Communication

First report of *Phaeoacremonium iranianum* causing olive twig and branch dieback

Elena Petrović [1], Karolina Vrandečić [2], Jasenka Ćosić [2], Gabriella Kanižai Šarić [2] and Sara Godena [2,*]

[1] Institute of Agriculture and Tourism, Karla Huguesa 8, Poreč 52440, Croatia; elena@iptpo.hr (E.P.); sara@iptpo.hr (S.G.)
[2] Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek, Osijek 31000; kvrandecic@fazos.hr (K.V.); jcosic@fazos.hr (J.Ć.), gkanizai@fazos.hr (G.K.Š.)
* Correspondence: sara@iptpo.hr

Abstract: In the olive orchard on the western part of Istria, Croatia, twig and branch dieback was observed on several olive trees from the local cultivar 'Buza'. In total, seven samples from symptomatic trees were collected. Samples were analyzed, and four fungal isolates showed morphological similarities to the species *Phaeoacremonium iranianum.* One isolate, chosen as a representative, was taken for molecular identification and pathogenicity tests. Based on DNA sequence data of ITS1/ITS4, βt2a/βt2b, EF1-728F/EF1-986R, the isolate was identified as *P. iranianum*. Pathogenicity tests were conducted on detached olive branches and olive trees in the greenhouse, and identified fungal species was pathogenic on olive. To the best of our knowledge, this is the first report of twig and branch dieback on olive causing by phytopathogenic specie of fungus *Phaeoacremonium iranianum*.

Keywords*: Phaeoacremonium iranianum*, olive, dieback

1. Introduction

Olive (*Olea europaea* L.) is one of the most important crops in the Mediterranean part of Croatia. According to the latest statistical data, Croatian national production of olives is approximately 23 800 tones [1]. Olive trees are known to be drought-resistant and hardy, and susceptible to several major diseases [2], but recently olive is becoming more susceptible to diseases caused by phytopathogenic fungi. The main reasons are changes in cultivation methods, planting of infected plant material, increasing resistance of pathogens to fungicides, climatic extremes, etc. In recent years, there have been various occurrences of new diseases on olive trees in Istria that were unknown even to the experienced olive growers. In order to create a plant protection strategy (within the framework of sustainable olive production), the detection of the causative agents of this unusual olive diseases is crucial.

2. Materials and Methods

2.1. Sampling and fungal isolation

In 2021, olive trees, which showed signs of twigs and branches dieback (Figure 1), discoloration of the bark, and necrotic lesions, were spotted in olive orchard on western side of Istria, Croatia. The surface of the orchard was 0.43 ha and contained approximately 70 olive trees. Olive trees of the orchard (100% local cultivar 'Buza') were over 30 years old and grown on the soil where grapevine was grown beforehand. In total, seven samples of branches from symptomatic trees of 'Buza' were collected and brought into the laboratory for analysis. Small pieces of branches were rinsed under tap water, surface sterilized in 70% ethanol for one minute, rinsed two times in sterile distilled water, and placed on a sterile paper sheet in laminar flow cabinet till dry. Pieces of branches were plated on PDA

 doi:10.20944/preprints202212.0104.v1

amended with 35 mg/L of penicillin and incubated. After five days of incubation at 25 °C under dark condition, isolates were transferred onto the fresh PDA medium.

Figure 1. Disease symptoms on olive branches in the orchard near Rovinj in Istria, Croatia, in the year 2021.

2.2. Morphological and molecular identification

After 14 days of incubation at 25 °C in dark conditions, pure fungal cultures were taken to examination. The four isolates showed morpological similarities to the species *Phaeoacremonium iranianum*. One isolate (R18 B4), choosen as representative, was taken for molecular identification. Total DNA from the isolate was extracted with Maxwell® RSC Plant DNA Kit (Promega, Madison, Wisconsin, USA). The PCR reaction was performed using ITS1/ITS4 [3], βt2a/βt2b [4], and EF1-728F/EF1-986R [5] pair of primers. The PCR reaction mixture was composed of 12,5 µL of EmeraldAmp® GT PCR Master Mix, 0,5 µL of each primer, 6,5 µL of nuclease-free water, and 5 µL of genomic DNA. Polymerase chain reactions (PCR) (Table S1), were conducted in a SureCycler 8800 Thermal Cycler (Agilent Technologies, Santa Clara, California, USA**)**. The PCR products were visualized on 1% agarose gel light using an iBright CL1000 Imaging System (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Purification of PCR products was done with the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich®, Burlington, Massachusetts, USA), and sequencing (with EZ-Seq) of the PCR products was done by Macrogen Europe (Amsterdam, Netherlands). Sequences were edited in Sequencher® (Gene Codes Corporation, Ann Arbor, Michigan, USA) and compared with sequences from GenBank®.

2.3. Pathogenicity tests of isolate

Two pathogenicity tests were conducted to determine pathogenicity of isolate on olive tree: one on detached branches from cultivars 'Buza' and 'Rosinjola' in the laboratory, and another one on the four-year-old olive tree of the cultivar 'Rosinjola' in the greenhouse. Detached branches were washed with water, surface sterilized in 10% sodium hypochlorite solution for 10 minutes, rinsed with sterile distilled water for 10 minutes, and placed in laminar flow cabinet, on sterile paper, till dry. Branches were inoculated by placing a 4-mm-diameter mycelium plug from a 14-day-old PDA culture of R18 B4 isolate in a wound made with a 4-mm-diameter cork-borer. Wounds were sealed with vaseline and protected with Parafilm. Ten branches in total, per cultivar, were used. Fungal treatments were compared to the control treatment inoculated only with PDA plugs without mycelia, sealed with vaseline, and protected with Parafilm. Inoculated branches had been kept in laboratory conditions for 20 days.

Randomly chosen branches from olive trees from the greenhouse were inoculated the same way as previously described for detached branches. Inoculated plants had been kept in a greenhouse for three months, from March to July 2022, and monitored for the presence of symptoms. After incubation, samples were collected, and in an attempt to fulfill Koch's postulate, small pieces of necrotic tissue from the edge of each lesion were cut and placed on PDA to recover inoculated fungus.

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3. Results

3.1. Sampling and fungal isolation

In the field the symptoms of the disease on 'Buza' olive trees were wilting and dieback of twigs and branches, the same as brown internal necrosis. The symptoms such as dieback were observed on lateral branches, on one side of the trees. When the outer layer of bark from the branches was scraped away, it has been revealed that the brownish discoloration has extended on the surrounding tissue.

3.2. Morphological and molecular identification

Fungal isolates has been identified based on the colony characteristics (color, form, margin, elevation, surface, and opacity), and spores characteristics (color, presence or absence of septum, and shape). The developed fungal colonies were brownish on PDA, reverse darker brown; circular shaped with an entire edge, with aerial, opaque, and cottony mycelium, and branched septate hyphae (Figure 2). The isolate produced hyaline, unseptate, and ovoid conidia. These morphological characteristics identified the fungus as *Phaeoacremonium iranianum* L. Mostert, Gräfenhan, W. Gams & Crous, 2006 [6]. For molecular identification, consensus sequences of representative R18 B4 isolate were produced (GenBank accession numbers: OP627795 for ITS, OP684932 for TUB, and OP684933 for EF1α gene). BLAST analysis of the sequences showed 100% similarity with *P. iranianum* (reference number MG745842 for ITS, KF179086 for TUB, and KF764625 for EF1α gene). Phylogenetic analysis (Figure S4) was made using ITS sequence data from reference isolate, R18 B4, and isolates from GenBank. The sequences were aligned using ClustalX2 (UCD Dublin, Ireland) software, and a phylogenetic tree was made using MEGA11 (Pennsylvania State University, USA) software.

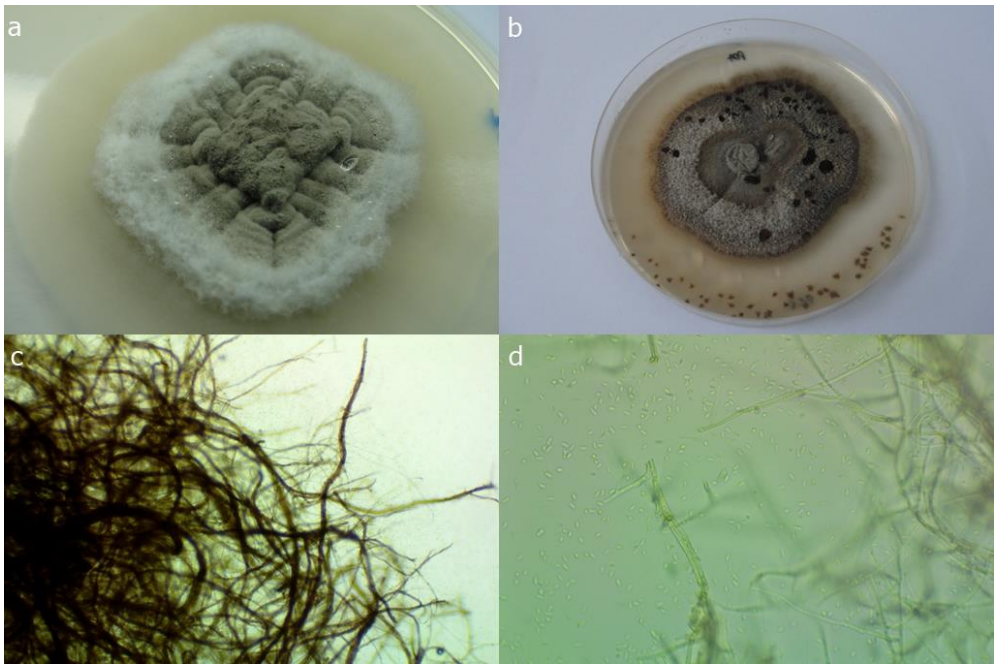

Figure 2. (**a**) *P. iranianum* colony, on PDA, after two weeks in the dark at 25 °C. (**b**) *P. iranianum* colony, on PDA, after two months. (**c**) Micrographs of *P. iranianum* isolate under the microscope. Scale bar = 10 μm. (**d**) Hyaline, ovoid conidia.

3.3. Pathogenicity tests of isolate

The symptoms of the disease on olive branches tested in the laboratory, and on the branches collected from olives in the greenhouse showed similar symptoms as the branch

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samples collected from the field survey. Brown streaking in cross-section was detected (Figure 3), and when the outer layer of bark from branches was scraped away, brown discoloration extended around the affected tissue. The pathogen had been consistently reisolated from affected pieces of wood, while the controls remained healthy. To fulfill Koch's postulate, one isolate, chosen as a representative, was carried out for molecular identification, as previously described, using an ITS5/ITS4 [7] pair of primers. Purification and sequencing of the PCR products were done by Macrogen Europe (Amsterdam, Netherlands). BLAST analysis of the ITS5 and ITS4 sequences showed 100% similarity with *P. iranianum* (reference number MG745842).

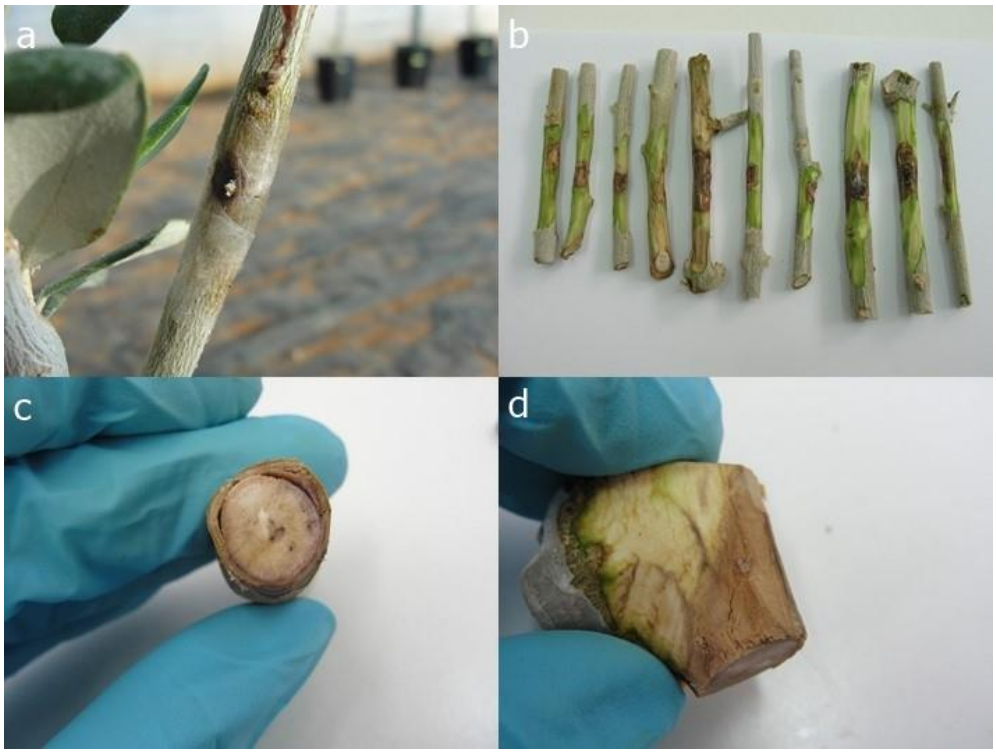

Figure 3. (**a-d**) Disease symptoms on branches used in pathogenicity tests from March to July 2022.

4. Discussion

There are several species from the *Phaeoacremonium* genus associated with olive diseases worldwide: *Phaeoacremonium africanum* [8,9], *P. alvesii* [10], *P. italicum* [10-12], *P. minimum* [8-14], *P. oleae* [8,9], *P. parasiticum* [8-11,15], *P. prunicola* [8,9], *P. scolytii* [8-12], *P. spadicum* [8,9], and *P. sicilianum* [10,11]. *P. iranianum* is a specie from the family *Togniniaceae*, named after the country, Iran, from which the majority of strains were collected [6]. It was previously described as a plant pathogen on several species of woody plants, including almond trees [16,17], citrus trees [18], cypress trees [19], forest trees [20], grapevine [20-27], pome fruit (apple, quince, hawthorn, pear) [28], and prunus trees [29]. It's mostly associated with Petri and Esca diseases, one of the most destructive declining diseases of grapevine [22]. As the observed infected olive trees were grown on the ground where grapevines were previously grown, and since olive and grapevine share common pathogens such as phytopathogenic fungi from the *Botryosphaeriaceae* family, there is a high risk of transmission of *P. iranianum* between grapevines and olives [26]. Aerial spores can be dispersed between vineyards that were near each other and those established in close proximity to fruit orchards, ornamental trees, or numerous other woody hosts [26]. This poses a danger to olive trees, especially in the Mediterranean part of Croatia, where vines and olives are often grown together. To the best of our knowledge, this is the first report of *Phaeoacremonium iranianum* causing olive twig and branch dieback on olive trees.

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Supplementary materials:

ITS4 and ITS5 sequences from Koch's postulate.

PIKOCHB_ITS_ITS4.txt PIKOCHB_ITS_ITS5.txt

Table S1. PCR amplification program set according to Alves et al. (2006) [30].

Hot Start 95 °C	Start Cycle	Denaturation 94 °C	Annealing 55 °C	Elongation 72 °C	End Cycle	Elongation 72 °C
5 minutes	30 times	30 seconds	45 seconds	1 minute and 30 seconds		10 minutes

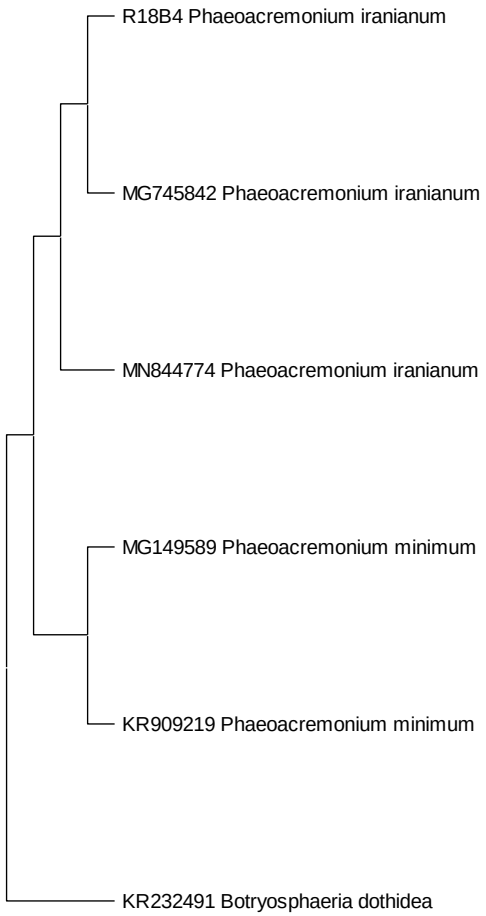

Figure S4. Phylogenetic tree based on internal transcribed spacer (ITS) sequences alignment generated from neighbor-joining tree.

Author Contributions: Conceptualization, E.P. and S.G.; methodology, E.P and S.G.; investigation, E.P. and S.G., writing—original draft preparation, E.P.; writing—review and editing, S.G., K.V., J.Ć., G.K.Š. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Croatian Science Foundation Installation Research Project "Natural bioactive compounds as a source of potential antimicrobial agents in the control of bacterial and other fungal pathogens of olives", Anti-Mikrobi-OL (AMO), UIP-2020-02-7413.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All sequences data for isolate R18 B4 are available in NCBI GenBank following the accession numbers in the manuscript. Sequences data from Koch's postulate, PCR amplification program, and phylogenetic tree are available in Supplementary materials.

Conflicts of Interest: The authors declare no conflict of interest.

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