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ORIGINAL ARTICLE

Pathogenicity of *Phytophthora* and *Halophytophthora* species on black alder and the host histological response

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Abstract

Riparian alder forests are threatened by *Phytophthora* across Europe. Comparative studies of the pathogenicity of *Phytophthora* species are crucial for developing effective management strategies. Although only a limited number of species, particularly *P.* × *alni*, lead to tree decline in natural environments, many species demonstrate pathogenicity in inoculation trials. *Phytophthora* species vary in their ability to infect different tissues, such as phloem and xylem, and trigger defence responses in the host through the formation of tyloses and callose. By comparing the histological responses of alder to various *Phytophthora* species, we can gain insights into the success of *P.* × *alni* and the potential damage that could be caused by other species. To investigate the defence strategies of black alder (*Alnus glutinosa*) against attack by *Phytophthora* and *Halophytophthora* species present in Catalonia (NE Spain), we conducted inoculation trials on saplings using nine potentially pathogenic species and compared the histological responses. *Phytophthora* × *alni* and *P. plurivora* were the most aggressive species followed by other exotic species such as *P.* × *cambivora* and *P. cactorum*. *Phytophthora* × *alni* and *P. plurivora* were equally damaging despite the higher prevalence of *P.* × *alni* in declining alders in natural settings. Although *P.* × *alni* mainly invaded the phloem, *P. plurivora* also invaded the xylem and triggered the production of tyloses. Histological analyses revealed a diverse range of plant responses to infection by *Phytophthora* species, providing a better understanding of their adaptability in natural environments compared with solely observing lesions. The low level of callose production in saplings inoculated with *P.* × *alni* compared with callose production in saplings inoculated with less pathogenic species suggests that *P.* × *alni* can evade recognition by the host and, hence, could partially explain its success.

Keywords *Alnus* · Callose · Decline · *P.* × *alni* · Riparian forest · Tyloses

Introduction

Black alder (*Alnus glutinosa* (L.) Gaertn.) is the most widespread alder species in European riparian forests, where it is considered a key species due to its role in nitrogen fixation, stabilization of banks, and biodiversity maintenance (Bjelke et al. 2016; Claessens et al. 2010; Haque et al. 2015). Riparian alder forests are threatened by emergent pathogen invasions, which add to the stress caused by changes in water regime in rivers (Schnitzler 1994; Rodríguez-González et al. 2014). Alder decline and mortality due to *Phytophthora* disease have been reported throughout Europe. Declining alders are usually characterized by canopy dieback, bleeding cankers, growth reduction, higher cone production, and small and yellowish leaves (Bjelke et al. 2016).

Alder seems to be susceptible to many *Phytophthora* species; however, only a few of them cause damage in the

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field (Jung et al. 2018). The *Alnus*-specific species complex *Phytophthora alni*—which includes *P. uniformis*, *P.* × *multiformis*, *and P.* × *alni*—is considered the main cause of alder decline in Europe (Husson et al. 2015; Jung et al. 2018). Among these three species, *P.* × *alni* is the most common, and *P.* × *multiformis* and *P.* × *alni* are the most aggressive (Haque et al. 2015). *Phytophthora alni* sensu lato was first reported in the 1990s in the UK (Gibbs 1995) but is now present throughout most of Europe (Redondo et al. 2015a; Bjelke et al. 2016). In the Iberian Peninsula, the most recent reports of *P.* × *alni* come from western locations in stands of *Alnus lusitanica* (Pintos Varela et al. 2010; Kanoun-Boulé et al. 2016); however, the presence of this pathogen in mountainous locations in eastern Iberia, such as the Spanish Pyrenees, is less known.

Besides *P.* × *alni*, other *Phytophthora* species have also been isolated from declining alder stands, but to a much lesser extent. For instance, *P. plurivora* has been isolated from bleeding cankers on several occasions (Redondo et al. 2015a; Trzewik et al. 2015), representing 4–23% of *Phytophthora* isolates (the majority of isolates being *P.* × *alni* or *P. uniformis*). There are also reports of decline in association with pathogenic species such as *P. cactorum*, *P. siskiyouensis*, *P. gonapodyides*, and *P. lacustris* (Navarro et al. 2015; O'Hanlon et al. 2020; Bregant et al. 2020; Feau et al. 2022), and pathogenicity tests (e.g., *P. cinnamomi*, *P. citrophthora*, *P. nicotianae*, *P. palmivora*, and *Halophytophthora fluviatilis*) (Caballol et al. 2021; Haque and Diez 2012; Santini et al. 2006) have revealed the breadth of species that could potentially damage alder.

Increasing our knowledge regarding which *Phytophthora* species can infect alder and why should help to direct management efforts to improve riparian forests. Pathogenicity tests represent the basis for risk assessments to determine the susceptibility of hosts to infection; however, particularly for alder, there is a mismatch between host susceptibility in pathogenicity tests and pathogen prevalence under field conditions. One reason for this could be the absence or low prevalence of certain *Phytophthora* species in rivers and streams. However, potentially pathogenic species such as *P. lacustris* or *P. gonapodyides* are prevalent in water bodies but are rarely found causing cankers (Redondo et al. 2018; Jung et al. 2018; Bregant et al. 2020). Furthermore, known plant pathogens, such as *P.* × *cambivora* and *P. plurivora*, are isolated quite frequently in nature (Català et al. 2015), pointing to causes other than inoculum load as potential explanations for the prevalence of *P.* × *alni* in diseased alders. Exploring susceptibility traits other than lesion length, such as those related to plant immunity or resistance in the cambium, could be informative.

Finding a common pathogenicity measure for the different *Phytophthora* species can be complex. Measuring lesion length in the stem following inoculation with mycelial plugs represents only part of the infection and may be more relevant for some *Phytophthora* species than for others. For instance, *Phytophthora* infections in alder commonly start in the roots; however, in the case of the *P. alni* complex, they can spread upwards to the trunk or directly infect the trunk during floods (Zamora-Ballesteros et al. 2017; Jung et al. 2018). Histological examinations can provide additional information on inter-specific differences in the spread and survival of pathogens in different tissues and on the defence response of the plant (Nave et al. 2021). For instance, *P.* × *alni* and *P. uniformis* can grow and reproduce within different organs of alder roots (e.g., epidermis, vascular cylinder), with a clear preference for the cortex (Nave et al. 2021). Therefore, both pathogens show a high capacity for infection and spread in roots (Nave et al. 2021). Similar studies on other tissues, such as stem xylem and phloem, can help to understand and compare the infection process of alder pathogens. For instance, several *Phytophthora* species, such as *P. palmivora*, can infect the phloem and cambial tissue of the stem, whereas others, such as *P.* × *cambivora* and *P.* × *alni*, can also colonize the xylem (Brown and Brasier 2007; Jung et al. 2018). The different affected tissues may then impact different physiological processes of plants. For instance, xylem infection of tanoak by *P. ramorum* can lead to a decrease in sap flow and hydraulic conductivity, hampering water and nutrient transport owing to the obstruction of xylem vessels by tyloses (i.e., the overgrowth of parenchyma cells into vessels) and by pathogen hyphae and chlamydospores (Parke et al. 2007). By contrast, Clemenz et al. (2008) did not find decreases in stem hydraulic conductivity in alder seedlings infected by *P.* × *alni*, apparently because the root system was not affected. However, the destruction of the phloem affected the translocation of assimilates to roots and led to leaf starch accumulation, stomatal closure, and reduced photosynthesis and growth rates. Therefore, differences between pathogens in terms of the type of tissue they preferentially colonize can affect the type of damage that is generated and, hence, determine which plant functions are impaired.

Differences in pathogenicity may reflect differences in the type of defence elicited by the host. Plants can use defence mechanisms (e.g., tyloses, lignin deposition, and callose deposition around sieve plates) to prevent the infection of vascular systems (Moerschbacher et al. 1990; Andrade-Hoyos et al. 2015). Several forest *Phytophthora* species can induce tyloses in xylem vessels (Jung and Blaschke 1996); however, little is known about the interspecific variation across species and the relationship between tylosis induction and necrosis. Sieve occlusion due to the production of callose, for example, has been associated with resistance to *P. cinnamomi* (van den Berg et al. 2018), although it did not impede phloem colonization by *P. cinnamomi* (Redondo et al. 2015b). By contrast, lignin production was only found

in roots susceptible to colonization by *P. cinnamomi* and was not able to hamper the development of hyphae in roots (van den Berg et al. 2018).

The aim of this study was to perform a comparative pathological test involving eight *Phytophthora* and one *Halophytophthora* species to determine the histological response of alder saplings following stem inoculation. Histological studies can provide information about the attack and defence strategies of the pathogen and the host and help us to understand the specificity and fitness of *P.* × *alni* in the field. Moreover, we report the presence of *P.* × *alni* and *P. plurivora* in symptomatic alder trees in Catalonia, NE Spain.

Material and methods

Phytophthora and *Halophytophthora* isolates

The *Phytophthora* and *Halophytophthora* isolates used in the inoculation study (three isolates of *P.* × *alni*, two of *P. plurivora*, one of *P.* × *cambivora*, three of *P. cactorum*, one of *P. castanetorum*, and two of *H. fluviatilis*) were recovered by soil baiting and taking bark samples from several tree species in Catalonia. *Phytophthora* isolates are described by Štraus et al. (2023), except for the three *P.* × *alni* isolates and one of the *P. plurivora* isolates, which are described in Table 1. *Halophytophthora* isolates are described by Caballol et al. (2021).

Pathogenicity tests

The pathogenicity of the 12 *Phytophthora* and *Halophytophthora* isolates was assessed using 2-year-old *Alnus glutinosa* saplings (1.0–1.5 m tall) bought from a commercial plant nursery. Each of the 12 *Phytophthora* or *Halophytophthora* isolates was used to inoculate a total of six saplings. In addition, six saplings were mock inoculated with sterile V8 agar, which served as controls. Each sapling was inoculated at three separate inoculation points to account for within-sapling variation. Inoculations were carried out by removing small sections of bark at approximately 5.0, 6.5, and 8.0 cm from the bottom of the stem, and then, 5-mm mycelial plugs from 3-week-old cultures grown on V8 agar were placed onto the exposed stem at each inoculation point and covered with Parafilm. Plants were kept under controlled conditions (25 °C climate chamber with 80% relative humidity and a 16/8 h day/night photoperiod) and regularly watered for 7 weeks. The length of the stem lesions was recorded after 7 weeks. Re-isolation of the inoculated *Phytophthora* and *Halophytophthora* isolates was attempted by placing small pieces of necrotic phloem from areas beyond the inoculation point onto selective CMA-PARPBH medium (Jeffers 1986). Growing *Phytophthora* colonies were sub-cultured onto V8 agar medium and incubated in the dark at 20 °C.

Wood anatomy and microscopy

For each species of *Phytophthora* and *Halophytophthora*, we selected five 3.0–4.5 cm long stem samples from five different saplings to perform xylem and phloem anatomical analyses and stored them in alcohol at 96% concentration. Wood samples were transversally cut using a sledge microtome (Gärtner and Nievergelt 2010) (Fig. 1A). Transversal wood Sects. (15–20 µm thick) were mounted on glass slides, stained with safranin (1%) and Astra blue (2%), dehydrated with ethanol (70%, 95% and 100%) and xylol, and mounted on microscope slides using Eukitt® (Sigma-Aldrich, St Louis, MO, USA) (Fig. 1B).

To quantify the response of the plant to inoculation with the different species of *Phytophthora* and *Halophytophthora*, in the 90°-quadrant of the section located closest to the lesion, we randomly selected three areas in the phloem and three areas in the xylem (Fig. 1C). In each area, we captured images at × 20 magnification with a digital camera mounted on a light microscope (Olympus BH2). The following data were recorded for each image (Fig. 2): (i) presence or absence of necrosis in the cambium; (ii) number of blue cells (potential pathogen hyphae) in phloem and xylem; (iii) number of red tyloses in xylem; (iv) number of altered (i.e., yellowish–orange or necrotized) ray parenchyma cells; (v)

Table 1 Location of *Phytophthora* isolated from symptomatic black alders growing in riparian forests in Catalonia (NE Spain), including coordinates (in decimal degrees) and bioclimatic variables (maximum temperature of warmest month, minimum temperature of coldest month, mean temperature of warmest quarter, mean temperature of coldest quarter)

Isolated species	Latitude (N)	Longitude (E)	Location	Max. temp. warmest month (°C)	Min. temp. coldest month (°C)	Mean temp. warmest quarter (°C)	Mean temp. coldest quarter (°C)
P. plurivora	42.380	1.343	Castellbó	24.9	− 1.7	17.8	3.3
P. × *alni*	42.588	1.134	La Guingueta d'Àneu	24.4	− 2.4	17.1	2.5
	41.910	0.934	Alòs de Balaguer	29.5	0.8	21.9	6.0
	42.102	2.218	S. Quirze de Besora	26.3	0.1	19.6	5.3

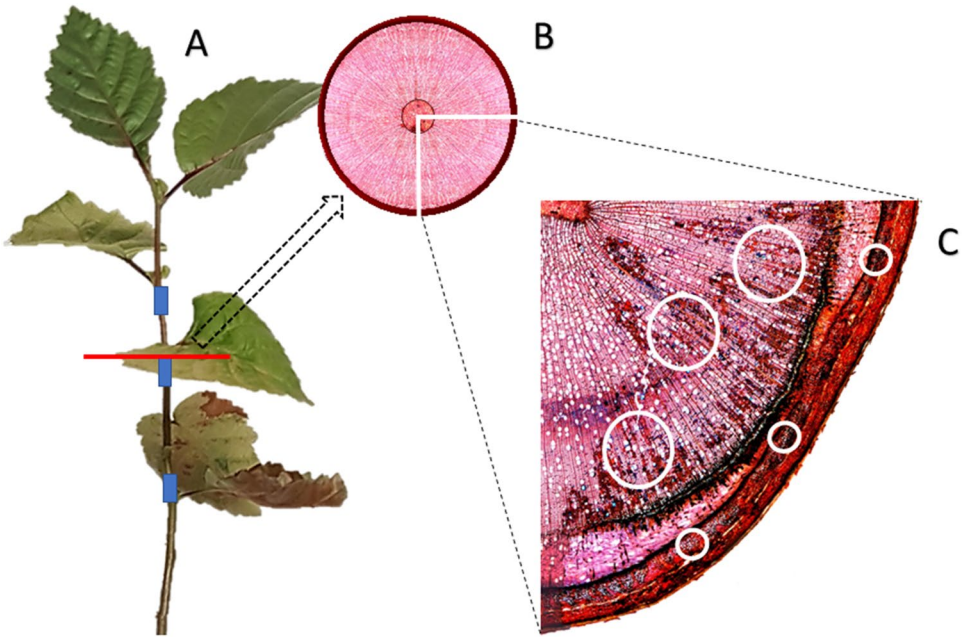

Fig. 1 Schematic of the approach used for taking wood samples for anatomical analyses. First, cross sections were taken above the three inoculation points (marked as blue rectangles) (**A**), stained with safranin (1%) and Astra blue (2%) and dehydrated with ethanol (70%, 95%, and 100%) and xylol and then divided into four 90°-quadrants (**B**). Images were taken of the quadrant adjacent to the necrotic lesion that developed from the inoculation point. White circles represent areas that were randomly selected for light microscopy observations of the xylem and phloem (**C**)

number of altered (i.e., damaged or decomposed wall) fibres or vessels; and (vi) number of altered (i.e., yellowish–orange or necrotized) axial parenchyma cells. Samples were also stained with a 1% aqueous solution of iodine–potassium iodide (Nakaba et al. 2006) to identify necrotized ray and axial parenchyma cells (Fig. 3). The different cell topologies were counted using the "multi-point" tool of the ImageJ v.1.40 software for image analysis (Schneider et al. 2012).

To examine the effect of each of the *Phytophthora* and *Halophytophtora* species on the histology of alder stems using fluorescence microscopy, we selected three alder stem samples at random. Fresh 15–20 µm thick stem sections were obtained using a sledge microtome (Gärtner and Nievergelt 2010). Half of these samples were stored by immersing them in distilled water. To detect modifications of the cell wall and the presence of hyphae in infected tissues, the fresh samples were stained with Calcofluor white M2R (Fluka 18909) and 10M KOH (1:1) (Ruzin 1999; Harrington and Hageage 2003). Samples were observed with epifluorescence under UV light (430 nm) using a U-MWBV2 (excitation filter 400–440 nm, dichroic mirror 455 nm, barrier filter 475 nm) cube. To detect the presence of callose in sieve plaques, the stored samples were stained with aniline blue (Merck 1275) and mounted with an antifade solution (Fluormount) (Ruzin 1999). Samples were observed with an Olympus U-ULS 100 HG epifluorescence system with a U-MWU2 (excitation filter 330–385 nm, dichroic mirror 400 nm, barrier filter 420 nm) cube, with callose appearing as a yellow/greenish fluorescence or bluish white (Fig. 4). In total, 40–50 stem cross sections were examined. The entire area of the xylem and the circumference of the phloem in each section were examined to identify the presence of putative *Phytophthora* hyphae, and, when present, the number of hyphae was recorded. Specifically, the following observations were recorded: (i) the presence or absence of putative *Phytophthora* hyphae, (ii) the presence of lignin, and (iii) the presence of callose in sieve plates (Figs. 4 and 5).

Data analysis

One-way analysis of variance was performed to compare the lesion length and histological measurements of saplings inoculated with different species, with isolates nested within species (in the case of *P. plurivora*, *P.* × *alni*, *P. cactorum*, and *H. fluviatilis*). For measures of the number of hyphae and the presence of lignin and callose in sieve plates, comparisons were only carried out among *H. fluviatilis*, *P. cactorum*, *P. plurivora*, *P.* × *alni*-inoculated saplings, and mock-inoculated controls. Dependent variables were subjected to transformations (i.e., $x^{0.3}$ for lesion length and altered axial cells (iodide stain) and $x^{0.5}$ for altered ray cells (iodide stain)) to meet the assumptions of normality and homoscedasticity of the residuals. Logistic regression was used for presence–absence variables, such as presence of hyphae (Calcofluor white stain), lignin, sieve plates, and necrosis in cambium. Post hoc analyses were performed using Tukey's honest significant difference (HSD) test. When analysing the presence of hyphae in the phloem (Astra blue and safranin stain), the assumptions of the analysis of variance were not met even when following variable transformation and when a non-parametric Kruskal–Wallis test followed by the Dunn post hoc test were performed. A principal component

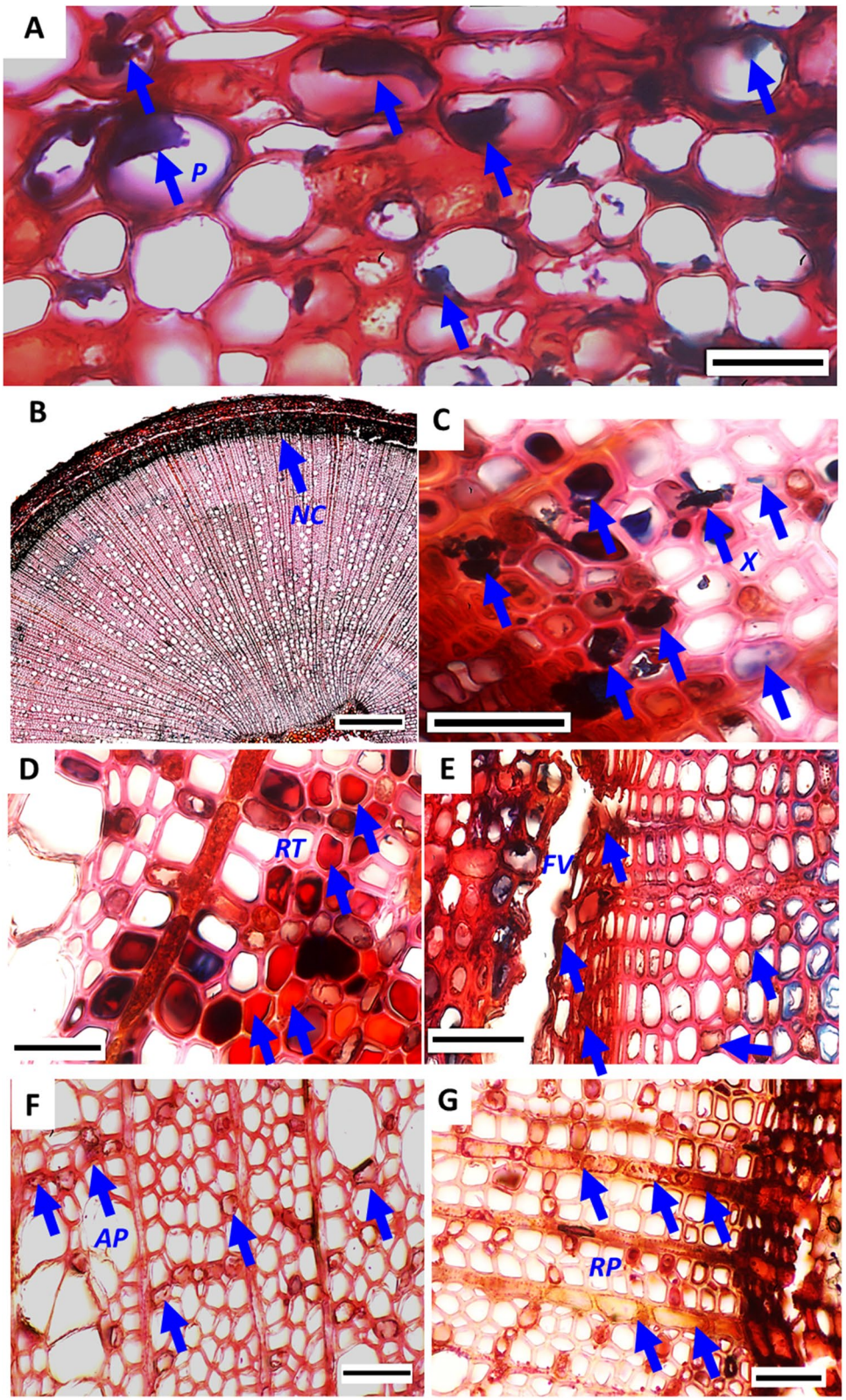

Fig. 2 Light microscopy images of cross sections of black alder stems inoculated with *Phytophthora* species and stained with safranin (1%) and Astra blue (2%) and dehydrated with ethanol (70%, 95%, and 100%) and xylol. Arrows indicate **A** the putative presence of hyphae in the phloem (P), **B** the presence of necrosis in the cambium (NC), **C** the presence of hyphae in the xylem cells (X), **D** the type of cells that we identified and counted as red tyloses (RT) in the xylem, **E** altered fibres or vessels (FV), **F** altered axial parenchyma cells (AP), and **G** altered ray parenchyma (RP). Scale bars represent 20 µm (**A**, **C**, **D**), 50 µm (**E**–**G**), and 1 mm (**B**)

analysis (PCA) was performed to determine the relationship among the measured variables and to compare the effect of inoculating alder with the different pathogenic species. The Kaiser–Meyer–Olkin (KMO) index was calculated as a measure of sampling adequacy. All statistical analyses were carried out using R software (R Core Team 2022).

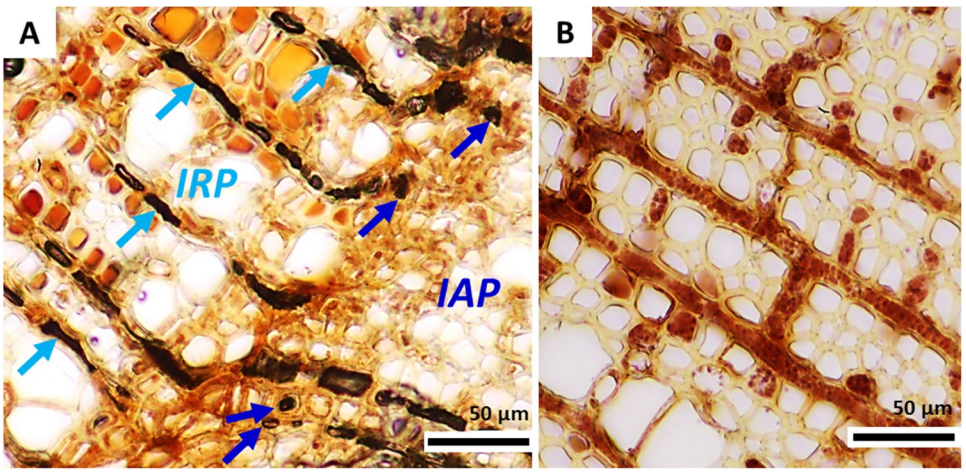

Fig. 3 Light microscopy images of cross sections of stems of black alder saplings inoculated with *P. plurivora* (**A**) or sterile agar (control) (**B**) and stained with a 1% aqueous solution of iodine–potassium iodide. Dark-blue arrows indicate altered axial parenchyma cells (IAP); light-blue arrows indicate examples of altered ray parenchyma cells (IRP)

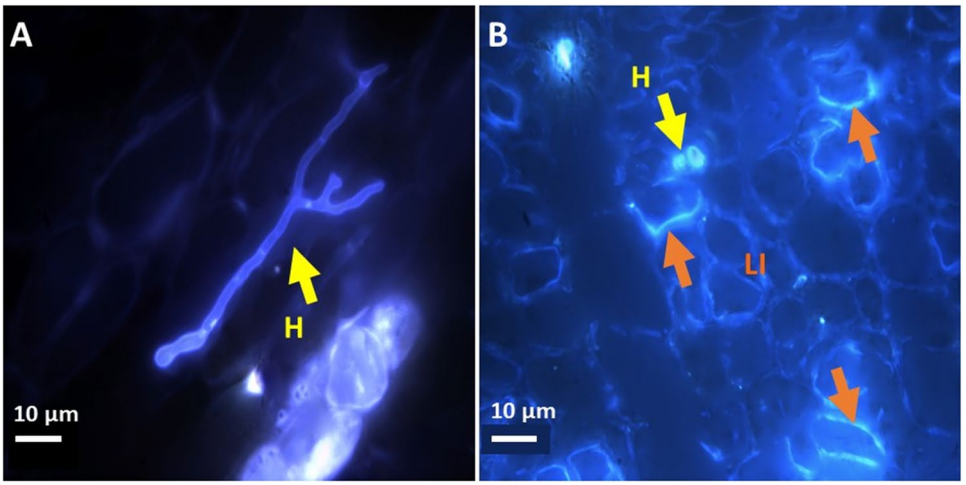

Fig. 4 Fluorescence micrographs of stem cross sections of black alder infected with *P.* × *alni*. **A** Hyphae of the inoculated pathogen (H, yellow arrow) in the phloem and xylem. **B** Hyphae (H, yellow arrow) and lignified phloem cell walls (LI, orange arrows), which are characterized by a higher fluorescence intensity

Field sampling and isolate typing

To obtain local and fresh isolates, a survey across several rivers in Catalonia was carried out from 2017 to 2021 that targeted symptomatic alders (i.e., necrosis in trunk and defoliation). To characterize the locations, climatic data for each site were obtained from Fick and Hijmans (2017). At each site, tissue from lesions was directly plated onto CMA-PARPHB selective medium. Plates were incubated at 20 °C in darkness. *Phytophthora*-like hyphae were transferred onto V8 agar medium and stored at 20 °C.

For molecular identification, fresh mycelium of each isolate was scrapped from the surface, added to water, and boiled at 96 °C for 5 min to extract DNA. Taxonomic identification was performed using the PA-F/R primer (Ioos et al. 2005) and the allele-specific primers RAS-PAM1-F/R, RAS-PAM2-F/R and TRP-PAU-F/R, and RAS-PAU-F/R (Ioos et al. 2006). When using PA-F/R and RAS-PAU-F/R primers, the cycling conditions consisted of an initial denaturation step at 95 °C for 3 min followed by denaturation for 35 cycles at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min (Ioos et al. 2005, 2006). When using the allele-specific primers RAS-PAM1-F/R, RAS-PAM2-F/R, and TRP-PAU-F/R, the cycling profile for PCR was the same as that described above except that the annealing temperature was 60 °C (Ioos et al. 2006).

Unknown *Phytophthora* isolates were identified using the ITS6 (Cooke and Duncan 1997) and ITS4 (White et al. 1990) primers. The cycling conditions consisted of an initial denaturation step at 94 °C for 5 min, denaturation for 35 cycles at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min (Samils et al. 2011). PCR products were sequenced by Macrogen.

Results

Pathogenicity tests

Phytophthora isolates were successfully re-isolated from bark lesions in 80% of inoculated saplings. By contrast,

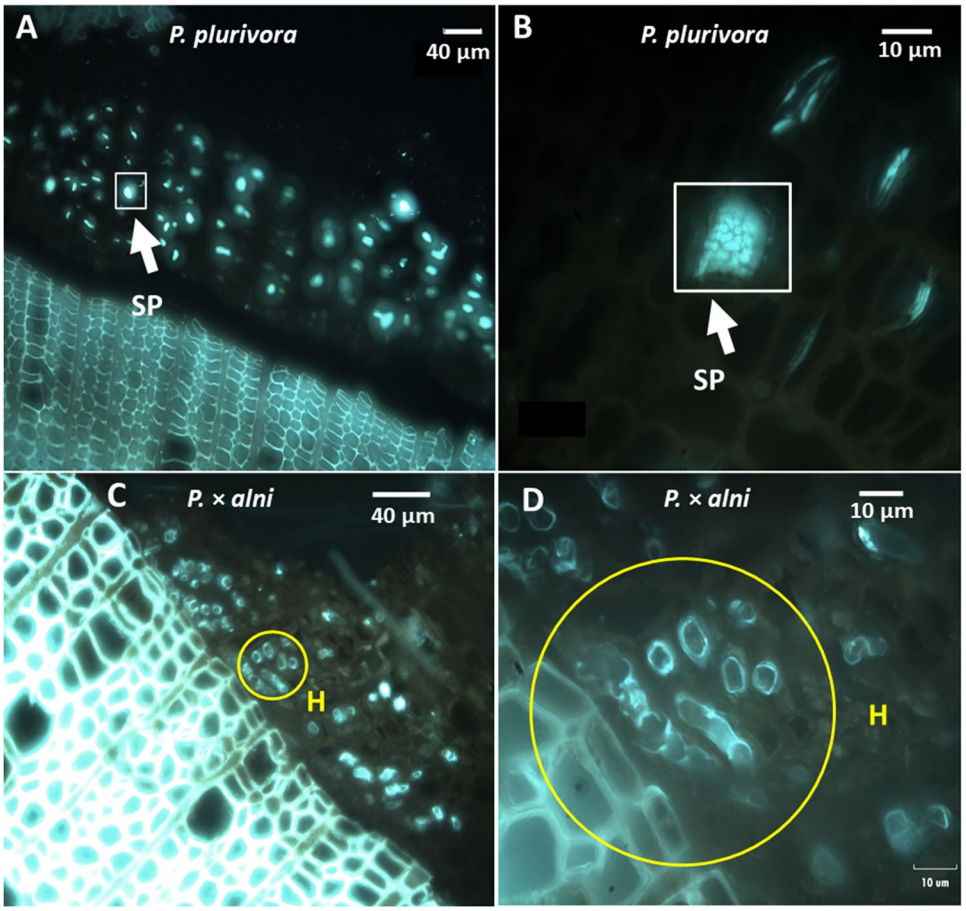

Fig. 5 Fluorescence micrographs at different magnifications of stem cross sections of black alder infected with *Phytophthora* and stained with aniline blue. **A**, **B** Callose in sieve plates (SP) of *A. glutinosa* inoculated with *P. plurivora*. **C**, **D** Presence of hyphae (H) and the absence of callose in sieve plates of *A. glutinosa* inoculated with *P.* × *alni*

the *H. fluviatilis* H1 isolate was only re-isolated from 33% of inoculated saplings, and isolate H2 was not re-isolated from any inoculated saplings. Average lesion lengths differed among inoculated species (Fig. 6, Online resource 1). Saplings inoculated with *Phytophthora* × *alni* developed the largest lesions, followed by saplings inoculated with *P. plurivora*, *P.* × *cambivora*, *P. cactorum*, or *H. fluviatilis*. Only saplings inoculated with *P. castanetorum* did not develop significantly larger lesions than the control (Fig. 6). Control saplings remained symptomless, with small lesions only detected in two of the plants.

Wood anatomy and microscopy

The putative presence of hyphae in phloem and xylem, the number of altered parenchyma, fibre and vessel cells, and potential defence responses (i.e., lignin, tyloses, callose) of saplings inoculated with different pathogenic species significantly differed among the inoculated species (Fig. 6, Online resource 1). Calcofluor white staining putatively detected hyphae of all inoculated species, except for *H. fluviatilis* (Fig. 6). Based on safranin and Astra blue staining, the number of hyphae in phloem observed in *P.* × *alni* and *P. plurivora*-inoculated saplings was higher than that of the control (Figs. 6 and 7A). The number of hyphae in xylem of *P. plurivora*-inoculated saplings was higher than that of the control (Fig. 6). All studied species produced necrosis in cambium, which was detected with Astra blue and safranin stain. Saplings inoculated with *P.* × *alni* or *P. plurivora* showed the greatest number of alterations in ray parenchyma cells, being significantly greater than that of *P. castanetorum* and the control (Fig. 6), and those inoculated with *P.* × *alni* or *P. cactorum* showed the most alterations in axial parenchyma, significantly higher than that of *H. fluviatilis* and control (Figs. 6 and 7B). Saplings inoculated with *P. castanetorum* had the fewest alterations in ray parenchyma, those inoculated with *H. fluviatilis* had the fewest axial parenchyma alterations, and those inoculated with *P. plurivora* had the fewest altered fibres and vessels (Figs. 6 and 7C, D).

Halophytophthora fluviatilis-infected saplings were the only saplings without lignin (Fig. 6). They also showed the highest frequency of callose in sieve plates, being significantly higher than *P.* × *alni*-infected saplings and control, and the lowest frequency of tyloses, being significantly lower than *P. plurivora*-infected saplings (Fig. 6). By contrast, *P.* × *alni*-infected saplings showed the lowest frequency of callose in sieve plates (Fig. 5), and *P.*

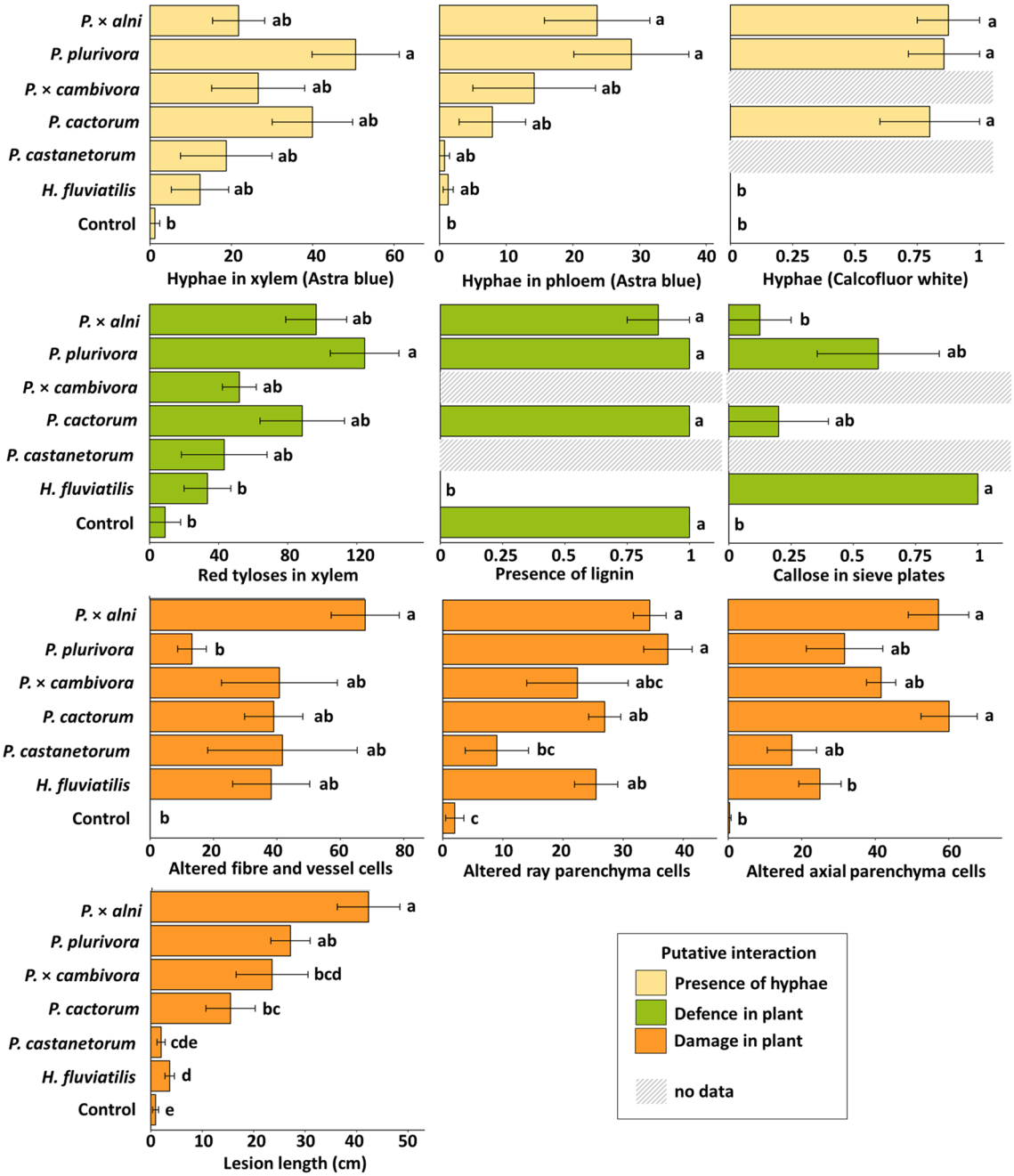

Fig. 6 Effect of inoculating black alder with different *Phytophthora* or *Halophytophthora* species on variables related to the presence of hyphae (detected using Astra blue and safranin or Calcofluor white staining), plant defence, and plant damage (stained with Astra blue and safranin). Different letters represent significant post hoc differences (Tukey HSD) among species at $p \leq 0.05$. Values are means ± SE

plurivora-infected saplings showed the highest frequency of tyloses (Fig. 6).

The two components of the PCA (KMO = 0.675) jointly explained 53% of the variance (Fig. 8). The first and second principal components accounted for 36% and 17% of the variance, respectively. The first principal component was mainly determined by callose in sieve plates (positive loading), lesion length, lignin, and hyphae (Calcofluor white staining) presence (negative loadings), whereas the second principal component was mainly determined by altered fibres, vessels, and axial parenchyma (positive loadings) and by hyphae in xylem (Astra blue and safranin staining) and tyloses (negative loading) (Online resource 2). The first component separated species with low pathogenicity such as *P. castanetorum* and *H. fluviatilis* (lower loadings) from the other species (Fig. 8). The second component differentiated

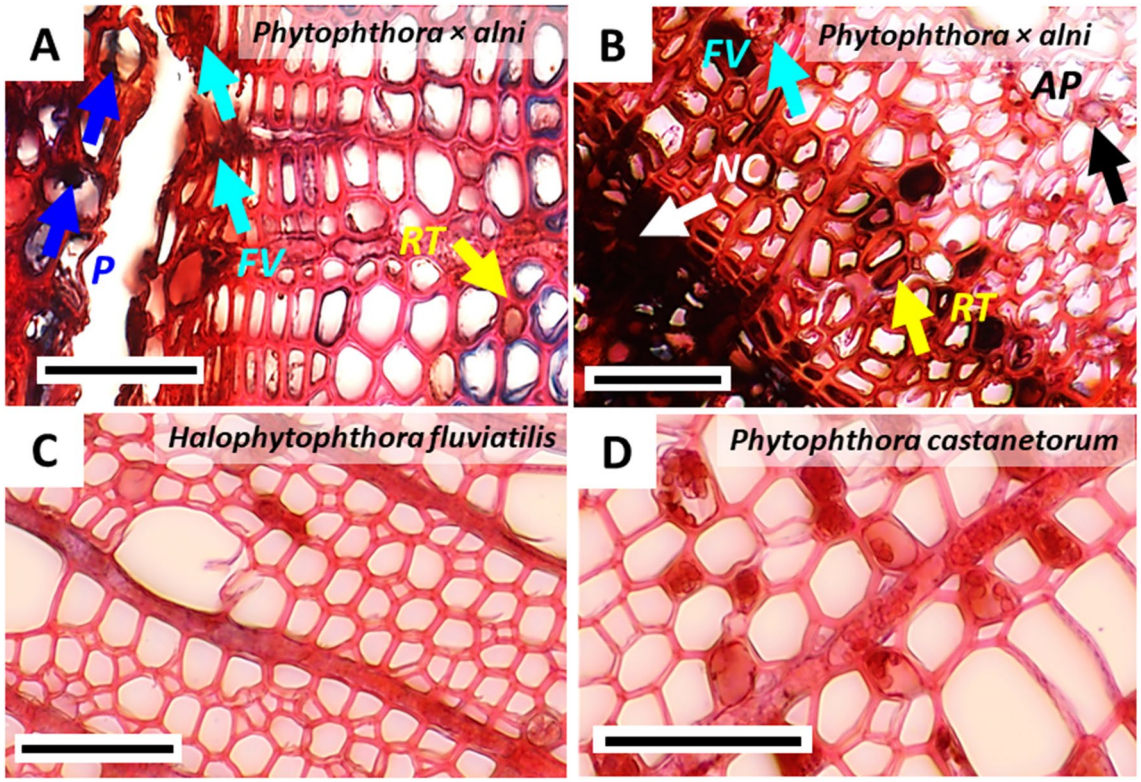

Fig. 7 Light microscopy stem sections of black alder saplings inoculated with *Phytophthora* × *alni* (**A**, **B**), *Halophytophthora fluviatilis* (**C**), or *P. castanetorum* (**D**) and stained with safranin and Astra blue. Arrows indicate hyphae in the phloem (*P*, dark blue), altered fibres/vessels cells (FV, light blue), red tyloses in xylem (RT, yellow), necrosis in cambium (NC, white), and altered axial parenchyma (AP, black). Scale bars represent 50 µm

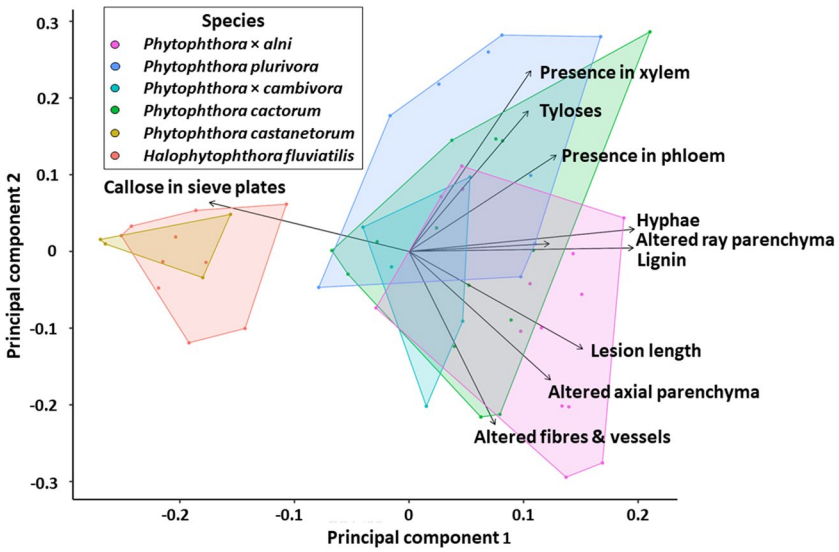

Fig. 8 Principal component analysis of variables measured in black alder saplings inoculated with different *Phytophthora* or *Halophytophthora* species

P. plurivora (higher loadings) from *P.* × *alni*, whereas the other species had intermediate positions (Fig. 8).

Individual correlations between variables showed that some variables were linked with lesion length whereas others were not related (Table 2, Online resource 3). For instance, lesion length was positively correlated with the presence of hyphae in phloem and alterations in vessels, fibres, and parenchyma cells (Table 2). Tyloses were positively correlated with the presence of hyphae (in xylem and phloem) and with alterations in parenchyma cells (Table 2).

Table 2 Correlations (Spearman coefficients) between lesion length, tyloses, and hyphae in phloem and xylem and altered fibres/vessels and axial and ray parenchyma cells (stained with Astra blue ('AB') and safranin ('S') or iodide ('I')) of black alder saplings inoculated with different *Phytophthora* or *Halophytophthora* species. Significance levels: ***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$. Significant values ($p \leq 0.05$) are shown in bold type face

	Lesion length	Tyloses	Hyphae in xylem	Hyphae in phloem
Tyloses	0.12	–	–	–
Hyphae in xylem (AB, S)	0.13	**0.57*** **	–	–
Hyphae in phloem (AB, S)	**0.53*** **	**0.42** **	**0.47*** **	–
Altered fibres and vessels	**0.33***	0.00	0.05	0.07
Altered ray cells (AB, S)	**0.40** **	**0.36** **	0.24	**0.35** **
Altered axial cells (AB, S)	**0.31***	**0.29***	0.12	0.03
Altered ray cells (I)	0.02	**0.43** **	0.17	0.01
Altered axial cells (I)	−0.02	**0.27***	−0.03	0.00

The presence of hyphae in phloem was positively correlated with altered ray cells (Table 2).

Phytophthora field isolations in alder

Altogether, *P.* × *alni* was detected in symptomatic alders at three locations, some of them with high average summer temperatures and maximum summer temperatures (Table 1, Online resource 4). Moreover, *P. plurivora* was isolated from one location (Table 1, Online resource 4). This is the first report of *P.* × *alni* in the Spanish Pyrenees.

Discussion

This study investigated the impact of *Phytophthora* infection on the stem xylem and phloem of alder trees to gain insights into why *P.* × *alni* is the dominant *Phytophthora* species in riparian ecosystems, even though alders are apparently susceptible to a large array of other *Phytophthora* species. Perhaps, the most iconic case concerns *P. plurivora*, which is very rarely found in riparian forests (Bjelke et al. 2016; Jung et al. 2018), even though this study, as well as previous ones (Trzewik et al. 2015; Zamora-Ballesteros et al. 2017), have shown that it can cause lesions in alder saplings that are similar to those caused by *P.* × *alni*. Other exotic *Phytophthora* species tested in this study (*P.* × *cambivora* and *P. cactorum*) presented intermediate levels of pathogenicity, whereas putative native species such as *P. castanetorum* and *H. fluviatilis* were the least aggressive.

Although saplings inoculated with *P.* × *alni* or *P. plurivora* developed the longest lesions, anatomical observations revealed differences in terms of the host response and the impact of these pathogens on plant structure. Alterations of parenchyma cells suggested that *P.* × *alni* (and *P. cactorum*) triggered both radial and axial responses, whereas responses to *P. plurivora* were mainly found along radial parenchyma, which was also seen in saplings inoculated with the other less pathogenic species. *Phytophthora* × *alni* and *P. plurivora* also showed differences in their capacity to colonize the xylem and phloem. Although *P.* × *alni* hyphae were mostly observed in the phloem, *P. plurivora* hyphae were abundant not only in the phloem but also in the xylem. Being able to attack the xylem could be seen as a competitive advantage for *P. plurivora* over *P.* × *alni*. Xylem infection is often associated with a high production of tyloses, which in turn cause vessel embolism, reducing hydraulic conductivity (Cochard and Tyree 1990; Parke et al. 2007).

Jung and Blaschke (1996) also found *Phytophthora*-induced tyloses that could potentially lead to water shortage and nutrient deficiency. One of the roles of tylosis is thought to be as a defence mechanism to hamper the advance of mycelium (Philips et al. 1986; Andrade-Hoyos et al. 2015). However, other studies have found no or even negative relationships between the presence of tyloses and plant resistance to *Phytophthora* species (van den Berg et al. 2018; Bufé 2019). In our study, tyloses were induced the most in plants inoculated with *P. plurivora*, one of the most aggressive species, and showed positive correlations with the presence of hyphae and damage in wood cells, suggesting that tyloses could be more indicative of the damage caused by the pathogen than of plant resistance. Likewise, lignification seems to be an indicator of damage, as it has also been associated with susceptible roots and abundant hyphae in *Phytophthora*-infected seedlings (van den Berg et al. 2018). Lignification was only absent in saplings inoculated with *H. fluviatilis*, the least pathogenic species.

Besides the lack of plant responses in the xylem to *P.* × *alni* infection, the lack of callose formation in the sieve plates was also a specific response to *P.* × *alni*. Callose formation in sieve plates has been related to plant resistance to *Phytophthora* pathogens (Vleeshouwers et al. 2000; van den Berg et al. 2018). For instance, van den Berg et al. (2018) found no callose formation in susceptible roots with invading hyphae. In our inoculation experiment, callose formation was almost absent in plants inoculated with the highly pathogenic *P.* × *alni* and was most abundant in plants inoculated

with the less pathogenic *H. fluviatilis*. By contrast, sieve plates with callose were almost five times more abundant in saplings infected with *P. plurivora* than in those infected with *P.* × *alni*, indicating a stronger defence response to *P. plurivora* than to *P.* × *alni*. We speculate that avoiding recognition by the host is a possible mechanism that could explain why *P.* × *alni* has become such a dominant pathogen in alder. By contrast, *P. plurivora* may be less pathogenic under field conditions, provided that the host is able to recognize the pathogen quickly enough and is able to mount a sufficiently strong defensive response.

High inter-specific variability was observed in terms of the impact of infection by the different *Phytophthora* species on the same host, which is similar to the observations reported by Clemenz et al. (2008). Significant correlations between lesion length, which is frequently used to evaluate pathogenicity, with damage and infection-related variables were found, in contrast to Zamora-Ballesteros et al. (2017). However, these correlations were rather low, highlighting that lesion length only represents a part of the pathogen–tree interaction and that histological analyses can provide a more comprehensive assessment of the actual damage caused by the pathogen. Of special interest are the variables that helped to differentiate *P.* × *alni* and *P. plurivora*, which caused lesions of similar lengths but which make very different contributions to alder decline in nature. As observed in the second component of the PCA, tyloses and hyphae in the xylem provided additional information not correlated with lesion length, which helped to differentiate *P.* × *alni* from *P. plurivora*. Callose in sieve plates and lignin, also not correlated with lesion length, allowed us to differentiate pathogens causing more disease in nature from less harmful species.

In the Spanish Pyrenees, the species complex *P.* × *alni* and, to a much lesser extent, *P. plurivora* seem to be the main *Phytophthora* species attacking alder trees in nature. The situation in the Pyrenees is somewhat similar to that observed in Bavaria (S Germany), Sweden, and Poland, where the frequency of *P. plurivora* is usually much lower than that of *P.* × *alni* (Redondo et al. 2015a; Trzewik et al. 2015; Jung et al. 2018). The presence of *P.* × *alni* in the Pyrenees is concerning because *A. glutinosa* populations might be especially vulnerable given that they are at the southern edge of the species distribution (populations further south have recently been reclassified as *Alnus lusitanica* Vít, Douda & Mandák) and given the low genetic diversity of alder populations in the Pyrenees (Havrdová et al. 2015).

Conclusions

Although most of the *Phytophthora* species tested were able to infect and damage the host alder species, histological observations showed that apart from *P.* × *alni* and *P. plurivora*, the tested isolates had only a feeble impact on plant tissues. The formation of less callose in sieve plates and fewer tyloses in saplings inoculated with *P.* × *alni* rather than *P. plurivora* suggests that the higher prevalence of *P.* × *alni* in declining alders in the field compared with *P. plurivora* seems to relate to a lack of recognition of *P.* × *alni* by the host rather than to the capacity of *P.* × *alni* to cause lesions. Histological analyses of xylem and phloem, two major tissues for woody plant functioning, can complement pathogenicity tests and ecophysiological measures and help to explain the impacts of *Phytophthora*–plant interactions in nature.

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Author contribution Jonàs Oliva designed the experiment. Material preparation and data collection were performed by Michele Colangelo, J. Julio Camarero, Maria Caballol, Francisco José García Breijo, and Dora Štraus. Cristina Vieites-Blanco performed the statistical analysis and interpreted the results. The first draft of the manuscript was written by Cristina Vieites-Blanco and Jonàs Oliva. All authors read and approved the final manuscript.

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Data availability Data obtained in this study are available at Figshare (10.6084/m9.figshare.23904495) (Vieites-Blanco et al. 2023).

Declarations

Competing interests The authors declare no competing interests.

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