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SHORT NOTES

Diplodia scrobiculata: a latent pathogen of *Pinus radiata* reported in northern Spain

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Summary. *Pinus radiata* is a tree species native to the Central Coast of California and Mexico, which has been widely introduced in Europe for wood production. In Spain, especially in the northern region, it was introduced in the nineteenth century. Plantations located in the Basque Country (northern Spain) showing symptoms of Diplodia shoot blight were studied to confirm the causative pathogen species. Symptomatic and asymptomatic trees were sampled, and more than 150 fungal isolates obtained were morphologically characterized, with identities confirmed by sequencing the internal transcribed spacer (ITS) and the translation elongation factor 1-α (EF1-α) regions. Species-specific primers for *Diplodia sapinea* and *D. scrobiculata* were used to differentiate these fungi. *Diplodia scrobiculata* was detected on samples from asymptomatic trees, and BLASTN comparison was performed using the NCBI database. Lesions on *P. radiata* seedlings under controlled conditions were proved to be more substantial from *D. scrobiculata* than from *D. sapinea*. This is the first report of virulent *D. scrobiculata* in asymptomatic *P. radiata* trees in Spain.

Key words: *Pinus radiata,* Diplodia shoot blight, asymptomatic, aggressiveness.

Introduction

Diplodia scrobiculata has been reported in Europe and Spain (Stanosz *et al*., 1999; Moret and Muñoz, 2007). This fungus is known to coexist and interact with *D. sapinea*, one of the most common fungi found on pine trees (Burgess *et al*., 2004), but *D. scrobiculata* has a much more limited distribution and host range (Bihon *et al*., 2010). In its native range, *Pinus radiata* D. Don (Monterey pine) has been suggested to be exclusively associated with *D. scrobiculata* (Burgess *et al*., 2004). Previous pathogenicity studies have shown *D. scrobiculata* to be less virulent than *D. sapinea* (Palmer *et al*., 1987; Blodgett and Stanosz, 1999; Blodgett and Bonello, 2003), and biocontrol experiments have proved its ability to reduce Diplodia shoot blight (Muñoz *et al*., 2008). Nevertheless, *D. scrobiculata* has been reported to be as virulent as *D. sapinea* in South Africa (Bihon *et al*., 2010).

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This paper presents results which identified the fungal species associated with Diplodia blight in northern Spain.

Material and methods

A survey for incidence of wood fungal pathogens was conducted in *Pinus radiata* plantations showing Diplodia shoot blight symptoms, located in the Basque Country (northern Spain, 42.989625°N, -2.618927°E). Samples were obtained from diseased and symptomless trees ranging from 9 to 52 years old. Wood cores were collected with a Pressler's increment borer (diameter = 5 mm) at 130 cm height (Grissino-Mayer, 2003). The cores were placed into sterilized tubes, labelled, transported to the laboratory, and stored at 4ºC.

The wood cores were surface-sterilized for 2 min with sodium hypochlorite (1% active chlorine) and rinsed with sterile deionized water. Thin disks cut from whole cross sections of the cores were placed on potato dextrose agar (PDA, Oxoid) and incubated

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in darkness for 7 d, and the cultures were placed into clear plastic boxes in an incubator at 20ºC. Developing fungal colonies were transferred to PDA and incubated in darkness for 7 d.

Botryosphaeriaceae isolates were incubated with sterilized pine needles according to the modified method of Smith *et al*. (1996) and were examined weekly for formation of pycnidia and conidia.

The growth rates and colour of the isolates growing on PDA at 23°C in darkness were measured using colonies generated from 5 mm^2 diam. mycelial plugs obtained from the margins of 5-d-old PDA colonies. Fungal species were identified by colony and conidium morphology (de Wet *et al*., 2003; Phillips *et al*., 2013).

Genomic DNA was extracted from mycelia cultured on PDA at 23ºC, using a commercial kit (Analytik Jena AG, Life Science). A total of two partial gene regions were used in this study: internal transcribed spacer (ITS) and the translation elongation factor 1-α (EF1-α). The ITS region was amplified with primer pairs ITS1 and ITS4 (White *et al*., 1990) as described by Alves *et al*. (2004). The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) were used to amplify part of the translation elongation factor 1-α (EF1-α), as described by Phillips *et al*. (2005), Alves et al. (2006) and Alves et al. (2008). PCR products were purified (Nucleospin®, Macherey-Napel) and sequenced (Eurofins. Genomics, Germany).

Related sequences of *D. scrobiculata* used in the dendrograms were downloaded from the NCBI database. BLASTN comparison of the sequences was performed using the NCBI database and E value, and identity percentages were determined. Sequences were aligned with multiple sequence comparison by log-expectation (MUSCLE), using Mega v 7.0.26 software (Tamura *et al*., 2016).

Phylogenetic analyses using Maximum Likelihood (ML) and Neighbour-Joining (NJ) (Saitou and Nei, 1987) were performed using MEGA v. 7.0.26 (Tamura *et al*., 2016) with a Kimura 2-parameter model and statistical bootstrapping procedure involving 500 replicates.

To confirm the identity of fungal strains, the specific primers BotR, DpF, and DsF were used. These were developed for differentiation of the fungal pathogens *Diplodia sapinea* and *D. scrobiculata*, as described by Smith and Stanosz (2006).

Pathogenicity of fungal strains was tested by stem inoculation of each isolate on five *P. radiata* seedlings (18 months) maintained in a greenhouse at 22 ± 4ºC and 55–60 % relative humidity (Figure 1a). For each inoculation, a mycelial plug (3 to 4 mm^2 diam.) taken from the margin of an actively growing colony on PDA was placed in a shallow wound made by cutting the apical meristem of the seedling, and removing the first 5 cm.

Results

More than 150 isolates were obtained from *P. radiata* plantations suffering from Diplodia shoot blight throughout the Basque Country. *Diplodia sapinea* was the most common fungus isolated from symptomatic trees and is considered a widespread pathogen (99% of the cultures). *Diplodia scrobiculata* was isolated from an asymptomatic tree located in Bizkaia (43.352889ºN, -2.929818ºE).

Five weeks after incubation of fungi with sterilized pine needles, conidia appeared. They were brown, internally roughened without septa, clavate

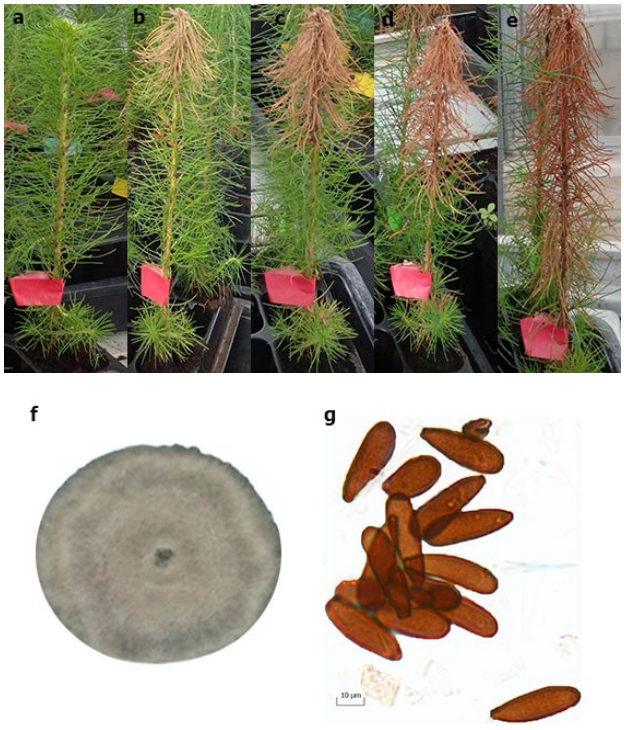

Figure 1. Disease caused by *Diplodia scrobiculata* on *Pinus radiata* seedlings. **a**: Uninoculated control. **b:** Symptoms produced 16 d after inoculation. **c:** at 30 d. **d:** at 55 d. **e:** at 75 d. **f**: Mycelium of the pathogen growing on PDA. **g**: Conidia of *Diplodia scrobiculata*.

with truncate bases (Figure 1g). Conidium dimensions were: length, 31 to 44 μm (mean = 38.1, standard error ± 3.06) and width 9 to 13 μm (mean = 11.1 ± 1.03), with a length/width ratio of 3.46 ± 0.41 (n=32).

When cultured on PDA at 20ºC, the colonies were white with sinuate edges and appressed mycelium that became dark grey after 4 d (Figure 1f).

The strain was submitted to the Spanish Type Culture Collection (CECT-Universitat de Valencia, reference number CECT 20966).

Phylogenetic relationships among *Diplodia scrobiculata* isolates were assessed using the Neighbour-Joining method from the DNA sequences of the ITS region and EF1-α gene, and were constructed with MEGA v.7.0.26 software. Bootstrap values (500 replications) are provided to indicate support levels for tree nodes (Figures 2 and 3).

These dendrograms are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the dendrogram. The analysis for ITS phylogenetic tree involved 15 nucleotide sequences (Figure 2) and eight for the EF1-α gene (Figure 3). GenBank accession numbers of each reference sample are provided.

BLAST analysis of the ITS sequences revealed an E value 0, and 99% of sequence homology, with the external *D. scrobicualta* sequences from the GenBank database included. The representative ITS sequence of *D. scrobiculata* obtained in this study was deposited in GenBank (Accession No. KX363798).

BLAST analysis of the EF1-α gene sequences have E values of KF766399,1.67 x 10^{-6}; EU392260 and DQ458884,1.41 x 10^{-4} ; AY624253,1.12 x 10^{-6}; AY624254, 1.11 x 10^{-8}; HM100270,1.07 x 10^{-3}; HM100268, 1.04 x 10^{-5}, respectively, and 98% of sequence homology with the external *D. scrobicualta* sequences from the GenBank database. The representative EF1-α gene of *D. scrobiculata* obtained in this study was deposited in GenBank (Accession No. KY962007).

In the pathogenicity test, infections first became visible 2 weeks after inoculation the needles in the top of the *P. radiata* seedlings became light brown. Although the inoculated plants produced resin, in almost all cases it was not enough to avoid the spread of the disease. The infections extended down through the main stems, the needles became dark brown, began to fall, and the stems turned tan.

Twelve weeks after inoculation, the seedlings displayed dark brown discoloration lesions (Figure 1e), located in both the bark and the wood tissues of the main stems ranging from 2.3 to 15 cm (mean = 6.3 ± 5.9 cm) for *D. scrobiculata* and from 0.6 to 11.5 cm (4.05 ± 0.99) for *D. sapinea.* The pathogens were successfully re-isolated onto PDA from symptomatic tissues and identified by the colony morphology and production of characteristic conidia, thus fulfilling Koch´s postulates. The control seedlings treated with sterile PDA plugs (non-inoculated) remained asymptomatic.

Discussion

This is the first report of *D. scrobiculata* affecting *P. radiata* in Spain. Although the fungus was isolated

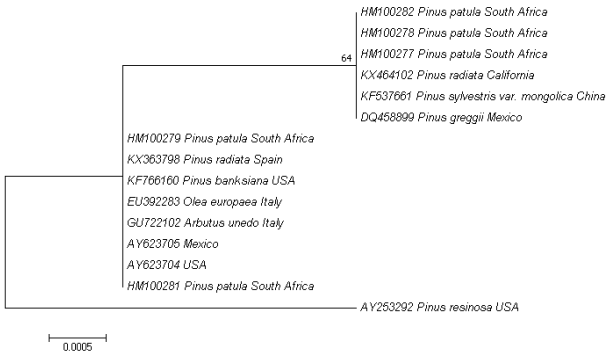

Figure 2. Dendrogram obtained from the dataset of 15 *Diplodia scrobiculata* isolates using the ITS region. Branches are labelled with the bootstrap values. Unlabelled branches have bootstrap values less than 50%.

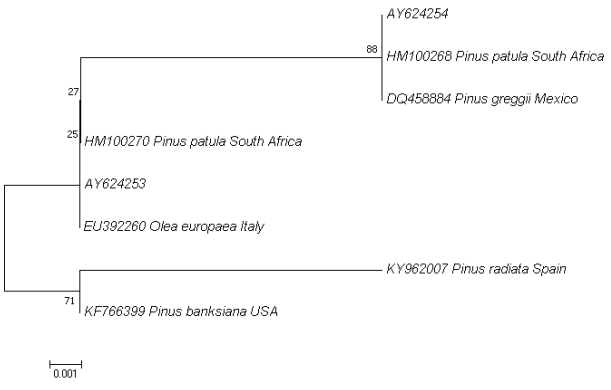

Figure 3. Dendrogram obtained from the dataset of eight *Diplodia scrobiculata* isolates using the EF1-α gene. Branches are labelled with bootstraps values. Unlabelled branches have bootstrap values less than 50%.

from an asymptomatic tree, in greenhouse conditions it showed a high aggressiveness. Further investigations should be undertaken to determine the distribution and impacts of this pathogen, and its interactions with *D. sapinea*. The roles of biotic and abiotic factors in the development of disease caused by *D. scrobiculata* on *P. radiata* should also be investigated.

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