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Short Notes

Characterization and pathogenicity of *Pleurostoma richardsiae* causing decline of mango trees in Southern Italy

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Summary. Mango trees (*Mangifera indic*a) showing symptoms of twig and branch dieback, internal wood necroses, and decline, were surveyed in an orchard in Palermo province (Eastern Sicily, Italy). A *Pleurostoma*-like fungus was consistently isolated from symptomatic wood tissues. Based on morphology and phylogenetic analysis of ITS and *tub2* sequences, the fungus was identified as *Pleurostoma richardsiae*. A pathogenicity test was conducted by inoculating stems of 2-year-old mango seedlings with mycelium plugs and conidium suspensions of a representative isolate. Two months after inoculation, necrotic lesions were observed around the inoculation points, and *P. richardsiae* was reisolated from the necrotic tissues. This is the first report of *P. richardsiae* causing dieback and decline of mango trees.

Keywords. Fungal diseases, *Mangifera indica*, wood necrosis, twig dieback, phylogeny.

Mango (*Mangifera indic*a L.; *Anacardiaceae*) is a fruit tree crop that is native to India and Southeast Asia (Mukherjee, 1953). Mango is widely cultivated especially in tropical and subtropical regions. In recent years, its cultivation has increased in the Mediterranean basin, due to the popularity of the fruit among European consumers and good productivity, and adaptability of the species to different environments. In Italy, the cultivation of subtropical crops is mainly concentrated in the coastal regions of Sicily (Palermo, Messina, and Catania provinces), where climate and soil conditions are suitable (Lauricella *et al.,* 2017). The growing interest in the mango fruit is due to their high content of bioactive compounds beneficial for human nutrition and health, which makes it attractive for direct consumption and useful for food and pharmaceutical industries (Maharaj *et al.,* 2022; García-Mahecha *et al.,* 2023).

In Italy, few pre- and post-harvest fungal diseases of mango have been reported. The most production-limiting diseases include canker and shoot blight caused by *Botryosphaeriaceae* spp. (Aiello *et al.,* 2022), and fruit decay and stem-end rot caused by *Colletotrichum* spp. (Ismail *et al.,* 2015). Stem-end rot of fruit caused by *Neofusicoccum* spp. has also been occasion-

Figure 1. Decline of mango trees 'Glenn' grafted on 'Gomera 3', caused by *Pleurostoma richardsiae* in an orchard located in Bagheria (Palermo, Italy). (a) Dieback of twigs and branches showing necroses of inner wood tissues. (b) Severe dieback of a mango tree.

ally observed (Ismail *et al.,* 2013a). Leaf spots caused by *Pestalotiopsis uvicola* and *P. clavispora* (Ismail *et al.,* 2013b), and wilt caused by *Verticillium dahliae* (Ahmed *et al.,* 2014), have also been reported.

Since 2021, dieback of twigs and branches was observed on 6-year-old mango trees ('Glenn' grafted on 'Gomera 3' rootstocks) during surveys of mango orchards in Bagheria (Palermo province, Italy). Affected trees had declining vegetation vigour during the year, followed by rapid dieback of twigs and branches, often leading to tree death (Figure 1). During a survey in May 2023, examination of cross-sections of branches and twigs of declining and low vigour trees revealed irregular, brown to black wood necroses, co-occurring in some cases with black spots and reddish-brown to black streaking of the inner wood tissue (Figure 2). Further observations detected canopy thinning and dry leaves hanging from the twigs. The sudden decline observed after years of slow and stunted growth made disease incidence variable, with few plants showing dieback at each season, and disease incidence based on these symptoms was approx. 10%.

Twigs and branches showing internal necroses were randomly collected from five plants, and were kept in plastic bags and taken to a laboratory for pathogen isolation and further analyses. Small wood fragments (3 × 3 × 3 mm, n = 160) from the margins of necrotic or apparently healthy tissues were surface sterilized in a 1.2% sodium hypochlorite solution for 60 s, and then rinsed once in sterile distilled water for 60s. The fragments were air dried in a laminar-flow cabinet on sterile paper, placed on potato dextrose agar (PDA, Lickson) amended with lactic acid (APDA; containing 1 mL L^{-1} of 98% [vol/vol] lactic acid), and were then incubated at approx. 25°C under natural light for 10 d. One type of fungal colony consistently grew from the symptomatic mango tissues, with isolation frequency of 60 to 72%. From twelve representative *Pleurostoma*-like colonies, single-conidium isolates were obtained, and were stored in the collection of the Dipartimento di Agricoltura, Alimentazione e Ambiente, section Patologia Vegetale, University of Catania.

Two representative isolates (CP23, CP28) were grown on acidified Malt Extract Agar (AMEA) at 25 ± 1°C for 21 d in the dark, to study colony morphology

Figure 2. Details of symptoms on a mango tree 'Glenn'. (a to d) Cross-sections of branches and twigs of declining trees with irregular, brown to black wood necroses. (e) Cross-section of a twig showing reddish-brown to black streaking in the inner wood tissue. (f) Longitudinal section of branch showing internal wood necrosis.

(texture, density, obverse and reverse colour, and margin), according to Vijaykrishna *et al.* (2004). Mycelium samples were mounted on microscope slides in lactic acid. Lengths and widths of conidia (n = 30) were measured at 100× magnification, using a Zeiss Axiolab 5 microscope and Zeiss Axiocam 208 color, using the software Zen Core (v.35.96.03000), and average dimensions and length/width ratios of the dimension means were calculated.

Colonies grown on AMEA for 20 d had white to off-white cottony appearance in the centres, with outwardly decreasing aerial hyphae extending to light grey, slightly uneven colony margins, as reported by Lawrence *et al.* (2021). The colonies produced two distinct types of conidia. One type were brown subglobose to spherical and thick-walled, with dimensions (min, average, max; length/width ratio ± standard error of the mean) of (1.5–) 2.2 (–3.1) × (1.5–) 2.0 (–2.8) µm; 1.1 ± 0.03. The other types were hyaline, cylindrical to oblong ellipsoidal, and thin-walled, with dimensions (4.5–) 5.3 (–6.8) × (1.8–) 2.6 (–3.5) µm; 2.4 ± 0.08.

All collected fungal isolates were grown on PDA for 14 d, and mycelium was then removed with a sterile scalpel. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corporation). The obtained DNA was stored at 4°C for further analyses. Two gene regions were amplified and sequenced. The internal transcriber spacer region (ITS) of the nuclear ribosomal RNA operon was amplified with the primers ITS5 and ITS4 (White *et al.,* 1990), and the primers Bt2a and Bt2b were used for the partial beta tubulin gene (*tub2*) (Glass and Donaldson, 1995). PCRs were carried out in a total volume of 25 µL, using One Taq® 2X Master Mix with Standard Buffer (BioLabs), according to the manufacturer's instructions. PCR conditions were set as follows: 30 s at 94°C; 35 cycles, each of 30 s at 94°C, 1 min at 52°C, and 1 min at 68°C; and 5 min at 68°C. PCR products were visualized on 1% agarose gels (90 V for 40 min) stained with GelRed® Nucleic Acid GelStain (Biotium), were purified, and then sequenced in both directions by Macrogen Inc. (Seoul, South Korea). The obtained forward and reverse DNA

Table 1. Fungal isolates from mango used in phylogenetic analysis. Isolates in bold font were obtained in the present study.

Fungal species	Isolate ID	Host	Location	GenBank accession number[b]	
				ITS	*tub2*
Calosphaeria africana	CBS 120870[a]	*Prunus armeniaca*	South Africa	EU367444	EU367464
Calosphaeria pulchella	CBS 115999[a]	*Prunus avium*	France	EU367451	KT716476
Flabellascus tenuirostris	CBS 138680[a]	*Fagus sylvatica*	Czech Republic	KT716466	KT716488
Jattaea algeriensis	STEU-6201[a]	*Prunus salicina*	South Africa	EU367446	EU367466
Jattaea ribicola	CBS 139779[a]	*Ribes petraeum*	Austria	KT716463	KT716480
Phaeoacremonium minimum	CBS 246.91[a]	*Vitis vinifera*	Yugoslavia	AF017651	AF246811
Phaeoacremonium novae-zelandiae	CBS 110156[a]	*Cupressus macrocarpa*	New Zeland	KF764572	DQ173110
Pleurostoma ochraceum	CBS 131321[a]	*Homo sapiens*	Sudan	JX073270	JX073271
Pleurostoma ootheca	CBS 115329[a]	Unknown	Thailand	MH862984	JX073272
Pleurostoma repens	CBS 294.39[a]	*Pinus* sp.	FL, USA	NR_135925	JX073273
Pleurostoma richardsiae	CBS 270.33[a]	Unknown	Sweden	AY179948	AY579334
P. richardsiae	EFA 317B	*Vitis* sp.	Spain	KX036522	KX036523
P. richardsiae	pr_GRAP	*Vitis vinifera*	Brazil	MG966406	MH053437
P. richardsiae	pr_OLIV	*Olea europaea*	Brazil	MG966416	MH053439
P. richardsiae	KARE488	*Prunus domestica*	Tulare County, CA	MT645621	MT734998
P. richardsiae	KARE1566	*Olea europaea*	San Joaquin County, CA	MT645625	MT735002
P. richardsiae	CP12	*Mangifera indica*	Bagheria (Sicily, Italy)	PP001252	PP025884
P. richardsiae	CP15	*Mangifera indica*	Bagheria (Sicily, Italy)	PP001253	PP025885
P. richardsiae	CP22	*Mangifera indica*	Bagheria (Sicily, Italy)	PP001254	PP025886
P. richardsiae	CP23	*Mangifera indica*	Bagheria (Sicily, Italy)	PP001255	PP025887
P. richardsiae	CP28	*Mangifera indica*	Bagheria (Sicily, Italy)	PP001256	PP025888
P. richardsiae	CP30	*Mangifera indica*	Bagheria (Sicily, Italy)	PP001257	PP025889
P. richardsiae	CP31	*Mangifera indica*	Bagheria (Sicily, Italy)	PP001258	PP025890
P. richardsiae	CP32	*Mangifera indica*	Bagheria (Sicily, Italy)	PP001259	PP025891
P. richardsiae	CP33	*Mangifera indica*	Bagheria (Sicily, Italy)	PP001260	PP025892
P. richardsiae	CP35	*Mangifera indica*	Bagheria (Sicily, Italy)	PP001261	PP025893
P. richardsiae	CP36	*Mangifera indica*	Bagheria (Sicily, Italy)	PP001262	PP025894
P. richardsiae	CP37	*Mangifera indica*	Bagheria (Sicily, Italy)	PP001263	PP025895

[a] Isolates linked to type specimens.
[b] ITS = internal transcribed spacer; *tub2* = beta-tubulin.

sequences were assembled, edited and aligned using MEGA X (Kumar *et al.,* 2018). All sequences of the ITS and *tub2* gene regions obtained were deposited in the National Centre of Biotechnology Information (NCBI) GenBank database (Table 1).

The obtained sequences were first compared with those available in GenBank. BLASTn searches of ITS and *tub2* sequences showed 99% similarity with the sequences of *Pleurostoma richardsiae* (Nannf.) Réblová & Jaklitsch (*Pleurostomataceae*, *Calosphaeriales*) isolate CBS 270.33 (GenBank accession No. MT153151.1), and 100% similarity with *P. richardsiae* isolate KARE1881 (GenBank accession No. MT735027.1). Phylogenetic analysis based on Maximum Parsimony (MP) was conducted on a concatenated dataset of ITS and *tub2,* including a total of 29 taxa, based on Lawrence *et al.* (2021). Multiple alignment was conducted in MEGA X. Maximum Parsimony analysis was carried out using Phylogenetic Analysis Using Parsimony (PAUP*) version 4.0a (Swofford, 2002), and *Phaeoacremonium minimum* CBS 246.91 and *Phaeoacremonium novae-zelandiae* CBS 110156 were used as the outgroups. The MP parameters were set as follows: heuristic search function and tree bisection and reconstruction (TBR) as branch swapping algorithms, with the branch swapping option set on "best trees" only. Gaps were treated as "missing", the characters unordered and of equal weight, and Maxtrees were limited to 100. MP scores including tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated. A total of 1,000 bootstrap replicates were performed to test the robustness of the tree topology. The MP analysis of the combined dataset showed that of 1574 total characters, 512 were parsimony informative, 268 were parsimony-uninformative, and 794 were constant. In total, 100 trees were retained. Tree scores were: TL = 1874, CI = 0.688, RI = 0.649, and RC = 0.446. The MP analysis showed that the isolates from Bagheria clustered within the group of *P. richardsiae*, with strong support (bootstrap support 100) (Figure 3).

To assess pathogenicity of *P. richardsiae*, isolate CP28 was inoculated onto 2-year-old potted healthy plants of mango 'Gomera 3', which were maintained in a growth chamber at 25 ± 1°C with a 12 h photoperiod. The stem of each plant was surfaced disinfected with 70% ethanol and wounded with a sterilised 5 mm diam. cork borer. Agar plugs (5 mm diam.) taken from 30-d-old fungal cultures growing on AMEA at 25 ± 1°C, or 20 μL of conidium suspension (1×10^5 conidia mL^{-1}), were placed into each stem wound. A total of six plants were inoculated, with three plants per inoculation method and two inoculation sites per plant along the stem. Control plants were inoculated with AMEA plugs and sterile distilled water. After inoculation, the wounds were sealed with Parafilm®, and the plants were maintained at 25 ± 1°C in a growth chamber. After 2 months, bark was removed with a sterile blade, and lengths of lesions (upward and downward from inoculation points) were measured, and means were calculated. Re-isolations were carried out (as described above) to determine fulfilment of Koch's postulates, and proportions (%) of *Pleurostoma*-like colonies were determined.

Pathogenicity tests confirmed that *P. richardsiae* was pathogenic to mango trees, causing reddish-brown to black necrotic lesions in the wood of all the inoculated plants, and these lesions were visible 2 months after inoculation (Figure 4, b and c). Control plants did not show any symptoms, except those due to wound oxidation (Figure 4, a and d). Mean lesion length from *P. richardsiae* isolate CP28 was 2.25 ± 1.29 from conidium suspension inoculations, and 2.85 ± 0.47 cm from mycelium plug inoculations. Re-isolation frequency was 80%, and these fungal colonies matched the originally inoculated *P. richardsiae* isolate as indicated by colony and conidium morphology.

Pleurostoma richardsiae has been reported from many countries as the cause of diseases in important crops. The fungus is a severe vascular pathogen of olive in Brazil, California, Croatia, Greece, Italy, Spain and South Africa (Carlucci *et al.,* 2013; Nigro *et al.,* 2013; Markakis *et al.,* 2017; Ivic *et al.,* 2018; Canale *et al.,* 2019; Spies *et al.,* 2020; Agustí-Brisach *et al.,* 2021; Lawrance *et al.,* 2021; van Dyk *et al.,* 2021). This fungus was also reported as a pathogen of grapevine in Brazil, California, Italy, Spain, and South Africa (Halleen *et al.,* 2007; Rolshausen *et al.,* 2010; Carlucci *et al.,* 2015; Pintos Varela *et al.,* 2016; Canale *et al.,* 2019). It was sometimes isolated from esca-affected vines, although its role in the disease has not yet been confirmed (White *et al.,* 2011). Avocado and nut crops have also been reported to be hosts of this pathogen (Olmo *et al.,* 2015; Markakis *et al.,* 2017; Sohrabi *et al.,* 2020). Several species of *Botryosphaeriaceae*, several *Phaeoacremonium* spp. and *Fomitiporia mediterranea*, have been isolated with *P. richardsiae* from woody tissues of grapevine (Pintos Varela *et al.,* 2016; Raimondo *et al.,* 2019), almond (Sohrabi *et al.,* 2020), and olive (Spies *et al.,* 2020; van Dyk *et al.,* 2021), showing the same symptoms. However, only *P. richardsiae* was isolated from symptomatic twig and branch tissues of mango trees in the present study.

This is the first report of *P. richardsiae* causing disease on mango trees. Future epidemiological studies are needed to assess the presence of *P. richardsiae* in mango

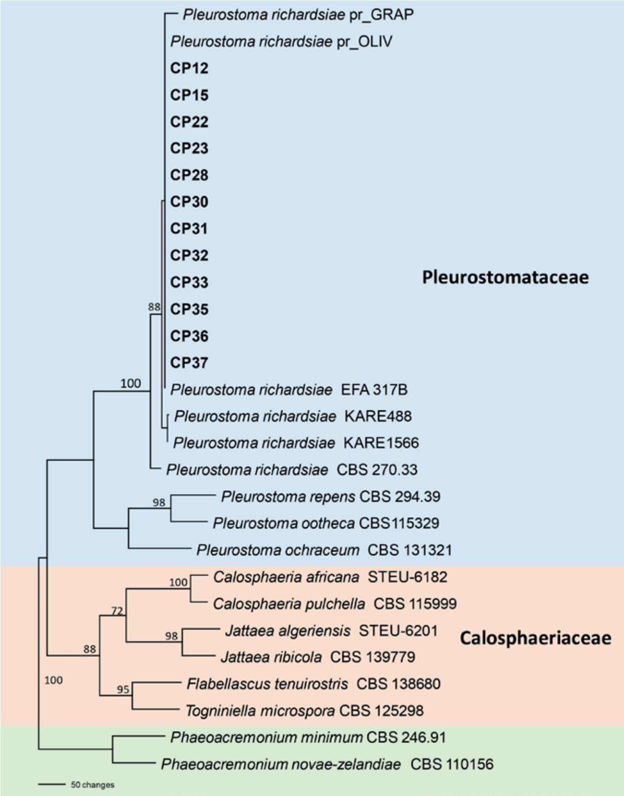

Figure 3. A parsimonious tree generated from maximum parsimony analysis of the two-gene (ITS + *tub2*) combined dataset. Numbers beside the clades represent parsimony bootstrap values from 1,000 replicates. Isolates in bold font were obtained in the present study. The bar indicates the number of nucleotide changes.

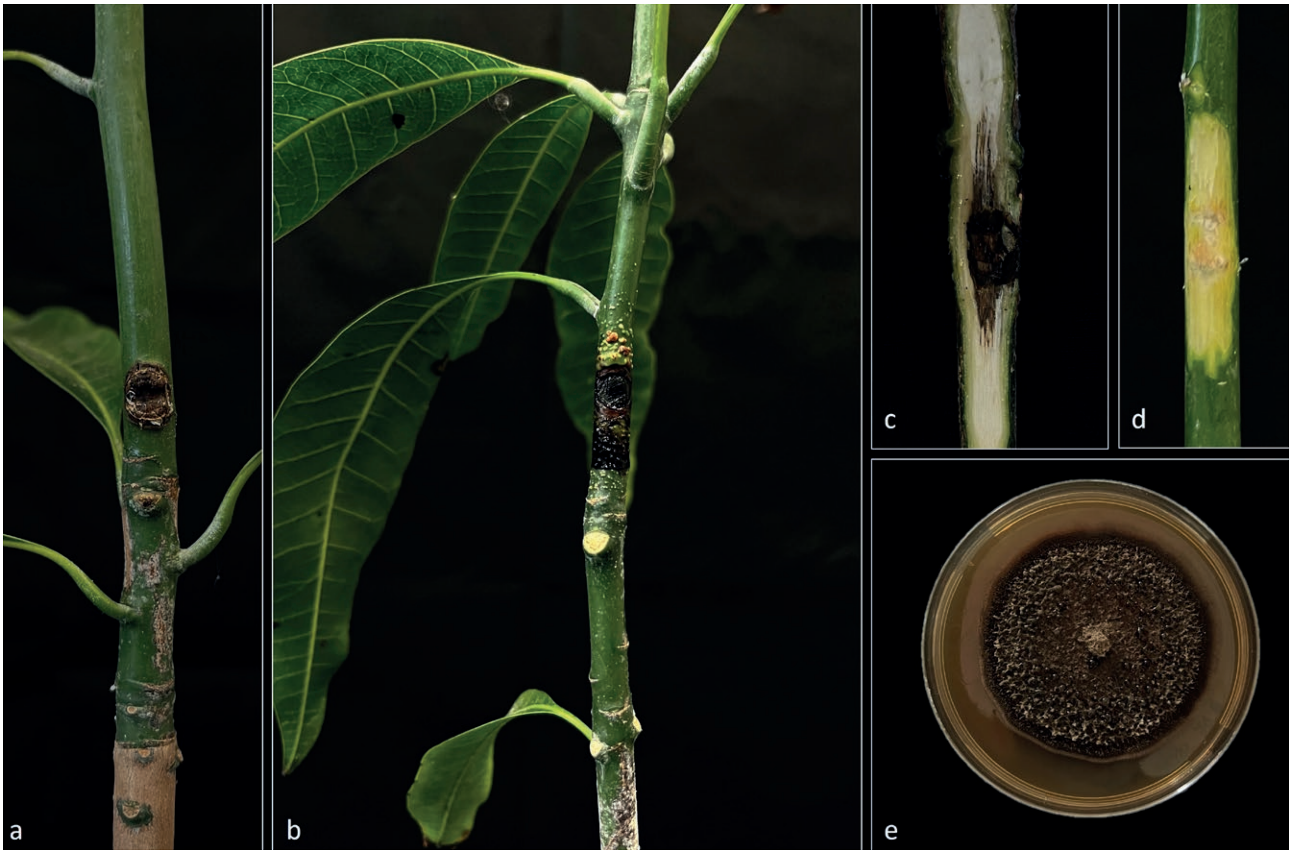

Figure 4. Pathogenicity test on mango seedlings 'Gomera 3'. (a) Agar plug inoculation control. (b) Stem inoculated with *Pleurostoma richardsiae* isolate CP28, showing a brown to black lesion. (c) An internal lesion produced by *P. richardsiae* isolate CP28 around the inoculation point. (d) Internal tissue of a control stem with no symptoms around the agar inoculation point. (e) Colony of *P. richardsiae* isolate CP22 grown on acidified malt extract agar for 30 d at 25°C.

orchards in other areas of Italy, and to evaluate possible effects of climate change on the emergence of new pathogen-host interactions. It is known that climate change may alter plant physiology and increase susceptibility to disease, and may also widen plant pathogen host ranges (Guarnaccia *et al.,* 2023; Joachin *et al.,* 2023). To date, *Botryosphaeriaceae* that cause canker and shoot blight of plants are considered the most damaging trunk pathogens for mangoes in Italy (Aiello *et al.,* 2022). The present record of severe damage of mango caused by *P. richardsiae* deserves attention for the risks it could pose to this important fruit tree crop.

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DATA AVAILABILITY

Nucleotide sequences from this study are deposited in NCBI GenBank with the accession numbers reported in the paper text.

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