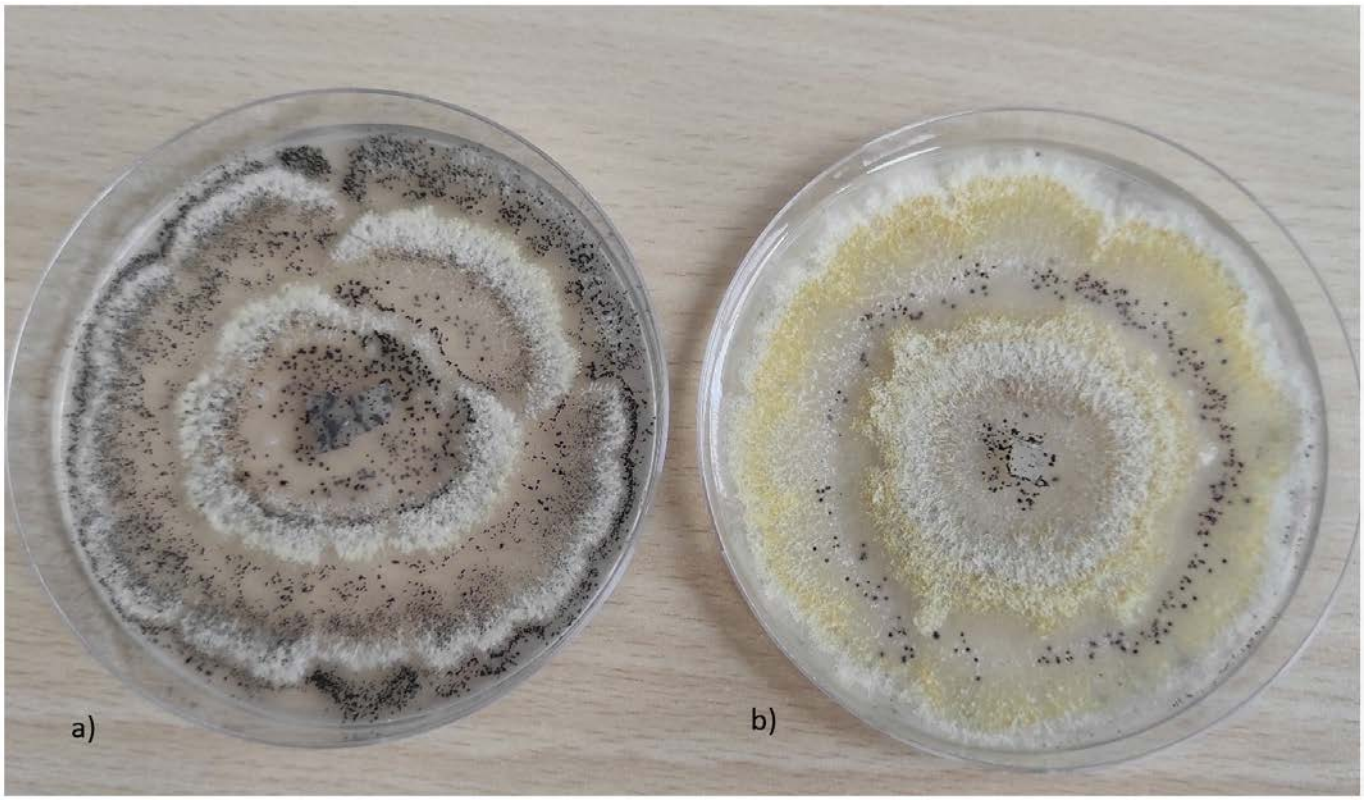

Figure 2. Colonies of Cg HR1 isolate (a) and Cg HR4 (b) of *Coniella granati* on PDA after 20 days of incubation

CONCLUSIONS

The study revealed the presence of *Coniella granati* in pomegranate orchards in Neretva Valley. To our knowledge, this is the first report of this serious pathogen on pomegranates in Croatia. Identification of *C. granati* was carried out on the basis of cultural and morphological characteristics, and subsequently confirmed by PCR and pathogenicity testing. Although only pathogenicity on fruits was proven in the present study, the isolation of the fungus from twigs and trunks of declining trees is indicating that *C. granati* is causing more extensive damage in Croatian pomegranate orchards, threatening the production of this fruit crop. Further studies are needed to assess the extent of *C. granati* damage and distribution, but also to investigate the effective measures for the control of this pathogen.

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