

First report of *Bursaphelenchus xylophilus* on *Pinus sylvestris* in Spain

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Abstract

Aim of the study: To determine if the decline of a *Pinus sylvestris* L. stand is due to pine wilt disease.

Area of study: This research was conducted in the Agricultural and Phytopathological Laboratory of Galicia, LAFIGA (AGACAL, Xunta de Galicia).

Material and methods: Six decaying *P. sylvestris* trees were sampled for *Bursaphelenchus xylophilus* detection. Morphological and molecular tools were applied for nematode identification according to the EPPO protocols.

Main results: The nematode *B. xylophilus* was detected for the first time in *P. sylvestris* trees under natural conditions in Spain.

Research highlights: It is confirmed the serious threat posed by pine wilt disease to *P. sylvestris* stands.

Keywords: molecular identification, morphological identification, pine wood nematode, surveys.

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Resumen

Objetivo del estudio: Determinar si el decaimiento de un rodal de *Pinus sylvestris* L. es debido a la enfermedad del marchitamiento del pino.

Área de estudio: Esta investigación se llevó a cabo en el Laboratorio Agrario y Fitopatológico de Galicia, LAFIGA (AGACAL, Xunta de Galicia).

Materiales y métodos: Se recogieron muestras de seis árboles de *P. sylvestris* con síntomas de decaimiento para la detección de *Bursaphelenchus xylophilus*. Para la identificación del nematodo, se emplearon herramientas morfológicas y moleculares según los protocolos de la EPPO.

Resultados principales: El nematodo *B. xylophilus* fue detectado por primera vez en árboles de *P. sylvestris* en condiciones naturales en España.

Aspectos destacados de la investigación: Se confirma la grave amenaza que la enfermedad del marchitamiento del pino representa para las masas de *P. sylvestris*.

Palabras clave: identificación molecular, identificación morfológica, nematodo de la madera del pino, inspección.

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Pine forests worldwide are under threat by pine wilt disease, which is caused by the invasive nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle and transmitted by insects of the genus *Monochamus*.

At the start of the 20th century, this North American native species spread to Asian forests and was first reported in Europe, specifically Portugal, in 1999 (Mota et al., 1999). The nematode appeared in Spain in 2008 (Robertson et al., 2009) but for now, it is restricted to three regions near the Portuguese border. In both countries, the first detections of the pinewood nematode were done in *Pinus pinaster* Ait. trees. Since then, in Portugal, it was also detected in *Pinus nigra* Arnold in 2013 (Inácio et al., 2015), and more recently in *Pinus sylvestris* L. (Fonseca et al., 2024).

In Spain, only *P. pinaster* (Robertson et al., 2009; Abelleira et al., 2011) and *Pinus radiata* D. Don. (Zamora et al., 2015) have been identified as hosts for the pinewood nematode under natural conditions. However, recently, samples of *P. sylvestris* trees showing advanced wilting symptoms compatible with pine wilt disease were collected as part of the surveys of susceptible trees within the Action Plan for *B. xylophilus* in the Autonomous Community of Galicia in the demarcated area of As Neves (Pontevedra), a Spanish-affected area. These surveys are conducted by the Health and Plant Production Service of the Galician Government (Servicio de Sanidad y Producción Vegetal, Dirección General de Ganadería y Agricultura e Industrias Agroalimentarias, Consellería de Medio Rural, Xunta de Galicia) Specifically, 6 trees were sampled in a mixed plantation of *P. pinaster* and *P. sylvestris* at an altitude of 560 m, located in Tui, 6 km from the Portuguese border (-8° 41' 8.25" N, 42° 5' 17.03" W).

In October 2023, wood samples of *P. sylvestris* were collected using a drill and stored in plastic bags.

Both morphological and molecular identification of *B. xylophilus* were performed at the Agricultural and Phytopathological Laboratory of Galicia (Laboratorio Agrario y Fitopatológico de Galicia - LAFIGA, AGACAL, Xunta de Galicia), using protocols established by the European and Mediterranean Plant Protection Organization (EPPO) in standard PM7/4 (4).

The wood samples (a minimum of 50 g) were incubated for 14 days at 25 °C; subsequently, the nematodes were extracted using a modification of the Baermann funnel method. The individuals present in the suspension obtained were observed using an OLYMPUS SZX-7 stereomicroscope. Adults with morphological characteristics compatible with the genus *Bursaphelenchus* were transferred to a microscope glass slide. Using a NIKON microscope model LABOPHOT-2, individuals with features characteristic of *B. xylophilus* were selected, and their identity was confirmed by molecular techniques. The main features used for its morphological identification were the shape of the female tail, the presence of a vulva flap at 70-80% of body length from the anterior end and the shape of the male spicule (Ryss et al., 2005; Braasch et al., 2009).

Nematode DNA was obtained using the method described by Iwahori et al. (2000), included in the EPPO standard on diagnostics regarding *B. xylophilus* (PM7/4(4)). A nematode individual is soaked into 1 µL of water and crushed with filter paper once dry. The paper containing the nematode residues was transferred to a tube and 5 µL of ultrapure water was added. This extract was used as the PCR (Polymerase Chain Reaction) template.

For detection, real-time PCR was performed based on François et al. (2007) using the SLAN-96P Real-Time PCR System (Sansure Biotech); 1 µL of genomic DNA was added in 25 µL reaction containing 12.5 µL of Blastaq™ Probe 2X qPCR MasterMix (abm, Richmond, Canada) and 200 nM of each primer and probe.

Primers designed for the specific detection of the determined sequence of *B. xylophilus* were used, with an amplicon size of 77 bp that can be found in GenBank (accession number L09652, Tarès et al., 1993): SatF (5'-TGA-CGG-AGT-GAA-TTG-ACA-AGACA-3') and BSatRV (5'-AAG-CTG-AAA-CTT-GCC-ATG-CTA-AA-3') and the fluorogenic probe TaqMan BSatS (5'-FAM-ACA-CCA-TTC-GAA-AGC-TAA-TCGCCT-GAG-A-BHQ1-3'). The real-time PCR was performed following the recommendations of the EPPO. The PCR cycling specifications included an initial denaturation at 95 °C for 10 minutes, 30 reaction cycles of 15 seconds at 95 °C and 30 seconds at 59 °C and simultaneous hybridization/elongation at 59 °C.

Data were analyzed using SLAN-96P Real-Time PCR System (Sansure Biotech) software, according to the manufacturer's instructions. The cycle threshold values were calculated to determine the PCR cycle number at which the reporter fluorescence exceeded the background. Four controls were also included: (i) Negative isolation control: DNA from 5 nematodes of other species isolated from wood, for contamination monitoring during DNA extraction; (ii) Positive isolation control: DNA extracted from five cultured *B. xylophilus* nematodes to ensure that sufficient quantity and quality of DNA was isolated; (iii) Negative amplification control: molecular grade water previously used to prepare the reaction mixtures so that false positives due to contamination during reaction mixture preparation can be ruled out; and (iv) Positive amplification control: DNA extracted from *B. xylophilus* culture to monitor amplification efficiency.

The present study reports the first detection of the pinewood nematode in *P. sylvestris* in Spain, constituting a significant finding in the region. Furthermore, this discovery represents the second confirmed case in *P. sylvestris* in western Europe, highlighting the importance of continued monitoring and research on this and other pine species across the European continent.

Competing interests: The authors have declared that no competing interests exist.

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