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Anatomical Defence Responses of Resistant and Susceptible 12-Years-Old Pine Species Infected with *Bursaphelenchus xylophilus*

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ABSTRACT

Pine wilt disease (PWD) is caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, an endoparasite that infects *Pinus* species. While some pine species are susceptible to PWD, others seem to be resistant or resistant under field conditions, through mechanisms still not understood. In this study, we evaluated the damage and anatomical response of PWN-susceptible (*Pinus sylvestris* and *Pinus pinaster*) and resistant (*Pinus halepensis* and *Pinus pinea*) pine species, through histological analysis along five time-periods following nematode inoculations on 12-year-old trees. We observed a time-framed sequence of defensive responses, with the initial formation of wound-periderm, the accumulation of calcium oxalate (CaOx) crystals, and an enhanced activity of polyphenolic parenchyma (PP) cells, particularly in the resistant species. The presence of phenolic compounds, lignification of cell walls, and the formation of traumatic resin canals were also observed in the resistant species. Additionally, the development of *callus* tissue in response to nematode invasion may represent a potential barrier to nematode progression, emphasising the role of rapid tissue regeneration in nematode-resistant pines. These trees exhibited slower damage progression and more effective nematode containment compared to the susceptible *P. pinaster* and *P. sylvestris*, which suffered extensive tissue disruption, apparently due to the absence of critical physical barriers. We did not find evidence of early dissemination of *B. xylophilus* through the resin canals. Overall, our study highlights the importance of early anatomical and biochemical responses in preventing the dissemination of the PWN, providing a deeper understanding of the defence strategies regulating the susceptibility of pine species to PWD.

Keywords: histochemistry; wood anatomy; phenolic compounds; pine wilt disease; *Pinus*

INTRODUCTION

Native to North America, the pine wood nematode (PWN) *Bursaphelenchus xylophilus,* has emerged as a threat to both Asian and European pine forests, being the causal agent of Pine wilt disease (PWD) (Webster and Mota 2008).

Several native European pines are among the most susceptible species to PWD, including *Pinus pinaster*, *P. nigra* Arnold and *P. sylvestris* L., along with non-native species such as *P. radiata* D. Don. Inversely, *P. pinea* L. and *P. halepensis* Miller have shown resistance to PWN under field conditions (Evans et al. 1996, Mota et al. 1999, Naves et al. 2008a, Sousa et al. 2011, Inácio et al. 2015, Nunes da Silva et al. 2015, Zamora et al. 2015, Silva et al. 2021, Fonseca et al. 2024), by mechanisms which are still not understood.

Transmission of the PWN between hosts is mediated by insect vectors of the *Monochamus* genus, known as pine sawyer beetles, with *Monochamus galloprovinciallis* Olivier being the only vector in Europe (Sousa et al. 2001, Naves et al. 2007). When the beetles feed on the bark and phloem of healthy pine trees, the nematodes invade the branches through the wounds and, once inside, are reported to feed on the epithelial parenchyma cells lining the cortex resin canals, with nematodes believed to disperse within the host via the resin canals of the xylem (Ichihara et al. 2000, Kawaguchi 2006, Mamiya 2008, Son et al. 2010, Futai 2013, Nunes da Silva et al. 2015).

In susceptible pine species, this process induces extensive tissue damage, eventually leading to a reduction in resin production and damage to cambium and phloem tissues, resulting in the death of the trees within a few months.

Several publications have described the within-host distribution of the PWN and its detrimental effects on pines, including denaturation and necrosis of parenchyma cells (Ichihara et al. 2000, Hara and Takeuchi 2006), cavitation and embolism in the tracheids (Kuroda 2008, Umebayashi et al. 2011, Yazaki et al. 2018), biochemical responses (Rodrigues et al. 2021, Trindade et al. 2022) and differential expression of genes (Kuroda et al. 2011, Hirao et al. 2012, Gaspar et al. 2017, Liu et al. 2017). However, these studies have been primarily focused on pine seedlings inoculated with the PWN, while the anatomical response of adult pine trees has yet to be studied. In this study, we aimed to characterise the defence responses of European pine species by examining 12-year-old susceptible and resistant pine trees, providing a more realistic approach that simulates natural infection of adult trees by *Monochamus* vectors in the field. To achieve this, we artificially inoculated the PWN into the pine hosts by simulating a feeding wound from the insect vectors, avoiding extensive tissue damage or direct injection of the nematode into the pine tissues. We then evaluated the within-host distribution, damage, and impacts of PWN at five time points after nematode inoculation.

MATERIALS AND METHODS

Plant Material

Experiments were conducted on 12-year-old potted (80Lt vases) trees of four pine species, selected for their contrasting susceptibility to pine wilt disease: *P. sylvestris* and *P. pinaster* as susceptible, and *P. pinea* and *P. halepensis* as resistant (Evans et al. 1996, Naves et al. 2008a, Sousa et al. 2011, Nunes da Silva et al. 2015, Zamora et al. 2015, Silva et al. 2021). The trees, approximately 3 m high, were maintained under natural conditions at the INIAV campus in Oeiras, Portugal (38°41' N, 9°19' W; 39 m above sea level), with daily watering. The experiment took place in July 2017, under climatic conditions suitable for PWN development (mean temperature of 25°C, © WeatherSpark.com).

Nematode Culture and Tree Inoculation

Bursaphelenchus xylophilus isolate Bx013.003 GenBank database (NCBI) accession number MF611984.1) was provided by the INIAV's Nematology Laboratory (national reference laboratory for the PWN). The nematodes were reproduced in Erlenmeyer flasks filled with 10 g of barley seeds, 10 mL distilled water (autoclaved at 120 ◦C for 20 min) and *Botrytis cinerea* Pers., and maintained at 26 ◦C in the dark (Eppo 2020).

Inoculation trials were conducted in July 2017, aligning with the peak seasonal activity of *Monochamus* insect vectors and nematode transmission in the field (Naves et al. 2008b). For each *Pinus* species, five trees were inoculated with *B. xylophilus*, five trees were left untreated (control I), and five trees were inoculated with deionised water (control II). On each inoculated pine, a superficial scratching wound (~4 cm), simulating the feeding wounds made by the adult *Monochamus*, was made with a sterilised scalpel on a randomly selected, living branch (Figure 1).

An aqueous suspension of 400 µL containing ~2500 nematodes of different stages, kept under constant agitation, was slowly applied to each wound, which was then covered with sterile cotton and sealed with Parafilm to prevent dehydration. The same procedure was followed for control II trees, using deionized water only. In all the trees, branches of comparable age and diameter were used.

Histological Analysis

Wood samples were collected from five replicates (trees) of non-inoculated pines (control I) on the same day inoculations were made. In the water (control II) and nematode-inoculated treatments, five replicate (trees) samples for each were collected at 1, 3, 7, 16 and 28 days post-inoculation (dpi). For the sequential time-points 1/16 dpi and 3/28 dpi, samples were taken from different branches of the same tree.

Each replicate consisted of two ≈1 cm branch section (mean diameter of ≈1,2 cm), collected 2 and 4 cm below the inoculation site. Upon collection, samples were immediately fixed for 48 hours at room temperature in a 1:1:18 formaldehyde – acetic acid– alcohol solution (glacial acetic acid: 38% formaldehyde: 70% ethanol), and later dehydrated in a standard progressive ethanol series, embedded in paraffin and serial-sectioned at 10 µm (Leica, RM 2255). Sections were stained with Astra Blue + Safranin to allow observation of wood tissue anatomy and subsequently stained with Acid Fuchsin for nematode detection. All stained sections were cover-slipped with Entellan®. Wood sections were analysed for tissue damage and anatomical changes, which are presented separately.

Cross sections were examined with a light microscope (Leica, DM 2500 LED) and images obtained with the Leica Application Suite X 5.1.0.25446 software. Overall, we analysed a total of 205 replicate samples.

Statistical Analysis

A matrix was constructed using data from Table 1 (Suppl. File 2), excluding the presence of nematodes (N) and cortical (CRC) and xylem (XRC) resin canals.

The analysis considered the three tissues (cortex, phloem/cambium, and xylem) across the four *Pinus* species, factoring in the different time points after inoculation. The presence (1) and absence (0) of plant responses for each tree replicate was used to generate a binary matrix. Subsequently, a dissimilarity matrix was calculated using Jaccard distances (Paradis et al. 2004). To evaluate dissimilarities among species and inoculation times, Principal Coordinates Analysis (PCoA) was performed using the vegan R package (Oksanen et al. 2013). All analyses were conducted using R software (R Core Te 2013).

RESULTS

General Anatomy of Non-infected *Pinus* spp

Pine tissues exhibited the typical anatomical characteristics of healthy, adult *Pinus* trees (Figure 2). The periderm cells were arranged in a uniserial pattern and consisted of several layers of dead, flattened and suberized cells, which varied in thickness between the pine species.

Beneath the periderm, the cortex contained large vacuolate parenchyma cells, ranging in shape from rounded to irregular. The cortical resin canals were distributed in a single row, and the parenchyma cells contained granular and dense polyphenols. As expected, the secondary phloem consisted of three primary cell types: phenolic parenchyma cells (PP cells), sieve cells and ray parenchyma cells. The parenchyma cells were thin-walled, approximately circular in cross section and scattered with large vacuoles, prominent nucleus, and polyphenolic parenchyma cells (PP cells). Axial PP cells had a circular cross-sectional profile, containing vacuolar reddish phenolic bodies, and occurred in distinct rows separated by 5–10 rows of sieve cells and associated albuminous cells. Sieve cells were elongated

Figure 1. Example of the tree inoculation process in a 12-year-old *Pinus* spp. The images illustrate the experimental setup and steps involved in the mechanical wounding. **(a)** General view of the *Pinus* spp. trees used in the experiment; **(b)** Detail of the branch after mechanical wounding simulating *Monochamus* feeding activity; **(c)** Branch wound covered with Parafilm.

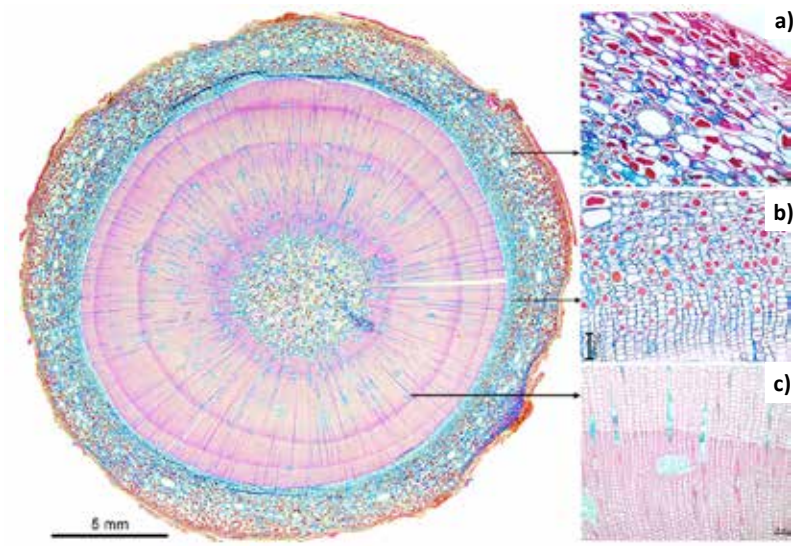

Figure 2. Example of the general anatomy of a non-infected *Pinus halepensis* spp branch sample (Bar 5 mm), with details for the **(a)** periderm and cortex, **(b)** phloem and **(c)** xylem, Bar 75 µm.

with unlignified thin walls, with many lateral sieve areas. The phloem rays were similar to the xylem rays, uniseriate and heterocellular, being continuous between the phloem and xylem tissues. At the time of sampling, the cambial zone was active, as expected during the spring/summer months. In the xylem, dispersed axial resin canals were found in all years of growth, but no polyphenols were observed.

Tissue Damage and Cellular Responses After PWN Infection

One Day After Infection

The first signs of tissue damage were observed in the cortical parenchyma tissue after one day (Figure 3), which was not observed in the control I (Figure 3a) and water-inoculated control II trees (Suppl. File 1). Rupture cells at the periphery of the cortex (Figure 3b), along with thicker cell walls and swollen cortical parenchyma cells, were generally observed in the nematode-inoculated trees, while the periderm remained intact in all species. Transverse cross-sections of stems showed an increase in phenolic content in the phloem tissue with new formation of polyphenolic parenchyma cells (PP), compared with the control I and II. In all species, some cellular variations became evident, particularly the swelling of PP cells, containing large inclusion bodies visible as red and blue aggregates in the photos (Figure 3c).

In general, ray parenchyma cells remained distinct and intact. The presence of phenolic contents in the xylem rays of the resistant species became noticeable (Figure 3d). Inversely, phenolic compounds were not observed in the susceptible species and the two control (I and II) samples. At this stage, no damages were observed in the tracheid in all species.

Three Days After Nematode Inoculation

The PWD-susceptible *P. sylvestris* and *P. pinaster* exhibited cortical parenchyma cells losing shape and rupturing, affecting the cell integrity at this time frame. As shown in Figure 4a, excretions or degraded cellular residues caused by *B. xylophilus* are already noticeable, with a clear distinction between intact and affected/necrotic cells, which appeared with deformed and cleaved red cell walls and without cellular content. In contrast, the PWD-resistant *P. halepensis* and *P. pinea* showed an increase in phenolic content and the presence of starch in cortical parenchyma, compared to the other two species and to the control II.

In all species, the integrity of the cortical resin canals was preserved, although occasional changes were observed, including the swelling of epithelial cells (Figure 4b), and a progressive increase in the phenolic content of the sheath cells in the resistant *P. halepensis* and *P. pinea*. In PWD-susceptible pines, a decrease in the phenolic content in the sheath cells was observed compared to Controls I and II. A mechanical barrier composed of calcium oxalate crystals (CaOx) in styloid form was observed in the *P. halepensis* cortex, along with an accumulation of phenolic compounds near the inoculation site, between the cortical parenchyma cells and the phloem tissue (Figure 4c). This pattern was observed exclusively in *P. halepensis*. Likewise, wound-periderms were formed in *P. halepensis* (Figure 4d) and, within one week, also in *P. pinea*, but were absent in *P. pinaster* and *P. sylvestris*. The wound-periderms developed around cortical resin canals, with necrosis observed in

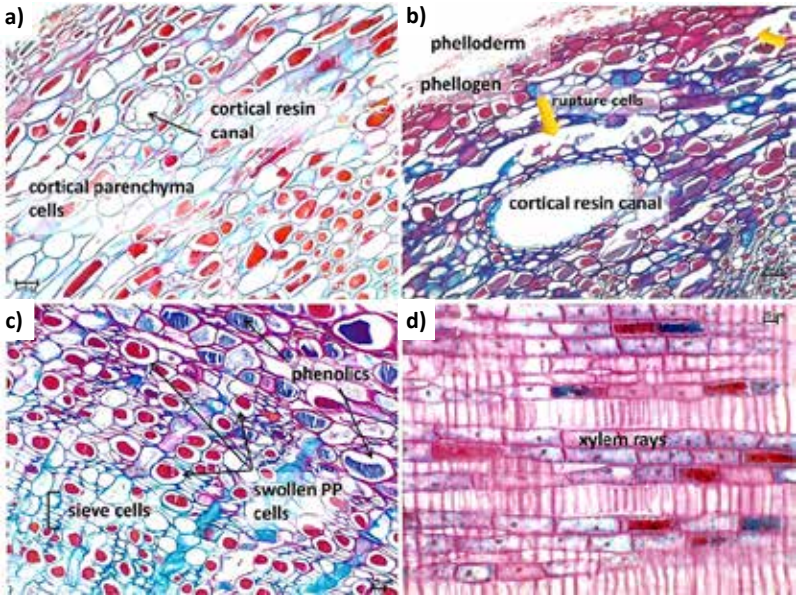

Figure 3. Light microscopy views of cross-sections of 12-year-old *Pinus* spp at one dpi. **(a)** control *P. halepensis*, Bar 75 µm; **(b)** first damages with ruptured cells at the periphery of the cortex in *P. halepensis*, Bar 75 µm; **(c)** phloem tissue showing polyphenolic parenchyma (PP) cells that have mobilized phenolic content and increased in size in *P. halepensis*, Bar 25 µm; **(d)** Presence of phenolic contents in the xylem rays of *P. pinea* visible as deep red and blue staining, Bar 25 µm.

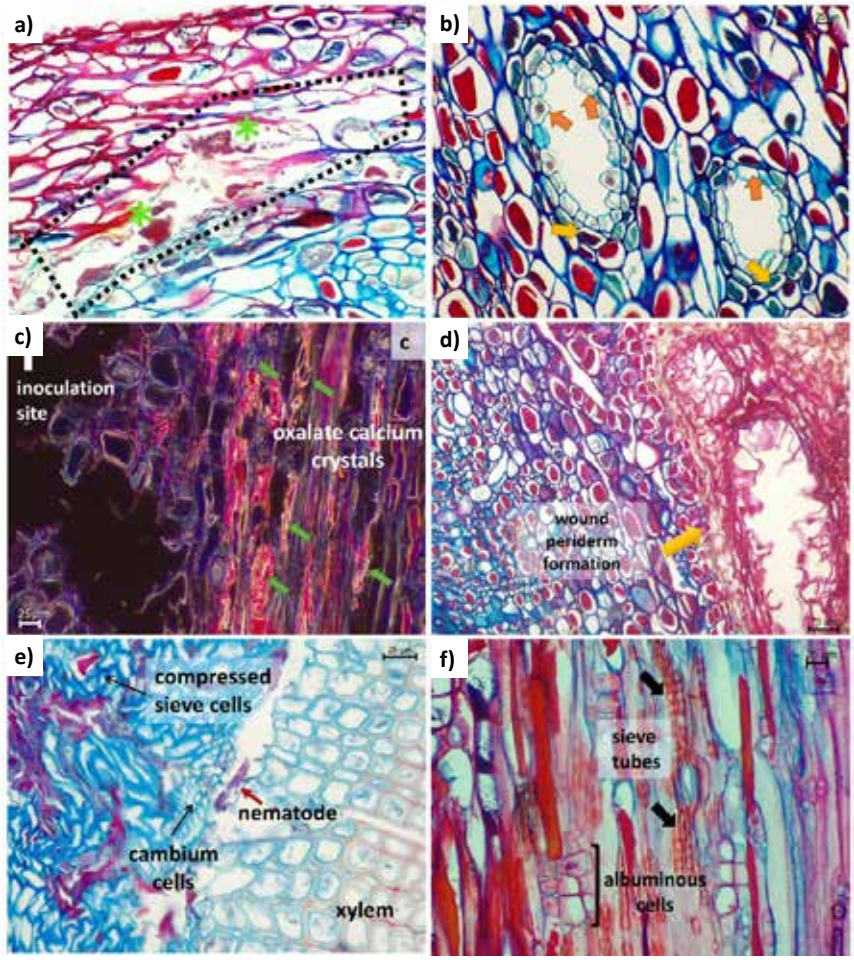

Figure 4. Cross-sections of 12-year-old *Pinus* spp. three days after PWN inoculation. **(a)** degraded cells with a clear separation between intact (blue cells) and necrotic (red cells) tissue in *P. sylvestris*. Nematode excretions or degraded cellular residues indicated by *, Bar 25µm; **(b)** swollen epithelial cells (indicated by yellow arrow) in cortical resin canal with increase of polyphenolic compounds in sheath cells (orange arrows) in *P. halepensis*, Bar 25µm; **(c)** longitudinal cut of cortex and phloem tissue, near to inoculation site, with presence of elongated calcium oxalate crystals (styloid), as bright spots along the parenchyma cells, with phenolic cell content in *P. halepensis*, Bar 75 µm; **(d)** induction of wound periderm and polyphenolic parenchyma cell in *P. halepensis*, Bar 75 µm; **(e)** compressed sieve cells and nematode presence in cambium tissue in *P. pinaster*, Bar 25 µm; **(f)** albuminous cells and sieve cells red stained, in a longitudinal section of phloem tissue in *P. halepensis*, Bar 25 µm.

the surrounding parenchyma cells. Rupture of phloem parenchyma cells was evident in *P. sylvestris*, and damage in the cambium tissue was also visible, leading to the formation of small cavities in the cambium region. In *P. pinaster*, the damage in the phloem and cambium was conspicuous, with compression of sieve cells and the presence of live nematodes in the cambium (Figure 4e). Sieve tubes and albuminous cells in *P. halepensis* stained red (Figure 4f), which could reflect a dynamic cellular response to infection, namely lignin accumulation in the radial walls of sieve cells (Vazquez-Cooz and Myer 2002, Yang et al. 2023). In *P. pinea*, irregularities in cell shapes with undulated cell walls were observed. At this stage, strands of phenolic clusters were observed in xylem tissue of *P. halepensis* (Figure 5a). In *P. pinea*, phenolic compounds were also detected, but in lesser amounts and more dispersed compared with *P. halepensis*. In susceptible species, the tracheids became flattened in earlywood tissues (Figure 5b), and the destruction of xylem rays in the presence of living nematodes was observed (Figure 5c and 5d).

Seven Days After Nematode Inoculation

In the susceptible *P. pinaster* and *P. sylvestris*, expansion of cell necrosis occurred 7 days after infection, characterised by disorganised parenchyma, loss of cell turgor pressure (Figure 6a), and compromised structural integrity. In the

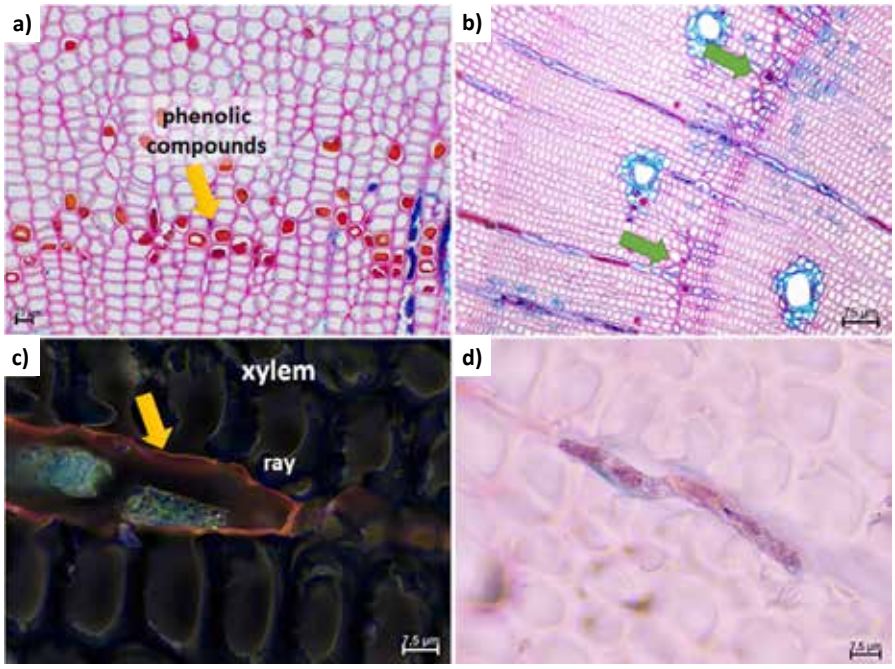

Figure 5. Histological features of stem xylem in inoculated 12-year-old *Pinus* species at three dpi; **(a)** strands of phenolic compounds in xylem tissues of *P. halepensis*, Bar 25 µm; **(b)** cross-section showing tracheids and ray parenchyma cells with abnormal shapes (arrows) in the earlywood in *P. halepensis*; Bar 75 µm; **(c)** and **(d)** longitudinal sections of xylem and ray parenchyma cells with nematodes in *P. sylvestris* and *P. pinaster*, respectively, Bar 7,5 µm

resistant species, the cortical parenchyma was densely packed with phenols and starch, as also found in control II; however, cell destruction became evident, with the rupture of cell walls in the nematode-inoculated pines. A conspicuous increase in size and number of PP cells was observed in the resistant pines, resulting in deformed and nonfunctional adjacent sieve cells, unlike control II (Figure 6b).

The direct passage of nematodes from the phloem to the xylem was visible in susceptible pines at this time point, with clusters of nematodes (composed of several individuals), and the collapse of the tracheids near the cambium (Figure 6c). In *P. halepensis*, styloid structures (elongated calcium oxalate crystals) were observed in xylem rays, with cell preservation (Figure 6d), while in *P. pinea* these structures were detected in the phelloderm (Figure 6e), being absent from the susceptible pines. In *P. pinaster,* tracheids exhibited inner walls with thread-like cracks, along with destruction of the ray parenchyma cells and epithelial cells of the xylemic resin canals. Traumatic resin canals (TRC) were present in *P. halepensis* along the new growth ring of the xylem (Figure 6f), a feature observed in the same pine species in the control II samples, but only at 28 dpi.

Sixteen Days After Nematode Inoculation

In the susceptible pines, the collapse of phelloderm cells, along with cell rupture, led to the death of inner bark tissue. This, combined with the disintegration of the phloem and cambium due to the direct feeding activity of the nematodes, resulted in the formation of intercellular spaces, causing generalised cell degeneration (Figure 7a). Although no nematodes were detected in these tissues, the damage is identical to that seen in Figures 4a and 6c. In contrast, the resistant species *P. halepensis* and *P. pinea* (Figure 7b) exhibited cells of varying size and shape, filled with different phenolic content. Additionally, some fissures were observed in the cortex. In phloem and cambium tissues, a linear, restricted cavity was visible. Inside, degraded cellular residues were observed, along with the formation of parenchyma cells with phenolic content in xylem tissue (Figure 7c). Oxalate calcium crystals in prism form were present in the phloem of *P. pinea*, while on *P. sylvestris*, crystals were only occasionally observed in the cortical parenchyma cells. No crystals were detected in *P. pinaster* or in the control samples.

In susceptible species, the denaturation of living cells in the xylem, such as ray parenchyma cells, was observed. At 16 days after infection, the nematodes had already destroyed most of the epithelial cells in the xylem resin canals (Figure 7d).

Twenty-Eight Days After Nematode Inoculation

Significant differences among pine species became even more apparent at this time point. For *P. halepensis* and *P. pinea*, the damage was not as extensive as in the other two species, but cell disorganization was evident in cortical tissue, along with cell deformation due to the breakdown of cell walls and the degradation of polyphenols (Figure

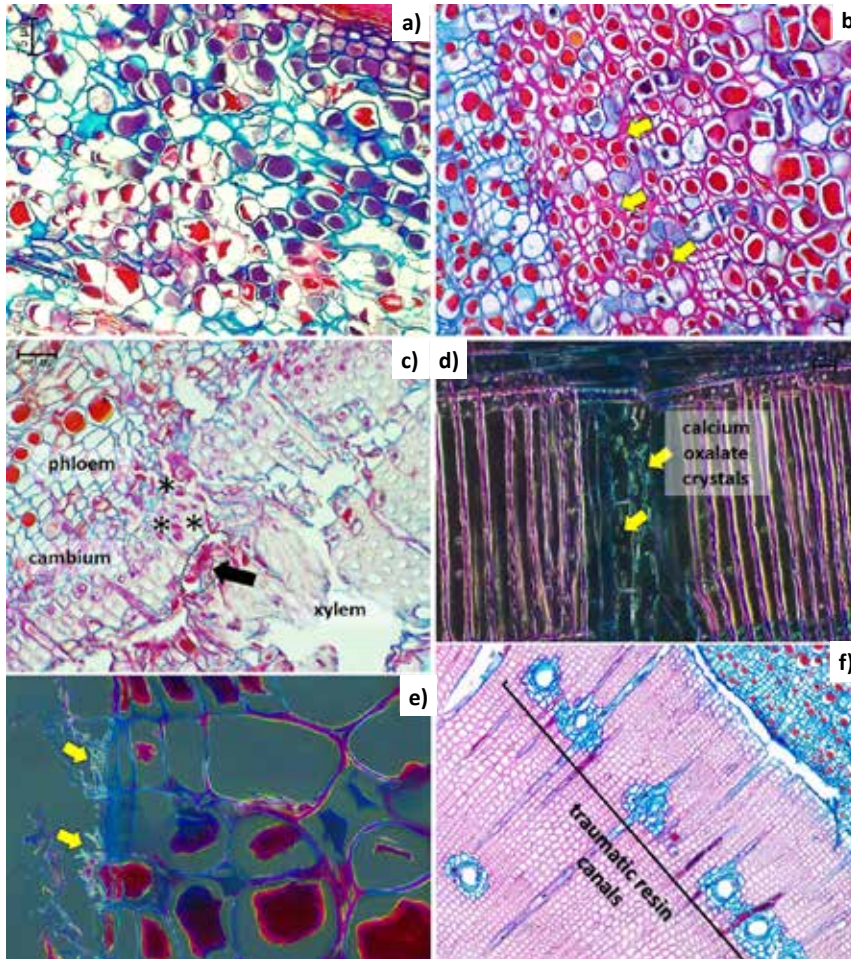

Figure 6. Transversal cross-sections of 12-year-old *Pinus* species after seven dpi. **(a)** disorganized cortical parenchyma cells with cell loss of turgor pressure in *P. sylvestris*, Bar 25µm*;* **(b)** *Pinus halepensis* showing enlargement of polyphenolic parenchyma (PP) cells while the adjacent elements appear to be crushed (arrows) with intense fuchsia stained, Bar 25µm; **(c)** nematodes (indicated by arrows and asterisks) migrate from the phloem to the xylem, causing tissue breakage in *P. pinaster*, Bar 50µm; **(d)** longitudinal sections of xylem ray parenchyma cells with elongated calcium oxalate crystals in *P. halepensis*, Bar 25 µm; **(e)** styloids in phelloderm in *P. pinea*, Bar 25µm; **(f)** development of traumatic axial resin canals in *P. halepensis*, Bar 75µm.

8a). In *P. pinaster* and *P. sylvestris*, browning and reddening of cortical parenchyma cells occurred in response to the nematode's presence (Figure 8b).

As in previous observations, xylem tissue damage was less extensive in *P. halepensis* and *P. pinea* than in the other two pine species. In both species, an increase in phenols was still occurring, and we observed the formation of callus in the xylem tissue of *P. halepensis*, consisting of cells with irregular shapes and thick, lignified walls (Figure 8c), and in the cambium of *P. pinea*. In the latter species, individual cells exhibited large vacuoles and a narrow layer of cytoplasm attached to a thin primary wall (Figure 8d), with reactivation of a new cambium above the *callus*. A clear lignification of the tracheids, ray parenchyma cells, and axial resin canal walls was evident for the resistant pines. At one day post-inoculation, these tissues had thin-walled epithelial cells packed with cytoplasm, while at 28 dpi, the cells were developing thick and lignified walls. The xylem resin canals also showed larger lumen with thick-walled sheath cells and thin-walled epithelial cells (Figure 8e) at this time point. In contrast, in *P. sylvestris* and *P. pinaster*, the entire phloem and cambium interface collapsed. Although no nematodes were directly detected on these sampled tissues at this time, the observations clearly suggest a response to the presence and feeding damages caused by *B. xylophilus*. In the control samples, no tissue damage or anatomical changes were observed in the cortex or cambium. In the phloem, disorganisation of PP cells and signs of traumatic resin canals formation were observed in response to wounding.

Nematodes were detected in the tracheids of *P. sylvestris* and *P. pinaster*, where they passed through the bordered pits, causing complete disruption of the xylem

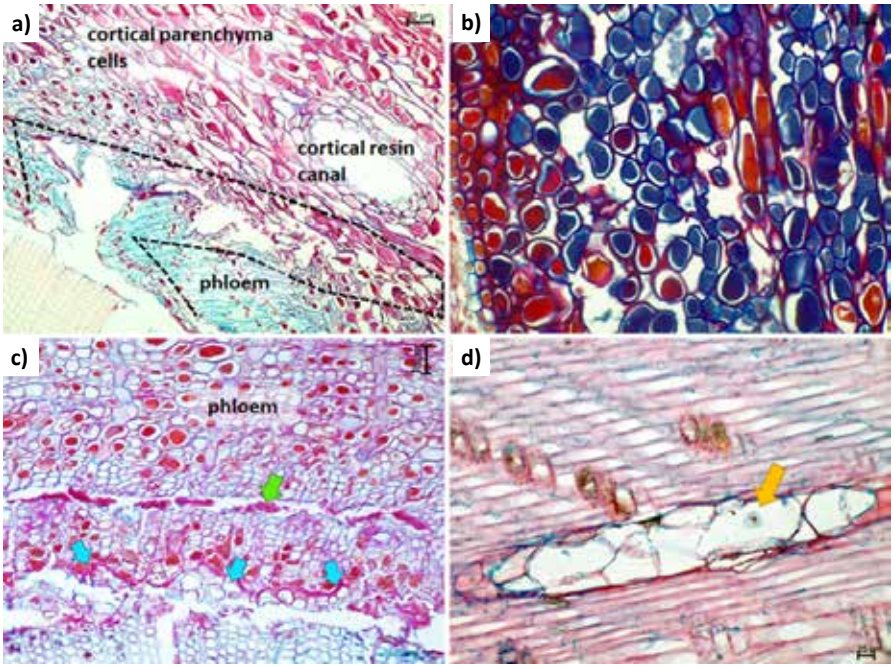

Figure 7. Cross-sections of 12-year-old *Pinus* species after 16 days. **(a)** cell degeneration originating intercellular spaces and collapse of the phelloderm in *P. sylvestris*, Bar 75 µm; **(b)** longitudinal cross-section of cortex with rupture of some parenchyma cell walls and cells filled with different phenolic content in resistant species (*P. halepensis*), Bar 25 µm; **(c)** cross-section stained with acid fuchsin showed cambium and xylem tissue with cavities filled with nematodes excretions or degraded cells. Formation of parenchyma cells with phenolic content in xylem tissue in *P. pinea*, Bar 75 µm; **(d)** longitudinal cut of xylem resin canal with epithelial cells destroyed and nematode inside the lumen (arrow) in *P. pinaster*, Bar 25 µm.

tissues, breakage of the lignified walls, and destruction of axial resin canals. At this point, fungi mycelium in both the tracheids and ray parenchyma cells (Figure 8f) appeared for the first time.

On the PCoA studying the matrix of responses across species and inoculation times, the first two axes accounted for 74,38% of the observed variance, with PCoA1 and PCoA2 explaining 52,16% and 22,22% of the variance, respectively (Figure 9).

Three main clusters were identified in the analysis. Cluster 1 predominantly includes early time points at one dpi of the four *Pinus* species, suggesting that early responses are relatively similar among species. The other two clusters represent the segregation between resistant (Cluster 2) and susceptible (Cluster 3) tree species at subsequent time frames.

Pinus pinea showed no significant differences between one and three days after inoculation, with all time points during this early period clustering within Cluster 1. Conversely, *P. halepensis* exhibited a striking divergence between one and three days post-inoculation. However, the later time points (3, 7, 16, and 28 days post-inoculation) for *P. halepensis* were more similar to each other and grouped within Cluster 2, along with the other time points for *P. pinea*.

Cluster 3 predominantly includes late time points (28dpi) and some intermediate samples from susceptible species. Notably, *P. pinaster* at 16 dpi stands out as an outlier, positioned far from other samples along PCoA1. This separation is attributed to the presence of calcium oxalate crystals in both the cortex and phloem/cambium cells.

DISCUSSION

This study revealed a clear and sequential timeline of contrasting and increasing tissue, anatomical and biochemical responses and damage among four pine species with different susceptibility to PWD.

Overall, damage to the cortex, phloem, cambium, and xylem parenchyma tissues was generally more extensive and severe in *P. pinaster* and *P. sylvestris* compared to *P. halepensis* and *P. pinea*, supporting the hypothesis of different degrees of susceptibility between these two groups of pine species.

Reactions to nematode presence at one dpi were common across all species. After 3 days, anatomical changes were observed only in replicates of resistant species, unlike the susceptible pine species, as detailed in Table 1 (Suppl. File 2).

The embedment of the samples with paraffin (instead of resin), because of the branches' relatively large dimensions, may explain the absence of nematodes on some tissues with observed damage/putative defences, as the nematodes may be dislodged during paraffin removal.

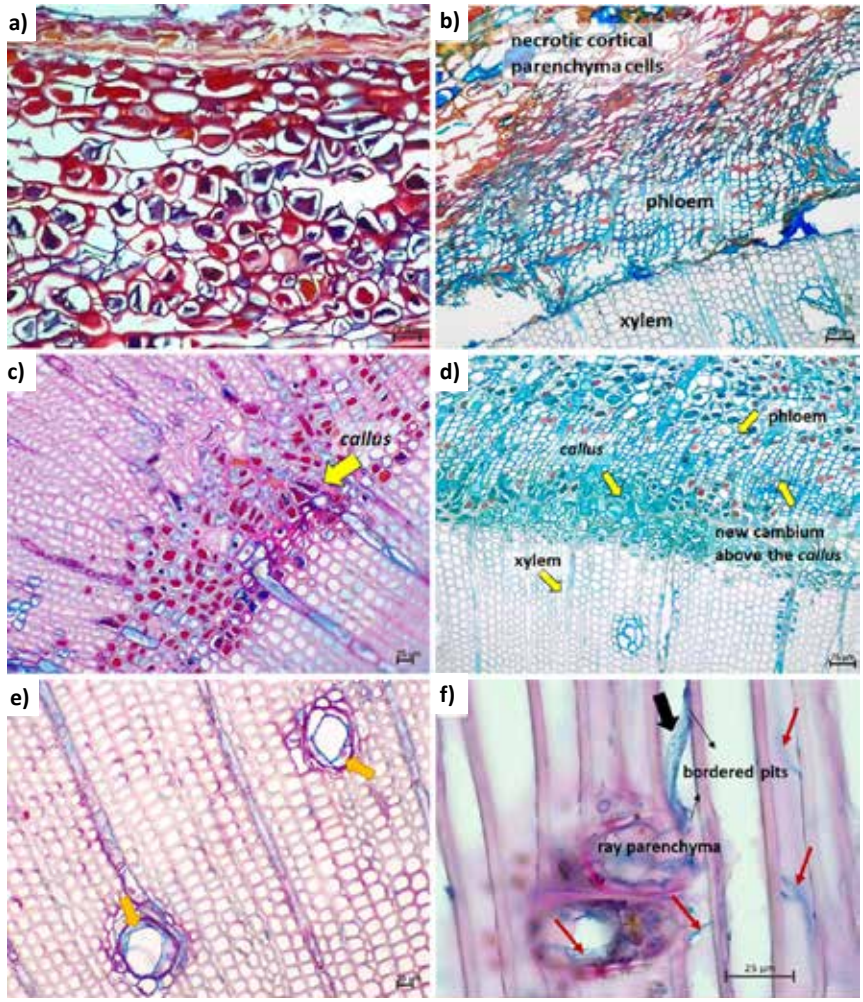

Figure 8. Cross-sections of 12 year-old *Pinus* species after 28 days. **(a)** Red cell deformation and cell disorganization in *P. halepensis*, Bar 75 µm; **(b)** cellular disruption with intercellular spaces and brownish cells stained in *P. sylvestris*, Bar 75µm; **(c)** *callus* formation from xylem in *P. halepensis*, Bar 25 µm; **(d)** *callus* formation from cambium tissues in *P. pinea*, Bar 75 µm, **(e)** transverse cross-section of xylem tissue showing thickening and lignification (intense fuchsia stain) of tracheids and xylem resin canals with thick-walled and lignified sheath cells in *P. pinea*; Bar 25 µm; **(f)** nematodes in tracheid's, with passage through bordered pits, with fungi mycelium (red arrows) in *P. sylvestris* (longitudinal cut), Bar 25 µm.

Our observations suggest that damage to pine tissues was caused by the presence and feeding activity of the PWN, as no tissue damages were observed in the control (I and II) samples. Furthermore, a response to wounding was observed only at 7 days post-wounding (dpw) in the phloem tissue of all species with the presence of CaOx crystals, and at 28 dpw with signs of traumatic resin canal formation in *P. halepensis*. No additional anatomical changes were observed in the other tissues or at other time points.

Nematode-induced damage began in the cortex near the inoculation site immediately upon inoculation and progressed to the phloem and cambium only in the susceptible species. During these early stages of infection, ruptured cells in the cortex periphery and thickened, swollen parenchyma cell walls were observed, suggesting a defensive mechanism shared by all four pine species. Similar observations of ruptured cells and changes in cortical tissue morphology following nematode infection have been reported in other pine species (Yamada 1993, Mamyia 2008, Koo 2013, Kusumoto 2014).

A shared trait in the two resistant pine species studied (*P. halepensis* and *P. pinea* was the formation of wounded periderm, a defence response triggered by the activation of polyphenolic cells in the secondary phloem. This response, induced by injury and pathogen invasion in bark tissue, has been described in previous studies (Ichihara 2000, Franceschi 2005, Krokene 2008, Menéndez-Gutiérrez 2018). Additionally, the accumulation of CaOx crystals in the first hours, observed exclusively in these two species, suggests a role as an initial physical barrier to nematode migration in

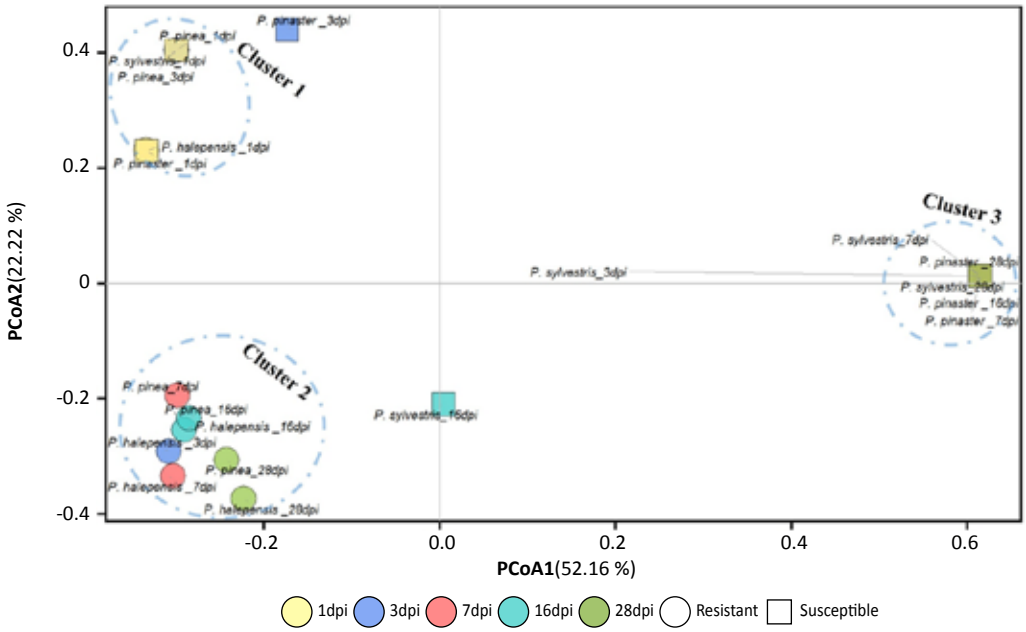

Figure 9. Principal Coordinates Analyses (PCoA) of the biochemical and cellular responses present in four *Pinus* species. Colours represent the five time points after inoculation, and symbols represent the susceptible and resistant species. All replicate pine trees inoculated with nematodes are included.

nematode-resistant pines, while in Control II samples, the crystals were observed only at seven dpw. Furthermore, secondary metabolite deposition was detected in the xylem ray cells and tracheids in *P. halepensis*, indicating another strategy for pathogen repression (Hara et al. 2006, Słupianek et al. 2021).

In both *P. halepensis* and *P. pinea*, lignification of sieve cell walls and albuminous cells appeared as a response to the nematode's presence. The lignification likely acts as a barrier to PWN, mitigating nematode damage and underscoring the importance of this process in defence strategies (Li et al. 2023). Also, in these two pine species, an increase of PP cells and phenolic content was observed in all tissues, but not in susceptible pines. In a previous work using these same pine individuals (Trindade et al. 2022), total phenolic compounds provided a clear distinction between resistant and susceptible pines. Similarly, Pimentel et al. (2017) demonstrated that higher levels of phenolics in the phloem correlated with reduced PWN population growth in resistant pine species.

The increase of the PP cells led to adjacent sieve cells appearing crushed and nonfunctional, creating a dense layer of cell walls. This layer probably represents a physical barrier to nematode penetration, as reported by Franceschi et al. (2005), Krokene et al. (2008) and Della Rocca et al. (2021) in studies of other pathogens.

We found no evidence of *B. xylophilus* disseminating through the cortical and xylem resin canals at early stages of infection. Nematodes or damage to the cortical canals were observed only after seven dpi in susceptible pines, and never in resistant species. These findings do not support previous reports suggesting that nematodes enter pine trees and lodge in the resin canals to feed on the epithelial and parenchymal cells (e.g., Fukuda and Suzuki 1988, Hara and Takeuchi 2006, Ichihara 2000, Kawaguchi 2006, Mamiya 2012). A similar pattern was observed in the xylem resin canals, which experienced damage only at 16 dpi, resulting in the total collapse of the canals in susceptible species only.

Traumatic resin canals were observed exclusively in *P. halepensis*. These canals have been reported as a response to insect attack, fungal invasion, and/or mechanical wounding (Eyles 2010, Nagy et al. 2000, Krokene and Nagy 2012), and have been linked to host responses following PWN inoculation (Mamiya 2008). Recently, Rodrígues-Garcia et al. (2023) found an increase in the number of xylem resin canals in susceptible pine seedlings after PWN inoculation, which contrasts with our observations. Moreover, we verified that resistant species had the highest density and relative area of resin canals in secondary xylem. The rapid induction of traumatic resin canals and the upregulation of terpene biosynthesis genes could contribute to reinforcing xylem defences and limiting PWN progression. When analyzing density of cortical resin constitutive canals on the control samples, we found higher density for *P. pinaster* than for *P. pinea*, as reported by Zas et al 2021; nevertheless, and unlike these authors, the diameters of the resin canals were similar for both species, which could be an artefact from studying adult trees and not pine seedlings as the previous authors. Therefore, our results do not support a relevant role of the resin canals' size and density in relation to PWN resistance, as no significant differences between resistant and susceptible pine species were found for these traits.

Over time, parenchymatous tissue with phenolic deposits developed in the earlywood of the current year, leading to *callus* formation in *P. halepensis*. In *P. pinea*, this occurred in the cambium. *Callus* formation can be an essential step in tissue regeneration, as it allows the replacement of damaged or infected tissues with healthy cells (Gričar 2007). It is important to note that this response cannot be attributed to wounding, not only because the changes were not observed post-wounding, but also because they were localised in tissues not affected by the injury.

The PCoA analysis identified three main clusters, with Cluster 1 indicating that early responses are relatively similar among species with varying tolerances to *B. xylophilus*. The period between 1 and 3 dpi appears to be a critical timeframe for distinguishing responses between resistant species (*P. halepensis* and *P. pinea)* and susceptible species (*P. sylvestris* and *P. pinaster*), as evidenced by the segregation of resistant species into Cluster 2 and susceptible species into Cluster 3. Overall, the most significant responses differentiating these two clusters were the increased presence of PP cells in the secondary phloem, lignification of sieve and tracheid walls, and the presence of phenols in the xylem, all of which were observed only in the resistant species.

Our results support previous findings that suggest a complex interaction of anatomical (Trindade 2012, Nunes da Silva et al. 2015, Zas et al. 2015, Canas et al. 2021) and biochemical defence mechanisms in response to PWN (Pimentel et al. 2016, Trindade et al. 2022, Nunes da Silva et al. 2025). According to Nunes da Silva et al. (2025), higher resistance of *Pinus pinea* to the PWN was associated with a more dynamic and effective activation of defence mechanisms, including phytohormone signalling, antioxidant activity, and expression of stress-related genes. Other factors, such as protein content (Modesto et al. 2022, Cardoso et al. 2024) and the susceptibility to water deficits at the tree level (Estorninho et al. 2022) may also play a role, highlighting the complexity of the various defence mechanisms.

CONCLUSIONS

This study is the first to compare the anatomical responses of adult pine trees with different susceptibility to PWD while mimicking nematode inoculation through insect-like feeding wounds. This trial provides a more realistic approach to simulating natural infection in the field, allowing an accurate characterization of the anatomical defense responses. Through anatomical observations, we identified distinct temporal response sequences, with 3 days emerging as a critical period for distinguishing reactions between resistant (*P. halepensis* and *P. pinea* and susceptible pine species (*P. sylvestris* and *P. pinaster*). The early formation of physical barriers, such as wound periderm, CaOx crystals accumulation, and the activation of polyphenolic parenchyma (PP) cells, may play a crucial role in the defence of pine species to *B. xylophilus* infection. These barriers develop almost immediately in resistant species, slowing or restricting the nematode´s establishment and spread (as seen by the absence of tissue damage distal to the inoculation site at the final time point), while other defence mechanisms are activated. Additionally, the accumulation of phenolic compounds, lignification, and the formation of traumatic resin canals further enhance these complementary defence strategies. A noteworthy observation is that we did not find evidence of *B. xylophilus* spreading through resin canals during the early stages of infection, unlike other studies conducted on pine seedlings. Along with the lack of structural damage to resin canals, this suggests that, once inside adult trees, *B. xylophilus* is primarily found on cortical parenchyma cells, with dispersal occurring through phloem, cambium tissue, and xylem rays. The development of *callus* tissue highlights the importance of tissue regeneration in the early recovery and survival of nematode-infested resistant pine trees, a phenomenon not previously reported for this pathogen. Future research should focus on identifying taxonomic, anatomical, biochemical, or phytohormonal markers to distinguish between susceptible and resistant pine species. Furthermore, we suggest that new studies should preferably be made with adult trees in order to achieve more realistic results, as the behaviour of the nematode in pine seedlings may differ from that in adult trees.

Author Contributions

Conceptualization, CST, TV, ES and PN; Methodology, CST, TV and PN; Formal analysis and investigation, CST and PN.; Writing—original draft preparation, CST, TV, ES and PN; Writing-review and editing, CST, TV, SPL, ES, JJ and PN; Statistics, CST and JJ; Funding acquisition, PN; Project Administration, PN. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Supplementary Files

Supplementary Files 1 - Representative transverse cross-sections of 12-year-old Pinus spp. showing the general anatomy of the different tissues and organisation of polyphenolic parenchyma (PP) cells at five time points post-wounding.

Supplementary Files 2 - Biochemical and cellular time-sequential responses in four Pinus species after B. xylophilus inoculation, organized by main tissues.

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