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Diplodia africana causing dieback on *Juniperus phoenicea*: a new host and first report in the northern hemisphere

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Summary. Branch dieback was observed on Phoenician juniper trees, in a natural growing area on Caprera Island (Italy), during 2009 and 2010. Fungal isolates obtained from symptomatic twigs and branches showing dieback and bark necrosis were identified as *Diplodia africana* by analysis of morphological features and genomic DNA sequences of the ITS region and translation elongation factor 1-α gene. Pathogenicity was verified by stem inoculation of 3-year-old saplings of Phoenician juniper. This is the first report of *D. africana* in the northern hemisphere, and of this fungus as a pathogen of Phoenician juniper.

Key words: juniper disease, *Botryosphaeriaceae*, Caprera Island.

Introduction

Phoenician juniper (*Juniperus phoenicea* L.) is a typical juniper found throughout the Mediterranean region. In Sardinia (Italy), this tree grows mostly at low altitudes close to the coast and in particular on the small islands of the La Maddalena Archipelago, including Caprera Island (Camarda and Valsecchi, 2008).

Since autumn 2009, a progressive dieback of shoots and branches on young and old trees of Phoenician juniper has been observed in an area (about 1 ha) in the centre of Caprera Island. In the summer of 2010 the affected plants showed extensive crown dieback (Figure 1), and severely infected plants eventually died. Following removal of the outer bark at the base of symptomatic twigs and branches on diseased plants, brown discoloration of wood and inner bark tissues was observed (Figure 2). Frequently, the necrotic lesions girdled the main stems, causing rapid death of the upper crowns. Leaves on affected branches turned yellow, then dull red and finally brown. They often remained attached for some time after branch death. Since there is no information about this unusual disease on Phoenician juniper, a survey was carried out to establish the causal agent.

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Materials and methods

Samples of dead and symptomatic twigs and branches were cut from seven randomly selected trees and transferred to the laboratory for analysis. Under microscopic examination, several pycnidia erumpent through the bark were observed on dead branches of all examined samples. Pathogen isolation was made from fragments of inner bark and wood tissues measuring approx. 5 mm^2 aseptically cut from live symptomatic branches. All samples were cultured in Petri dishes containing potato dextrose agar (PDA; Oxoid Ltd, Basingstoke, UK). After incubation at 25°C for 1 week, fungal colonies were sub-cultured onto PDA and incubated at room temperature until pycnidia developed. To induce sporulation, fungal isolates were also sub-cultured on PDA with sterile twigs

Figure 1. Early dieback symptoms on branches of a Phoenician juniper tree (a). Extensive twig and branch dieback (b).

of Phoenician juniper. Conidia were mounted in lactophenol for microscopic examination. Conidial dimensions were recorded using an Optika™ Vision Pro version 2.7 digital camera connected to a Leitz Diaplan (Leitz, Wetzelar, Germany) microscope, at ×400 magnification.

A Botryosphaeriaceous fungus was consistently isolated. One of the isolates (DA1) was used as a representative culture for further morphological and pathogenicity studies. Mycelial growth on 9 cm Petri dishes containing PDA was measured at seven different temperatures between 5 and 35°C (three replicate dishes for each temperature were made). After 60 h of incubation in the dark, the colony diameter in each dish was measured along two perpendicular axes and the two measurements were averaged. The colony appearance of cultures growing on PDA at 25°C in the dark for 1 week was also recorded. Isolates DA1 and BL27 were deposited in the culture collection of the Department of Plant Protection at the University of Sassari.

Genomic DNA was extracted from isolates DA1 and BL27 following the CTAB method of Doyle and Doyle (1987). The entire Internal Transcribed Spacer region (ITS) of the rDNA, including the 5.8S gene, was amplified by polymerase chain reaction (PCR), using the primers ITS1 and ITS4 (White *et al*., 1990). The primers EF1-688F (5'-CGGTCACTTGATCTACAAGTGC-3') and EF1-986R (5'-TACTTGAAGGAACCCTTACC-3') were used to amplify part of the translation elongation factor 1-alpha (EF1-α) gene. The PCR products were purified using the Eurogold extraction kit (Euro-Clone S.p.A., Italy) following the manufacturer's instructions. Both strands were sequenced by the

Figure 2. Brown discoloration of wood and inner bark tissues on a symptomatic branch of Phoenician juniper infected by *Diplodia africana*.

BMR Genomics DNA sequencing service (www.bmr-genomics.it). The nucleotide sequences were read and edited with FinchTV 1.4.0 (Geospiza, Inc.; http://www.geospiza.com/finchtv), aligned using ClustalW (www.ebi.ac.uk/clustalw/) and compared with sequences deposited in GenBank using the BLAST software (http://blast.ncbi.nlm.nih.gov).

Pathogenicity was verified by stem inoculation on ten 3-year-old saplings of Phoenician juniper. A mycelial plug (3 to 4 mm^2) taken from the margin of an actively growing colony on PDA was put in a shallow wound (~3 mm) made by a scalpel on the stem of each seedling. The inoculation point was covered with cotton wool soaked in sterile water and wrapped with Parafilm®. Five control seedlings were each inoculated with a sterile PDA plug. Inoculated seedlings were kept in the laboratory at 16 to 25°C in natural daylight for 2 months.

Results and discussion

The fungal isolates obtained in culture from symptomatic tissues were identified according to morphological features as *Diplodia africana* Damm & Crous (Damm *et al*., 2007). On PDA at 25°C, all isolates developed a moderately aerial mycelium, initially white and then turning dark grey from the centre to the margin of each colony after 5 to 6 days (Figure 3). After 30 days on PDA, all isolates sporulated well in culture and produced hyaline, aseptate, thick-walled, oblong to cylindrical conidia measuring 30.1±1.8 × 11.7±1.1 μm, with a length/width ratio of 2.6±0.3 (mean±SD, n = 50). Conidia from cultures were identical to those from diseased branches. The optimal temperature for colony growth of *D. africana* was near 25°C, and the mycelium grew slowly at 10°C (Figure 4). At 5 and 35°C mycelial growth was not observed after 60 h of incubation, but the fungus resumed growth when the plates were moved to 25°C. The isolate of *D. africana* grew better at 25 than at 20°C, in contrast to the observations of Damm *et al*. (2007).

Identification of the juniper pathogen was confirmed by analysis of the sequences of the ITS region (isolate DA1) and EF1-α gene (isolate DA1 and BL27). BLAST searches in GenBank showed from both regions 100% similarity with reference

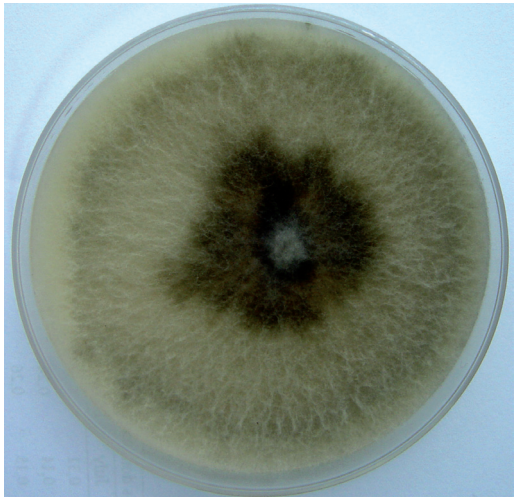

Figure 3. Isolate (DA1) of *Diplodia africana* grown on PDA for 7 days at 25°C in the dark.

sequences of *D. africana*, including the ex-type isolate (CBS 120835). Representative sequences of the isolate DA1 (ITS: accession number JF302648; EF1-α: accession number JN157807) and of the isolate BL27 (EF1-*α*: accession number JN157808) were deposited in GenBank.

Two months after inoculation, all saplings displayed bark necrotic lesions in the stems. Each lesion extended to girdle the stem, causing wilting of plants (Figure 5). Several erumpent pycnidia of *D. africana* were observed on the bark around the inoculation point. The pathogen was successfully reisolated from the symptomatic stem tissues, thus fulfilling Koch's postulates. Five control saplings inoculated with sterile PDA plugs remained symptomless.

The results obtained show that *D. africana* is directly involved in the aetiology of branch dieback and mortality of Phoenicean juniper trees observed in a natural area on Caprera Island. In the USA a similar disease was reported on *Juniperus* spp. and the canker and dieback causing agent was referred to as *Diplodia mutila* (Tisserat *et al*., 1988; Flynn and Gleason, 1993; Stanosz and Moorman, 1997). Recently, Alves *et al*. (2006), demonstrated that the juniper canker pathogen is a distinct species that they named *Diplodia cupressi* on the basis of the morphological and molecular sequence data. *Diplodia africana* differs from *D. cupressi* in conidium size, and morphology and radial growth rate of colonies in culture. In addition, both ITS and EF1-*α* sequence data clearly separate *D. africana* from *D. cupressi* and from any other *Diplodia* species.

Diplodia africana was originally associated with disease symptoms on stone fruit trees in South Africa (Damm *et al*., 2007). This is the first record of *D. africana* in the northern hemisphere and is also the first report of this fungus as a pathogen of Phoenicean juniper. Given the high ecological relevance of this outbreak and the abun-

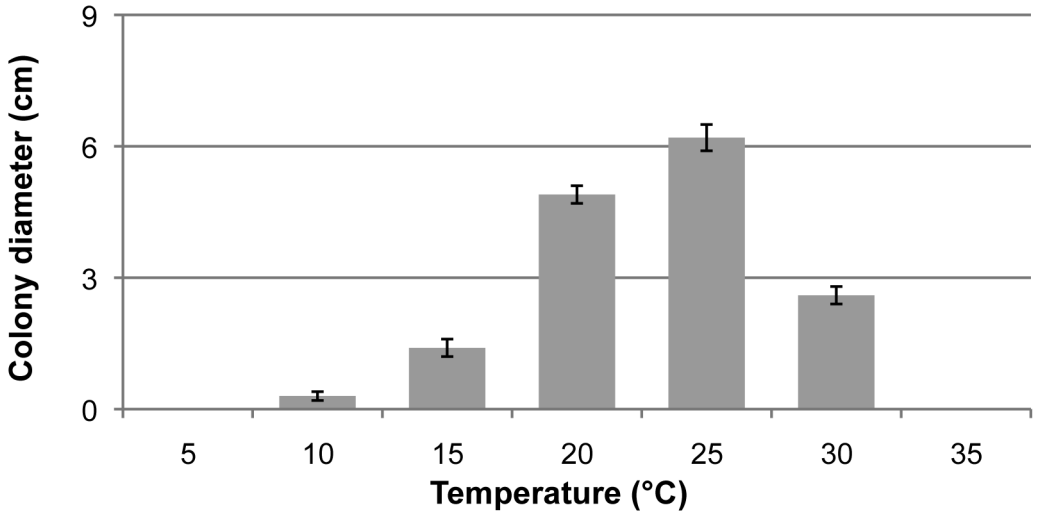

Figure 4. Mean colony diameters of *Diplodia africana* grown on PDA at different temperatures. Bar represents standard deviation.

Figure 5. Phoenicean juniper sapling showing crown dieback symptoms and inner bark necrosis in the stem, 60 days after inoculation with *Diplodia africana* (left). Asymptomatic control seedling (right).

dance of juniper ecosystems in Sardinia, further investigations are underway to monitor the health status of these ecosystems. In this regard, *D. africana* has recently been isolated from a symptomatic *Juniperus oxycedrus* L. ssp. *oxycedrus* tree in an archaeological area in southern Sardinia. Pathogenicity tests are underway to evaluate its aggressiveness on this host plant.

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