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Short Notes

Pleurostomophora richardsiae associated with olive tree and grapevine decline in Southern Brazil

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Abstract. Olive trees showing decline symptoms were reported in an orchard in Santa Catarina, Southern Brazil. The symptoms included foliar browning and leaf drop, die-back of twigs and branches, dark streaks in internal crown wood and necrotic lesions in bark. A grapevine sample exhibiting browning streaks in the vascular tissue of branches, trunk and roots, was also analysed. A *Phialophora*-like fungus was isolated from symptomatic tissues of both hosts, and monosporic cultures were established. Based on morphology and analysis of ITS, *tub2* and *tef* sequences, the fungus was identified as *Pleurostomophora richardsiae*. Isolates inoculated onto young nursery olive trees and grapevines produced brown streaking in the wood in most of the inoculated plants. This is the first report of *P. richardsiae* occurring in olive trees and grapevines in Brazil.

Keywords. Fungal pathogenicity, molecular systematics, *Olea europaea*, *Vitis* spp. wood decay fungus.

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INTRODUCTION

Olive cultivation is emerging in Brazil as an option for farmers. The production and cultivated area is restricted to the southeast and south of the country where the established olive groves are young and have been in production for only a few decades. In 2016, national production was 647 t from 573 ha of harvested area, having increased 138% since 2014 (FAO, 2018). Government agencies estimate that approx. 110,000 L of olive oil were produced in 2017. Viticulture is mainly carried out in the south and northeast of Brazil, and approx. 0.8 million t of grapes were produced in 2014 (FAO-OIV, 2016). The total area of vineyards in Brazil is 86,000 ha, and the south region is responsible for 75.3% of the production of table grapes and wines (IBGE, 2017). Olive orchard and vineyard production supply mostly to the Brazilian domestic markets.

Studies of pathogens associated with grapevine decline in Brazil are few, and reports of pathogens in olive trees are even fewer (Correia *et al.*, 2013; Chliyeh *et al.*, 2014). During a survey of an olive orchard in Santa Catarina (SC), a state in Southern Brazil, a decline disease of olive trees was noted. At the same time, a grapevine plant brought to our laboratory for diagnosis showed brown streaking in the vascular tissues of the branches. The purpose of the study reported here was to determine the cause of these diseases.

MATERIALS AND METHODS

Sampling and fungal isolation

Symptomatic olive trees, showing foliar browning and leaf drop, wilting of apical shoots, dieback of twigs and branches, dark streaks in the internal wood, and crown and necrotic bark lesions (Figure 1) were noted during a survey in an experimental olive orchard in Itá, a municipality in SC (-27.273913, -52.339676), in 2015. The orchard was approx. 2.5 ha and contained 3-year-old olive trees of cv. Arbosana. Symptomatic trees were in a 1,000 m² area where the soil was poorly drained. Samples were taken from two diseased plants. At the same time (in 2016), a grapevine plant, aged approx. 3 years, with dieback symptoms, was received by our laboratory for disease diagnosis. The branches showed brown discolouration in the vascular tissue and the bark was cracking (Figure 1). The grapevine was from Riqueza municipality, also in SC (-27.064126, -53.327396). Small pieces of the necrotic and discoloured tissues were removed, surface-disinfected for 3 min in 1.5% sodium hypochlorite solution, washed twice in sterile water and then incubated on 2% potato dextrose agar (PDA, Merck) amended with 100 mg L^{-1} streptomycin sulphate (Calbiochem). One fungal isolate was obtained from each sampled plant, *i.e.*, two isolates from the two sampled olive tree and one from the diseased grapevine. After sporulation, 20 µL of a spore suspension were plated on PDA, and after 2 d of incubation individual germinated spores were transferred to fresh PDA plates. The resulting cultures produced *Phialophora*-like fungi (Vijaykrishna *et al.*, 2004). One isolate from olive (pr_OLIV) and one from the grapevine (pr_GRAP) were used for further analyses.

Morphological characterization

Morphological characterization followed the procedures of Vijaykrishna *et al.* (2004) and Carlucci *et al.* (2015) with some modifications. Colony morphology was determined after 21 d of incubation on 2% malt extract agar (MEA) at 25 ± 2°C in the dark. Dimensions and morphology of conidia, conidiophores and phialides were determined from microscopic examination of fungal tissues in lactic acid preparations. For each structure, 30 measurements were made with a ×100 objective on a Zeiss Scope A1 Axio microscope equipped with AxioCam MRC, operated through Zen Lite Software 2012 (Zeiss). Radial growth of the isolates was determined by placing individual 3 mm diam. mycelial discs on MEA in Petri dishes. Two perpendicular measurements of colony diameters were made with a digital caliper after 8 d incubation in the dark at 4°C, 25°C or 37°C, with five replicates at each temperature.

DNA isolation and sequencing

Genomic DNA of the two isolates was extracted using the Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's instructions. The 5.8S rDNA gene and flanking internal transcribed spacers 1 and 2 (ITS), partial β-tubulin (*tub2*) and translation elongation factor 1-α (*tef1*) genes were sequenced to confirm fungal identification, using, respectively, the primer pairs ITS4 and ITS5 (White *et al.*, 1990), Bt2a and Bt2b (Glass and Donaldson, 1995), and EF1 and EF2 (O'Donnell *et al.*, 1998). After amplification, PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen). Sequencing was performed by ATCGene (Alvorada, Rio Grande do Sul, Brazil) in forward direction. Sequences of pr_OLIV and pr_GRAP isolates were deposited in GenBank. Matches with sequences in GenBank was performed with BLAST searches.

Pathogenicity of P. richardsiae *isolates*

The two isolates obtained in this study were inoculated onto the same plant species from which they were each isolated. Isolate pr_OLIV was also inoculated onto grapevine cultivars and isolate pr_GRAP was inoculated onto an olive cultivar. One-year-old nursery plants of cv. Arbosana olive trees (*Olea europaea*) and two rootstock varieties of grapevines (*Vitis vinifera*), 'Paulsen' (*V. berlandieri* Planch. × *V. rupestris* Scheele) and 'VR04343' (*V. rotundifolia* Michx. × *V. vinifera* L.) approx. 50 cm tall, were growing in 6 L capacity plastic bags of potting mix. The plants were each wounded to about 3 mm depth with a histological needle at 7 cm above soil level. Colonized mycelial agar plugs (3 mm diam.) were placed on the wounds, and each covered with a wet

Figure 1. (a–c) Disease symptoms in olive trees. (a) Severe dieback of twigs and branches, with browning of leaves, and premature plant death. (b) Transversal cross section of an affected trunk showing dark brown to black streaking in the crown. (c) Dark streaking in the core of wood and necrotic lesions affecting the bark. (d–e) Grapevine branches showing cracks and vascular discoloration. Scale bar = 1 cm. (f–g) Longitudinal sections showing acropetal and basipetal wood discolouration from inoculation points in (e) olive tree and (f) grapevine. Scale bar = 1 cm. (h) *Pleurostomophora richardsiae* colony, on MEA after 21 d in the dark at 25°C. (i–j) Micrographs of *P. richardsiae* structures. Scale bar = 10 µm. Arrows in (i) show a phialide, a conidiophore and hyphal bundles. Arrows in (j) indicate subglobose and oblong ellipsoid conidia, and coiled hyphae.

piece of cotton and sealed with Parafilm. Mycelial plugs were taken from fungal colonies grown on PDA for 21 d at 23°C (± 2°C). Non-colonised sterile PDA plugs were used for non-inoculated controls. Five non-inoculated or inoculated plants were prepared for each isolate. Pots containing the plants were randomly arranged on a bench in a greenhouse covered with an anti-aphid net, with temperature around 25°C, and watered three times each week. The inoculation points were analysed 4 months after inoculation. A longitudinal cut was made in the wood tissue of each plant through the point of inoculation, and the length of the discoloured wood above and below the point of inoculation was measured with a digital caliper. Re-isolations were made from all of the inoculated pieces of wood to recover the isolates, in attempts to fulfil Koch's postulates.

RESULTS AND DISCUSSION

The symptoms of disease on olive trees were foliar browning and leaf drop, wilting and dieback of twigs and branches, dark streaking in the inner wood tissues and necrosis, leading to a generalized decline of the trees. The grapevine received for diagnosis showed advanced symptoms of dieback, with severe discolouration of the vascular tissues affecting the whole plant from the roots to the branches, where the bark was cracking (Figure 1). Isolates of a *Phialophora*-like fungus was isolated from the diseased plants. Detailed morphological characterization and molecular analyses identified the isolates as *Pleurostomophora richardsiae* (Nannf.) Réblová & Jaklitsch. Voucher cultures of these isolates were deposited in the culture collection of the Centre of Biological Sciences, Federal University of Pernambuco (UFPE), with access numbers 7857 (pr_OLIV) and 7858 (pr_GRAP).

Pleurostomophora richardsiae is frequently found when searching for other pathogenic fungi in necrotic lesions in the periderms of plants (Vijaykrishna *et al*., 2004; Mostert *et al*., 2006; Olmo *et al*., 2015; Réblová *et al*., 2015). This fungus is an emerging plant pathogen and is gaining recognition as an important vascular pathogen involved in Esca disease complex of grapevine, and, more recently, associated with decline of olive trees (Carlucci *et al*., 2013; Varela *et al*., 2016). Pathogenicity of *P. richardsiae* in grapevine was confirmed by Halleen *et al.* (2007) in South Africa, and this pathogen has been recorded in California (Rolshausen *et al*., 2010), Italy (Carlucci *et al*., 2015), Spain (Varela *et al*., 2016) and Turkey (Özben *et al*., 2017). Reports of *P. richardsiae* causing decline in olive trees are more recent. Carlucci *et al.* (2013) found *P. richardsiae* associated with olive tree decline in surveys conducted in Italy, and remarked on the aggressiveness of the fungus to this host, since it can frequently be the only pathogen isolated from affected trees.

After 21 d in the dark on MEA, colonies of pr_GRAP and pr_OLIV were cottony, light brown and paler towards the periphery, with entire margins (Figure 1), which agrees with the description provided by Carlucci *et al.* (2015). The mycelium is composed of branched septate hyphae, sometimes in bundles or forming coils (Figure 1). Neither of the isolates grew in cultures at 4°C. According to Carlucci *et al.* (2015) the minimum temperature for growth of *P. richardsiae* is 10°C. Mycelia of *P. richardsiae* developed at 25°C (mean colony diam. = 32.7 cm for pr_ OLIV and 30.2 cm for pr_GRAP), and at 37°C (mean colony diam. + 11.4 cm for pr_ OLIV and 8.5 cm for pr_GRAP). Morphology of the isolates agreed with the previous description of *P. richardsiae*, producing cylindrical and oblong ellipsoidal conidia, short, unbranched conidiophores and predominantly type II phialides. The phialides were elongated, ampulliform and constricted at the bases (Vijaykrishna *et al*., 2004; Mostert *et al*., 2006; Carlucci *et al*., 2015).

ITS sequences of pr_GRAP and pr_OLIV were lodged in GenBank (NCBI), under accession numbers, respectively, MG966406.1 and MG966416.1. BLAST searches showed that these isolates had 100% identity with several *P. richardsiae* ITS sequences already deposited in GenBank, including the isolate that was associated with grapevine decline in Spain, access number JX258852.1 (Varela *et al*., 2016). The ITS sequences also presented 99% identity with the ex-type isolate CBS 270.33 (GenBank accession number NR135933; Mostert *et al*., 2003). Sequences of partial *tub2* and *tef1* genes were also deposited in GenBank. The accession numbers for pr_GRAP *tub2* and *tef1* sequences are MH053437 and MH053438. For pr_OLIV *tub2* and *tef1* sequences, the accession numbers are MH053439 and MH053440.

In the pathogenicity tests, both isolates produced brown streaking in the wood in most of the inoculated plants (Figure 1). Despite of the spread of the brown streaking, the isolates did not kill the inoculated nursery plants. Both isolates were re-isolated from inoculated plants.

This is the first report of *P. richardsiae* associated with diseased olive trees and grapevine in Brazil. With this report, we hope to contribute to knowledge of fungal phytopathogens that can be found in these plant species in Brazil. Further epidemiological studies are required to investigate the incidence of *P. richardsiae* in the south and other regions of Brazil, where olive trees and grapevines are cultivated, and determine

the impacts of the pathogen on productivity of olive orchards and vineyards.

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