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Short note

First report of canker and dieback caused by *Neofusicoccum parvum* and *Diplodia olivarum* on oleaster in Italy

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Abstract

Oleaster (*Olea europaea* subsp. *europaea* var. *sylvestris*) is a sclerophyllous forest tree occurring in the Mediterranean region including Sardinia (Italy). Oleaster suffers from few major diseases but, since 2017, a new and unusual disease leading to the death of both young and old trees has been observed in several woodlands of high ecological value in north-eastern Sardinia. Declining trees showed a variety of symptoms including leaf chlorosis, dieback and sunken canker on trunk and branches. The bleeding cankers exuded a white to orange sap, which gradually dried to a whitish gluey mass on the bark. Symptomatic wood samples yielded fungal isolates belonging to five fungal species: *Diplodia olivarum*, *Dothiorella sarmentorum*, *Neofusicoccum parvum*, *Rosellinia corticium* and unidentified *Pestalotiopsis* sp. In pathogenicity trials, *Neofusicoccum parvum* proved to be the most aggressive species. Our results provide the first evidence for a combined involvement of different Botryosphaeriaceae species in the aetiology of a new oleaster disease.

Keywords
Sardinia; emerging diseases; Botryosphaeriaceae; *Pestalotiopsis*; *Rosellinia*

Introduction

Oleaster (*Olea europaea* subsp. *europaea* var. *sylvestris*) is a long-living tree economically, socially, and culturally intertwined with the populations of Mediterranean region as well as a key component of the rural landscape (Marcuzzi, 1996; Gianguzzi and Bazan, 2019). In the Mediterranean region, it is genetically divided into seven areas that could overlay glacial refuges (Breton et al., 2006).

Oleaster is commonly considered a tree tolerant to environmental stresses, pathogens and insect pests (Jiménez-Fernández et al., 2016; Sesli and Yegenoglu, 2017). However, during the last years an increase in root rot symptoms caused by *Phytophthora* spp. was reported in southern Spain (González et al., 2017, 2019).

In Sardinia oleaster is a typical component of the Mediterranean maquis. It is considered as a species of high ecological and economic value, given its high adaptability to different environments and the good quality of its wood (Bacchetta et al., 2003). Since 2017, a new and unusual severe disease leading to the death of both young and old oleaster trees has been observed in several woodlands in north-eastern Sardinia. Since there is no information about this new disease, a survey was conducted to establish the causal agents.

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Materials and Methods

Field surveys, sampling procedure and fungal identification

From spring 2017 to winter 2018, the health status of oleaster trees was monitored in fourteen woodlands located in north-eastern Sardinia. Samples of branches showing whitish exudations, canker and dieback were collected from 38 symptomatic trees of different age (Table 1). All samples were taken to the laboratory to be inspected and then immediately processed. The outer bark surface was removed with a sterile scalpel. Longitudinal and transversal cuts were made to observe any internal symptoms. Isolations were made from approx. 5 mm^2 chips of inner bark and xylem cut aseptically from the margin of infected tissues.

All plant samples were placed on petri dishes containing potato dextrose agar (PDA 39 g l^{-1}, Oxoid Ltd) growing medium. After incubation at 25 ± 1 °C for 5-7 days in the dark, hyphal tips from the emerging fungal colonies were sub-cultured onto half-strength PDA supplemented with autoclaved holm oak twigs and incubated at room temperature under natural daylight to enhance sporulation.

Fungal isolates were initially grouped in morphotypes on the basis of colony growth characteristics, including surface and reverse colony appearance, observed after 7 days of incubation on PDA at 25 °C in the dark and morpho-biometric data of conidia. Measurements of conidia were taken with the software Motic Images Plus 3.0 paired with a Moticam 10+ camera connected to a Motic BA410E microscope.

Table 1 - Study sites information and number of symptomatic branches sampled

Study sites	Coordinates (N, E)	Elevation (m a.s.l.)	Number of branches sampled
1	40°57’58”N - 9°33’40”E	74	1
2	40°58’00”N - 9°33’97”E	44	1
3	40°58’00”N - 9°33’97”E	36	1
4	41°00’20”N - 9°35’27”E	31	1
5	40°59’96”N - 9°32’92”E	27	1
6	41°00’41”N - 9°30’18”E	7	1
7	41°03’74”N - 9°28’00”E	25	1
8	41°06’47”N - 9°27’57”E	53	1
9	41°07’11”N - 9°26’11”E	84	1
10	41°09’89”N - 9°24’12”E	38	1
11	41°10’35”N - 9°17’47”E	41	6
12	40°58’56”N - 9°31’45”E	103	17
13	40°28’29”N - 9°08’74”E	327	1
14	40°26’10”N - 9°11’44”E	226	4

DNA extraction, PCR amplification and sequencing

Molecular analysis was used to confirm the identity of all isolates at species level. InstaGene Matrix (BioRad Laboratories, Hercules) was used to extract genomic DNA from 5-day-old cultures grown on PDA and incubated at 25 °C in the dark. The primers ITS1 and ITS4 (White et al., 1990) were used

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to amplify and sequence the internal transcribed spacer (ITS) regions, including the complete 5.8S gene. Polymerase chain reaction (PCR) mixtures and amplification conditions were as described by Linaldeddu et al. (2016a). The PCR products were purified using a EUROGOLD gel extraction kit (EuroClone S.p.A.) following the manufacturer's instructions.

The ITS regions were sequenced by the BMR Genomics DNA sequencing service (www.bmr-genomics.it), in both directions, with the primers used for amplification. The nucleotide sequences were read and edited with FinchTV 1.4.0 (Geospiza, Inc. http://www.geospiza.com/finchtv) and then compared with reference sequences (type material) retrieved from GenBank using the BLASTn algorithm. ITS sequences from representative isolates obtained in this study were deposited in GenBank (www.ncbi.nlm.nih.gov/genbank).

Pathogenicity test

To verify the pathogenicity of the species isolated, a field inoculation trial was conducted in June-August 2018 on asymptomatic branches of oleaster growing in a natural stand in Sardinia (40°26'10"N - 9°11'44"E). During the experimental period, the daily mean air temperature was 13–36 °C. Six branches (6-9 cm in diameter) were inoculated with a representative isolate of each fungal species, and six uninoculated branches were used as controls. The inoculated region of the branch was surface-disinfected with 70% ethanol and a piece (0.7 mm in diameter) of outer and inner bark was removed with a flamed cork borer and replaced with an agar-mycelium plug of the same size taken from the margin of an actively growing colony on PDA. The inoculation point was covered with cotton wool soaked in sterile water and wrapped in a piece of aluminium foil secured with masking tape. Controls were inoculated with a sterile PDA plug applied as described above. After 3 months, the outer bark was carefully removed with a scalpel and the size of necrotic lesion surrounding each inoculation point was measured.

Re-isolation of inoculated species was attempted by transferring 10 pieces of inner bark and wood taken around the margin of each lesion onto PDA. Cultures were grown in daylight and room temperature and then identified by analysis of ITS sequences.

Statistical analyses

Pathogenicity assay data were checked for normality and then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined using Fisher's least significant differences multiple range test ($P \leq 0.05$) after one-way ANOVA using XLSTAT software (Addinsoft).

Results

Symptomatology

Field surveys have shown that symptomatic oleaster trees are widespread along the north-eastern coast of Sardinia. Sampled trees displayed a variety of symptoms including leaf chlorosis, a progressive dieback of twigs and sunken canker on trunk and branches. These cankers often exuded a white to orange sap, giving them the appearance of bleeding, which gradually dried to a whitish gluey mass on the bark. After removing the outer and inner bark from cankers, dark brown necrotic lesions of variable size were visible on the xylem tissue (Figs. 1a-h). In cross-section, necrotic lesions appeared with the characteristic wedge-shaped aspect typical of Botryosphaeriaceae infections (Figs. 1i-k).

Aetiology

Isolations performed from 38 cankered branch samples yielded a total of 32 fungal colonies. On the basis of morphological features and DNA sequence data (ITS region), five distinct species namely *Diplodia olivarum* A.J.L. Phillips, Frisullo & Lazzizera (16 isolates from 5 sites), *Neofusicoccum*

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parvum (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (11 isolates from 4 sites), *Pestalotiopsis* sp. (2 isolates from 2 sites), *Rosellinia corticium* (Schwein.) Sacc. (2 isolates from 2 sites) and *Dothiorella sarmentorum* (Fr.) A.J.L. Phillips, A. Alves & J. Luque (1 isolate) were identified (Figs. 1l-p).

Fig. 1 - Main disease symptoms detected on oleaster trees: branch dieback and leaf chlorosis (a); active sunken cankers on the trunk and branches wet due to the emission of a whitish gluey mass and extensive necrotic lesion of xylem tissues in correspondence of the cankers (b-h); cross-section of cankers on branches and main stem with wedge-shaped necrotic sectors (i-k). Colony morphology of *Diplodia olivarum* (l), *Dothiorella sarmentorum* (m), *Neofusicoccum parvum* (n), *Pestalotiopsis* sp. (o) and *Rosellinia corticium* (p) after 7 days growth at 25 °C on PDA in the dark. Symptoms observed on oleaster branches 90 days after inoculation with: *Diplodia olivarum* (q), *Dothiorella sarmentorum* (r), *Neofusicoccum parvum* (s), *Pestalotiopsis* sp. (t), *Rosellinia corticium* (u). Asymptomatic control branch (v)

For each species BLAST searches against GenBank showed 100% identity to reference sequences of ex-type isolates and/or representative strains. The ITS sequence of a representative isolate of each species was deposited in GenBank (*D. olivarum* MT509988, *D. sarmentorum* MT509989, *N. parvum* MT509990, *Pestalotiopsis* sp. MT509991 and *R. corticium* MT509992).

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Pathogenicity

All five species proved to be pathogenic on oleaster. At the end of the experimental period, all branches inoculated with *D. olivarum*, *D. sarmentorum*, *N. parvum*, *Pestalotiopsis* sp. and *R. corticium* displayed dark brown bark lesions that spread up and down from the inoculation site (Figs. 1q-v). The average lesion length differed significantly among species ($F_{4,25} = 105.332$, $P < 0.001$; Tab. 2). The lesions caused by *N. parvum* were significantly larger than those caused by other species. In addition, branches inoculated with *N. parvum* displayed wilting symptoms and a wedge-shaped necrotic sector in cross-section, congruent with field observations.

Control branches inoculated with sterile PDA plugs remained symptomless. All five fungal species were successfully re-isolated from symptomatic wood and inner bark tissues from inoculated branches, thus fulfilling Koch's postulates.

Table 2 - Mean lesion length ± standard deviation caused by fungal species on branches of oleaster

Study sites	Identification code	Mean lesion length (cm)	Leaf chlorosis	Re-isolation frequency (%)
Diplodia olivarum	DM1	3.2 ± 0.41b	no	100
Dothiorella sarmentorum	DM2	1.3 ± 0.17d	no	100
Neofusicoccum parvum	DM3	8.1 ± 1.47a	yes	100
Pestalotiopsis sp.	DM4	2.2 ± 0.18c	no	100
Rosellinia corticium	DM5	2.1 ± 0.52c	no	83.3
Control		0.00		
LSD critical value		2.06		

Discussion and Conclusion

The results obtained have allowed us to clarify the aetiology of a new disease of oleaster in different natural ecosystems in Sardinia. In particular, two Botryosphaeriaceae species, *D. olivarum* and *N. parvum* were isolated and identified as the main causal agents of this emerging disease.

Diplodia olivarum was originally described from rotting olive drupes in Apulia (Italy) (Lazzizera et al., 2008) and it was subsequently reported associated with cankered branches of carob and declining lentisk shrubs in Italy (Granata et al., 2011; Linaldeddu et al., 2016b) and declining almond trees in Spain (Gramaje et al., 2012).

Neofusicoccum parvum is a plurivorous pathogen reported on more than 100 plant hosts worldwide (Sakalidis et al., 2013; Dissanayake et al., 2016). In Italy *N. parvum* has previously been reported as a pathogen on several hosts such as cannabis, cork oak, grapevine and pomegranate (Linaldeddu et al., 2007, Carlucci et al., 2015; Riccioni et al., 2017; Alberti et al., 2018). It has also been reported associated with a decline of olives in southern Italy (Carlucci et al., 2013). Interestingly, on four branches *D. olivarum* and *N. parvum* were isolated from the same canker, suggesting a potential synergistic interaction in the pathogenesis process.

Given the low frequency of isolation of *D. sarmentorum*, *Pestalotiopsis* sp. and *R. corticium*, it is not possible to establish the exact role of these species in the aetiology of the disease. All three species have proven to be weak pathogens on oleaster. It remains to establish a possible synergic interaction with *D. olivarum* and *N. parvum*. All five species are reported here for the first time as oleaster pathogens.

Members of the Botryosphaeriaceae family represent a growing threat to agricultural crops, urban and forest ecosystems in the Mediterranean region (Phillips et al., 2005; Alves et al., 2013;

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Dissanayake et al., 2017). In particular, over the last few years there has been an exponential increase in the occurrence of diseases caused by species of this family in natural ecosystems (Piškur et al., 2011; Bragança et al., 2016; Linaldeddu et al., 2017; Shami et al., 2017).

The site factors that may have contributed to the onset of this new oleaster disease remain unknown. However, given the high susceptibility of oleaster to *N. parvum*, great caution should be taken in the use of oleaster as a source of genetic resistance against fungal pathogens in olive trees breeding programmes.

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