New hosts for *Lecanosticta acicola* and *Dothistroma septosporum* in newly established arboreta in Spain

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Abstract

A historical outbreak of needle blight disease was recorded during 2018 to 2019 in plantations of *Pinus radiata* and *Pinus nigra* in the North of Spain. The main pathogens involved in this historical outbreak were identified as *Lecanosticta acicola* and *Dothistroma septosporum*. Recently, a variety of tree species in three arboreta planted between 2011 and 2013 in the Basque Country as part of the European project REINFFORCE were showing symptoms of needle blight and defoliation. The aim of this study was to determine which pine species were affected with these pathogens. Tree species sampled included several provenances of *P. brutia*, *P. elliottii*, *P. nigra*, *P. pinaster*, *P. pinea*, *P. ponderosa*, *P. sylvestris* and *P. taeda*. Using molecular identification methods, *Lecanosticta acicola* was confirmed infecting *Pinus brutia* (Provenance: Alexandropolis, Greece and var. *eldarica*, Crimea) and represents a new host species for this pathogen. *Pinus elliottii* (Provenance: Georgia, USA) and *P. ponderosa* (Provenance: Central California, USA) are new host reports of *L. acicola* for Spain. *Dothistroma septosporum* was found for the first time on *P. brutia* (Provenance: Marmaris, Turkey) and *P. ponderosa* (Provenance: Oregon, USA) in Spain and was also detected infecting *P. nigra* (Provenance: Sologne Vayrières, France).

Keywords: brown spot needle blight; defoliation; Dothistroma needle blight; invasive; forest pathogens; *Mycosphaerella dearnesii*

1 INTRODUCTION

The incidence of *Lecanosticta acicola* (Thüm.) Syd., causal agent of brown spot needle blight (BSNB), and *Dothistroma septosporum* (Dorog.) Morelet and *D. pini* Hulbary, the causal agents of Dothistroma needle blight (DNB), have increased significantly during the last few decades in Europe (van der Nest, Wingfield, Janoušek, et al., 2019). In Spain, serious disease outbreaks due to these pathogens have occurred since 2008, especially in new plantations of *Pinus radiata* D. Don and *P. nigra* Arnold (Ortíz de Urbina et al., 2017). High defoliation levels and an unusual mortality rate have caused an economic, environmental and social alarmism that has questioned the efficacy of current management strategies carried out in forest plantations.

Between 2011 and 2013, 38 different arboreta were established under the European project REINFFORCE (https://www.efi.int/projects/reinfforce-resource-infrastructures-monitoring-adapting-and-protecting-european-atlantic) with the aim of understanding tree species' capacity to adapt to changing climates, by planting the same genetic material across different landscapes in four countries. Three of these arboreta located in northern Spain (Basque country) were planted in harvested *P. radiata* plantations and are currently surrounded by plantations of *P. radiata* and *P. nigra*, infected with *L. acicola* and *D. septosporum* (Ortíz de Urbina et al., 2017).

The fungal pathogens of BSNB and DNB have similar life cycles and symptoms, and they are not easily differentiated by their conidial morphology (van der Nest, Wingfield, Janoušek, et al., 2019). Their spread is associated with conidial dispersion and ascospore dissemination by air, rain splash and also by accidental human mediated introductions (Janoušek et al., 2016). Pine needles become infected by spores in spring causing initial symptoms of chlorotic spots. These later develop into red or brown bands causing needle necrosis and eventually defoliation.

In 2019–2020, needles displaying symptoms and defoliation comparable with those caused by *Dothistroma* spp. and *L. acicola* were observed on *Pinus* species in the arboreta. The main aim of the study was to determine which pine species were affected with these pathogens in the arboreta using a molecular approach.

2 MATERIALS AND METHODS

Pine seedlings, produced in nurseries in France and subjected to phytosanitary controls, were planted in the winters of 2011, 2012 and 2013 in three arboreta located in Albina (Araba), Irisasi (Gipuzkoa) and Umbemendi (Bizkaia) (Figure 1). The three arboreta contained the same eight pine species and consisted of between 3 and 11 trees of each provenance (42 provenances in total) as follows: *P. brutia* Ten. (4 provenances), *P. elliottii* Engelm. (2 provenances), *P. nigra* (9 provenances), *P. pinaster* Ait. (7 provenances), *P. pinea* L. (6 provenances), *P. ponderosa* Douglas ex C. Lawson (3 provenances), *P. sylvestris* L. (8 provenances) and *P. taeda* L. (3 provenances).

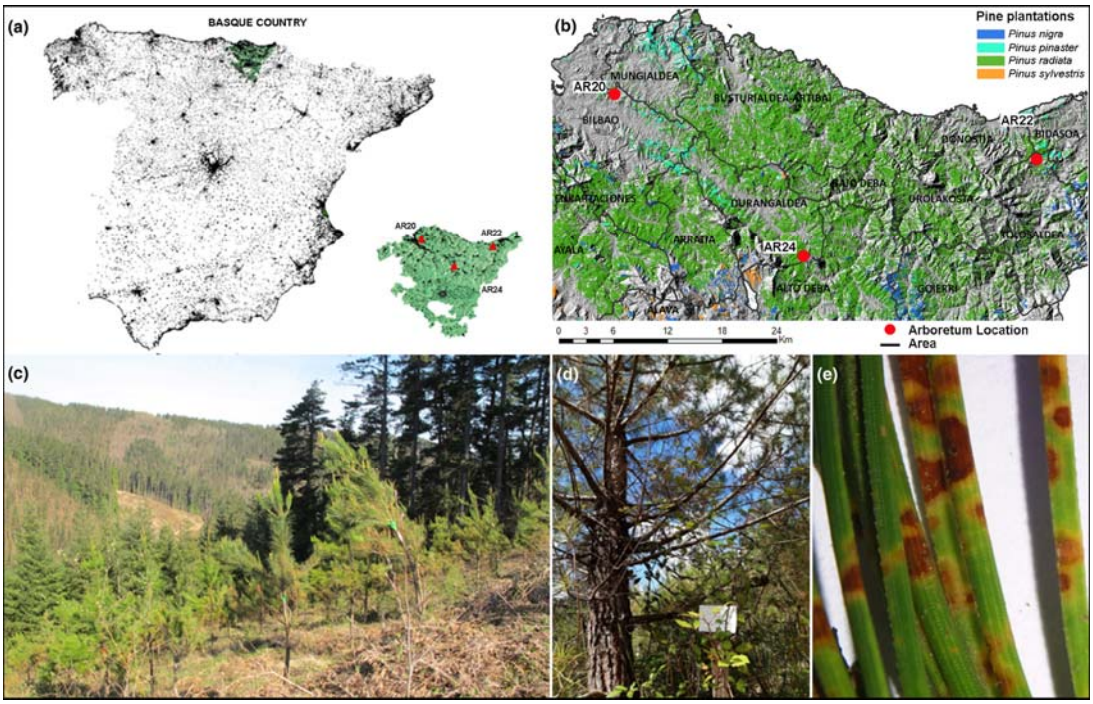

FIGURE 1. Locations of the three arboreta affected by a severe needle blight outbreaks in the Basque Country, Spain. (a) The location of the three arboreta (AR20, AR22 and AR24) from which samples were collected for this study. (b) Species of pine plantations surrounding the arboretum environment. (c) Severe needle blight outbreaks in adult and young *Pinus radiata* pine plantations located next to the arboretum. (d) *Pinus brutia* affected by needle blight. (e) Symptoms of needles blight observed on *P. brutia* needles collected in this study

Trees were visually inspected in the spring of 2019–2020 for any symptoms resembling brown or red spots or bands (Figure 1). For those that displayed symptoms, one sample of needles per tree (approx. 8 g per tree) was taken from the base and the central part of the crown. In this way, 113 samples were collected. All the samples were visually inspected after collection for the presence of acervuli on the needles. Isolates were obtained, when possible, directly from acervuli by streaking spores on *Dothistroma* sporulating medium (Mullett & Barnes, 2012).

DNA was extracted directly from 100 mg of symptomatic needles from all collected samples, and from fungal cultures obtained, with the innuPREP Plant DNA Mini Kit (Analytic Jena AG, Jena, Germany). Conventional PCR was carried out using species-specific primers for *L. acicola* and both *Dothistroma* spp. (Ioos et al., 2010). The reaction consisted of 0.4 µM of each primer (LAtef.F/LAtef.R, DStub2-F/DStub2-R or DPtef-F/DPtef-R), 10× buffer (Complete II KCl Buffer, IBIAN technologies), 200 µM of each dNTP, 0.5 U IBIAN-Taq DNA Polymerase (IBIAN technologies) and 1.5 µl DNA template in a total volume of 20 µl. The PCR conditions were as follows: 10 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 60°C and 45 s at 72°C and a final 10 min at 72°C. PCR amplicons were visualized on a 2% agarose (Conda) gel stained with GelRed® (Biotium Inc., California, USA). The reactions were considered positive for *L. acicola*, *D. septosporum* and *D. pini* if amplicon sizes measured 237 bp, 231 bp and 193 bp respectively.

For the needle samples that did not amplify with conventional PCR, a triplex real-time-PCR as described by Ioos et al. (2010) was used with slight modifications to increase the sensitivity of detection. Dual probes were labelled as DStub2-P1 – ABY-

TGGAATCCACAGACGCGTCA-QSY for *D. septosporum*; LAtef-P1 – FAM-CAAGCACTCTTGGAACACACCGC- BHQ1 for *L. acicola*, and 18S uni-P1 – VIC-ACGGAAGGGCACCACCAGGAGT- MGBNFQ for the 18S ribosomal DNA region. All qPCR reactions were performed in a QuantStudio™ 5 real-time PCR system (Applied Biosystems).

Identification of the fungal isolates was supported, additionally, by PCR amplification and sequencing of the internal transcribed spacer (ITS) region, and the translation elongation factor 1-α (TEF1) using the primers ITS1 and ITS4, and EF1-728F and EF1-986R respectively, as described in van der Nest, Wingfield, Ortiz, et al. (2019). PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and sequenced by Eurofins (Genomics). Sequencing data were edited and aligned using MEGA X software version 10.0.4 (https://www.megasoftware.net/). BLAST searches for the fungal taxa were conducted on the NCBI database (National Center for Biotechnology Information NCBI), and the consensus sequences deposited in GenBank.

3 RESULTS AND DISCUSSION

A total of 113 pine trees displayed symptoms of needle blight disease when visually examined; 42 in Albina, 18 in Irisasi and 53 in Umbemendi. From these, *P. ponderosa* was by far the species with the most positive detections (68%) followed by *P. brutia* (38%), *P. nigra* (12%) and *P. elliottii* (7.7%). *Pinus pinaster*, *P. pinea*, *P. sylvestris* and *P. taeda* were negative in all three arboreta. From the 113 trees sampled, 23 tested positive for either *L. acicola* or *D. septosporum* using conventional PCR (19 positives) and qPCR (4 positives) (Table 1). Of the 15 *P. ponderosa* trees infected, three were positive for *L. acicola* (13.6%) and 12 for *D. septosporum* (54.5%) followed by five trees of *P. brutia*, with four positive detections of *L. acicola* (30.7%) and one for *D. septosporum* (7.9%). Only one *P. elliottii* tree tested positive for *L. acicola* (7.7%). In the case of *P. nigra*, both species were detected but in two different trees. Host species distribution was as follows: *L. acicola* was detected in 44% of the *P. brutia* trees, 33% of *P. ponderosa* and 11% each in *P. nigra* and *P. elliotii*. *Dothistroma septosporum* was detected in 86% of the *P. ponderosa* trees and in 7% of the *P. brutia* and *P. nigra* each (Table 1). Only one pathogenic species was found in a sample (per tree). This is in contrast to the results of Ortíz de Urbina et al. (2017) where both pathogens were detected, although uncommon. In addition, although *Dothistroma pini* was not detected in the arboreta, this species has been detected before, rarely, on *P. nigra* in this region (Ortíz de Urbina et al., 2017).

The identity of the isolates was confirmed through sequence comparisons. All the ITS sequences for *D. septosporum* and *L. acicola* matched 100% to the sequences of the ex-neotype (KU948400.1) and ex-epitype (NR_120239.1) cultures for the two species, respectively. Similarly, the TEF1 sequences for all four *L. acicola* isolates were identical and had a 99.54% identity match with the ex-epitype culture of *L. acicola* from the USA (KC013002.2), and 100% identity with isolates from France (JX901633.1, KT737239.1, FJ868502). The eight *D. septosporum* isolates sequenced contained three TEF1 haplotypes. These sequences had an identity match of between 99.79%–100% to the ex-neotype culture of *D. septosporum* (KX364410.1) from Russia. Two TEF1 haplotypes were 100% identical to other sequences available in GenBank from France (JX901630.1, KF253251.1). The isolates were mainly obtained from *P. ponderosa* samples (nine isolates), but also from *P. brutia* (two isolates) and *P. nigra* (one isolate).

Table 1. Sites and *Pinus* host species affected by *Dothistroma septosporum* or *Lecanosticta acicola,* identified by conventional PCR, qPCR or ITS and TEF1 sequencing, in the three arboreta planted in northern Spain

Sample ID	Arboretum location	Host species	Provenance	*L. acicola*	*D. septosporum*	Identification method	ITS GenBank accession no.	TEF1 GenBank accession no.
37	Albina (AR24)	*P. elliottii*	Georgia, USA	1	0	Conventional PCR		
40	Albina (AR24)	*P. ponderosa*	Central California, USA	1	0	Conventional PCR		
AR13	Albina (AR24)	*P. ponderosa*	Oregon, USA	0	1	qPCR		
AR18	Albina (AR24)	*P. ponderosa*	Oregon, USA	0	1	qPCR		
AR20	Albina (AR24)	*P. ponderosa*	Oregon, USA	0	1	qPCR		
Pipo1c	Albina (AR24)	*P. ponderosa*	Oregon, USA	0	1	Conventional PCR, gene sequencing	MT160333	MT269909
Pipo1e	Albina (AR24)	*P. ponderosa*	Oregon, USA	0	1	Conventional PCR, gene sequencing	MT160336	MT269910
Pipo2a	Albina (AR24)	*P. ponderosa*	Oregon, USA	0	1	Conventional PCR, gene sequencing	MT160337	MT269911
Pipo3c	Albina (AR24)	*P. ponderosa*	Oregon, USA	0	1	Conventional PCR, gene sequencing	MT160330	MT269912
Pipo4a.1	Albina (AR24)	*P. ponderosa*	Oregon, USA	0	1	Conventional PCR, gene sequencing	MT160334	MT269913
Pipo4a.2	Albina (AR24)	*P. ponderosa*	Oregon, USA	0	1	Conventional PCR, gene sequencing	MT160335	MT269914
Pipo4b	Albina (AR24)	*P. ponderosa*	Oregon, USA	0	1	Conventional PCR, gene sequencing	MT160332	MT269915
Pipo4d	Albina (AR24)	*P. ponderosa*	Oregon, USA	0	1	Conventional PCR, gene sequencing	MT160331	MT269916
11	Irisasi (AR22)	*P. brutia*	Alexandropolis, Greece	1	0	Conventional PCR		
15	Irisasi (AR22)	*P. brutia*	Marmaris, Turkey	0	1	Conventional PCR		
16	Irisasi (AR22)	*P. nigra*	Sologne Vayrières, France	0	1	Conventional PCR		

Sample ID	Arboretum location	Host species	Provenance	*L. acicola*	*D. septosporum*	Identification method	ITS GenBank accession no.	TEF1 GenBank accession no.
h16a	Irisasi (AR22)	*P. nigra*	unknown	1	0	Conventional PCR, gene sequencing	MT160341	MT269917
5	Irisasi (AR22)	*P. ponderosa*	Oregon, USA	0	1	Conventional PCR		
h5a	Irisasi (AR22)	*P. ponderosa*	unknown	1	0	Conventional PCR, gene sequencing	MT160340	MT269918
32	Umbemendi (AR20)	*P. brutia*	var. *eldarica*, Crimea	1	0	Conventional PCR		
id697a	Umbemendi (AR20)	*P. brutia*	var. *eldarica*, Crimea	1	0	Conventional PCR, gene sequencing	MT160338	MT269919
id697d	Umbemendi (AR20)	*P. brutia*	var. *eldarica*, Crimea	1	0	Conventional PCR, gene sequencing	MT160339	MT269920
AR10	Umbemendi (AR20)	*P. ponderosa*	Oregon, USA	1	0	qPCR		

In this study, we found *P. brutia* infected by *L. acicola,* which means the first report on this *Pinus* species based on EPPO information and review data collected by van der Nest, Wingfield, Janoušek, et al. (2019). *Pinus elliottii* and *P. ponderosa* for *L. acicola* and *P. brutia* and *P. ponderosa* for *D. septosporum* represent new hosts in Spain. *Pinus elliottii* and *P. ponderosa* are considered susceptible species to BSNB (van der Nest, Wingfield, Janoušek, et al., 2019). Neither of these three pine species is widely planted in Spain, but the presence of needle blight pathogens on them suggests that environmental conditions in northern Spain are conducive for this disease. This should be taken into consideration for future plantings of these species.

Pinus brutia is closely related to *P. halepensis* Mill. which is adapted to Mediterranean regions where it has been extensively planted. *Dothistroma septosporum* was reported on *P. brutia* in Greece and Turkey (Tsopelas et al., 2013; Tunali et al., 2018). The Mediterranean nature of this host could be affecting its adaptability to the Atlantic area where the arboreta are established and predisposing it to fungal infections. Albina arboretum, for example, which is located in a transitional climatic zone between the Atlantic climate and the continental Mediterranean climate, showed the highest number of positive pathogen detections, especially of *D. septosporum* (26.2% of the samples) on *P. ponderosa* samples and *L. acicola* at a presence of 4.8% on *P. ponderosa* and *P. elliotii*. Both species were also confirmed in Irisasi (*L. acicola* in 11% and *D. septosporum* in the 16% of the total samples analysed). In the case of Umbemendi, the arboretum closest to the Atlantic coast, only *L. acicola* was detected (7.5% of the samples). A wider survey needs to be accomplished in order to determine the link between pathogen species presence and climatic regions.

Arboreta were mainly surrounded by *P. radiata* and *P. nigra* plantations that were severely infected with *Dothistroma* spp. and *L. acicola* being the most likely source of inoculum of these pathogens. As not all trees/provenances were damaged, this could indicate some level of resistance to the pathogens. It would be interesting to monitor these pine species and provenances during the next few years and to establish further collaborative research at a global scale to gain a better understanding into the response of the forest ecosystems to these pathogens.

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