

Article

Resin Canal Traits Variation in *Pinus* spp. with Different Susceptibility to the Pine Wood Nematode

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Abstract: Different studies have emphasized the influence of resin canal traits in the susceptibility of pine trees to the pine wood nematode (PWN) *Bursaphelenchus xylophilus*. Resin canals can facilitate the PWN migration through the stem and are involved in the accumulation of volatile terpenes in the xylem in response to the pathogen, inducing tracheid embolisms. In this work, we conducted a PWN inoculation experiment under greenhouse conditions to investigate the anatomical traits of constitutive resin canals among seven *Pinus* species with different degrees of susceptibility to the PWN: *P. canariensis*, *P. halepensis*, *P. pinea* and *P. taeda* were grouped into a ‘low-susceptible group’, and *P. pinaster*, *P. radiata*, and *P. sylvestris* were grouped into a ‘high-susceptible group’. The high-susceptible group presented higher xylem radial growth, wider constitutive canals in the cortex, lower frequency of constitutive canals in the cortex, and smaller constitutive canals in the xylem than the low-susceptible group. The size of constitutive cortical canals was positively related to the number of seedlings colonized by the PWN, suggesting that wider canals facilitated migration. The inoculation of the PWN increased the frequency and diminished the mean area of canals in the xylem, and the high-susceptible group showed more frequency of induced xylem canals than the low-susceptible group. Additionally, the high-susceptible group presented larger radial growths in the xylem than the low-susceptible group. These results suggest a role of resin canal traits on *Pinus* spp. susceptibility to the PWN. Nevertheless, the high interspecific variability found in these traits within each susceptibility group evidences the importance of other factors in the susceptibility to the PWN.

Keywords: pine wilt disease; resin ducts; defense system; induced resistance; *Bursaphelenchus xylophilus*



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1. Introduction

The pine wood nematode (PWN) *Bursaphelenchus xylophilus* has been described as a major threat to Eurasian pine forests [1,2]. Native to North America, it causes a severe wilt disease outside of its native region known as pine wilt disease, killing trees of several *Pinus* species in a matter of weeks or months [3]. The PWN has significantly harmed the pine forests in Asia, impacting various pine species [4]. In Europe, the nematode was first detected in 1999, in the Setubal Peninsula in Portugal [5], and has since spread all over continental Portugal and Madeira [6,7]. The first outbreak in Spain was declared in 2008 in Cáceres province [8], and other foci have been declared to date in southern Pontevedra (Galicia) and Salamanca (Extremadura). Although nematode infection is confined to these areas, the disease could spread throughout the Iberian Peninsula reaching the Pyrenees from where it could advance towards France [9]. The PWN is a European Union quarantine pathogen and its predicted economic impact in Europe could reach between 16.3 and 36.0 billion euro during the 2008–2030 period [10]. Although the PWN is pathogenic

to many pine species [1,2], differences in susceptibility have been observed among pine species [1,11,12] and among individuals within the same species [13,14]. Initially, the pine wilt disease in Europe was thought to be restricted to *P. pinaster* [15], but in 2013 the nematode was also detected in decayed *P. nigra* and *P. radiata* trees in Coimbra, Portugal [16] and Sancti-Spiritus, Spain [17], respectively.

Conifer trees, particularly within Pinaceae, have a complex and extensive defense system against abiotic [18,19] and biotic stress [20,21] or mechanical wounding [22,23]. In pines, the primary defense mechanism is the production of resin, a complex mixture of volatile monoterpenes and sesquiterpenes, as well as non-volatile diterpene resin acids [24]. Resin is synthesized and stored in cellular structures called resin canals [25,26]. Pine species, which contain a developed network of interconnected axial and radial resin canals in wood and bark [25,26], form the first defense against a perturbation on the constitutive canals [21,25,27]. A second defense reaction is the induced synthesis of resin and new canals, often called traumatic canals [28,29]. Previous studies have reported a link between resin canal traits and tree resistance to insects and pathogens [22,30,31]. In some cases, higher abundance or size of constitutive or traumatic resin ducts are indicators of enhanced biotic resistance in conifers [31,32]. However, in cases where the resin canals are colonized and used as a pathway for pathogen spread, an abundance of these structures can be detrimental for the tree [31].

The PWN is transmitted when adult beetles of the genus *Monochamus* feed on the phloem of healthy trees or during female oviposition into dying trees, where *M. galloprovincialis* is the only confirmed PWN vector in Europe [7]. The infection continues with the migration of the PWN through the resin canal system [3,33,34]. The infection induces the production and accumulation of volatile terpenes within the stem, which provokes tracheid embolisms, blocks the ascent of xylem-sap and leads to water deficit in pine trees [35]. Different studies have highlighted the importance of resin canal traits in facilitating PWN migration and reproduction. Traits such as canal size and abundance could affect nematode migration, with cortex and xylem resin canals playing the most relevant role [33,36,37]. A recent study performed on four different pine species showed that wider axial resin canals could facilitate nematode dispersal through the stem, increasing tree susceptibility [12]. Additionally, pine trees susceptible to PWN were reported to have more resin canals than trees capable of restricting nematode migration [38]. Yet, other traits can be also involved in resistance, e.g., the accumulation of phytochemicals toxic for the PWN as an induced defense reaction [11,35,39]. The migration rate of nematodes within a pine tree varies with the nematode species [40,41], nematode isolate [40,42], and the resistance level of the host tree [43].

In the present work, we aim to investigate the association between the susceptibility of seven *Pinus* species to the PWN and the anatomical traits of their constitutive resin canals. Furthermore, we also aim to elucidate if the infection with the PWN induces the formation of resin canals or a shift in their size. We hypothesize that the most susceptible pine species have wider and more abundant constitutive axial resin canals, facilitating nematode migration through the stem, and that PWN inoculation increases the frequency or the area of axial resin canals in the most susceptible pine species. The studied group was formed by five pine species widely distributed and planted in Europe (*P. pinaster*, *P. pinea*, *P. sylvestris*, *P. radiata*, and *P. halepensis*), an American resistant species (*P. taeda*) and the endemic Canary island pine (*P. canariensis*).

2. Materials and Methods

2.1. Plant Material and Experimental Design

In 2011, seeds from seven different pine species were sown: *P. canariensis*, *P. halepensis*, *P. pinaster*, *P. pinea*, *P. radiata*, *P. taeda* and *P. sylvestris*. The inoculation experiment was conducted under greenhouse conditions with a randomized complete block design with seven species, four blocks, and three treatments. To study constitutive anatomical traits, the first treatment consisted of non-inoculated seedlings. To study anatomical traits induced by

the PWN, the second and third treatments consisted of seedlings inoculated with sterilized distilled water or with the PWN, respectively. A total of 56 PWN-inoculated seedlings (8 per species), 28 water-inoculated seedlings (4 per species) and 35 controls (5 non-inoculated seedlings per species) were used. The initial height of seedlings ranged from 68.83 ± 2.38 cm for *P. sylvestris* to 138.08 ± 3.04 cm for *P. radiata* (Table S1). The mean temperature in the greenhouse was 22.5°C , ranging from 18°C to 27°C owing to oscillations between day and night mean temperatures. Seedlings were watered twice a week by a drip irrigation system.

2.2. Pinewood Nematode Inoculation and Quantification

A detailed characterization of the susceptibility of the seven pine species to the PWN was previously published by Menéndez-Gutiérrez et al. [11], where the inoculation methodology followed in our work is described. In brief, we used a *B. xylophilus* isolate obtained from a *P. pinaster* in As Neves (Pontevedra, Spain) in 2010. The isolate was reared on a fungal mat of *Botrytis cinerea* growing on PDA medium. The inoculum was provided by the Areeiro Phytopathological Institute (Estación Fitopatológica Areeiro, EFA, Pontevedra, Spain). Nematodes were extracted from *B. cinerea* plates using the Baermann funnel technique [44] the day before inoculation and the inoculation suspension was prepared with sterilized distilled water. Seedlings were inoculated in a greenhouse at Lourizán Forest Research Centre (Pontevedra, Spain) on 18 June 2013. A longitudinal cut was made in the previous year's growth of the main stem. Then, 300 μL of the suspension containing 600 nematodes (mixed developmental stages) was pipetted onto a strip of bandage previously placed on the wound. Parafilm[®] was used to prevent tissue desiccation. Water-inoculated seedlings were inoculated with 300 μL of distilled water instead of the nematode suspension.

At 42 days after inoculation (dai), 8 PWN-inoculated and 4 water-inoculated seedlings per species were harvested for nematode quantification. Five out of the 8 PWN-inoculated and the 4 water-inoculated seedlings were used for the measurement of anatomical traits. Additionally, 5 non-inoculated seedlings per species were harvested for constitutive anatomical traits analysis. For nematode quantification, nematodes were extracted from the seedlings using a modified Baermann funnel technique at 42 dai, dividing the seedlings into roots and aerial parts. Nematodes in each part were extracted separately. Dried weight was determined for each seedling fraction to express nematode density (ND) in number of nematodes per gram of dry weight for stem (NDs), roots (NDr) and total seedling (NDt). Furthermore, incidence data, i.e., the percentage of seedlings per species with nematode presence in the stem, roots and total seedling (%Ns, %Nr and %Nt, respectively), was calculated.

2.3. Anatomical and Morphological Traits

To study induced responses to the PWN, one 3-cm-long segment of each inoculated individual was cut 5 cm above the inoculation point. For the study of constitutive traits (non-inoculated seedlings), we harvested one 3-cm-long segment of each seedling at a similar height to the inoculated seedlings.

For light microscopy observation, transverse sections (20–30 μm thick) were cut using a sliding microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany). The samples contained the cortex, xylem and pith tissues. Sections were stained with a solution of safranin 1% and alcian blue 1% and mounted after dehydrating through the ethanol series. Samples were photographed with a light microscope using a Nikon digital Coolpix 4500 camera and analyzed with WinCELL Pro (version 2004a; Regent Instruments Inc. 2004, Québec, Canada). The following parameters were measured (Figure 1): (i) radial growth variables: cortex (Cg, μm) and xylem (Xg, μm) growths, and (ii) axial canals variables in cortex (C) and xylem (X) tissues: mean size of axial canals in cortex and xylem (adjacent epithelial and parenchyma cells included) (CsC and CsX, respectively; μm^2), frequency of axial canals (CfC and CfX; number of canals per mm^2) and area of axial canals (adjacent

epithelial and parenchyma cells included) per mm^2 (CaC and CaX; $\mu\text{m}^2/\text{mm}^2$). On each preparation, a minimum of 3 random measurements of growth variables and all the axial canal variables in the complete transverse section were taken.

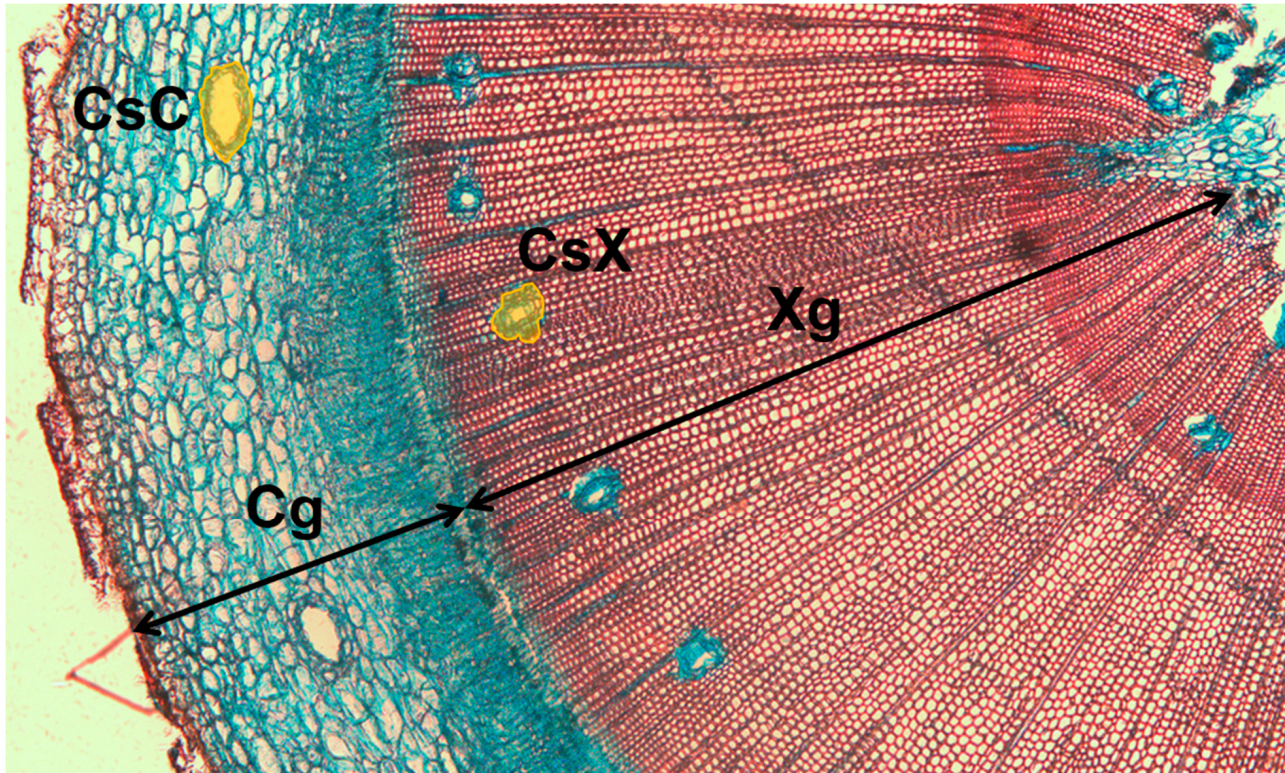


Figure 1. Transverse section of a *Pinus halepensis* seedling stained with safranin (red) and alcian blue (blue). Parameters measured in each one of the analyzed samples were: (i) radial growth variables; xylem growth (Xg, μm) and cortex growth (Cg, μm), and (ii) axial canals variables; size of axial canals (adjacent epithelial and parenchyma cells included) in cortex (CsC; μm^2) and size of axial canals (adjacent epithelial and parenchyma cells included) in xylem (CsX; μm^2). Other traits measured in xylem and cortex were the transverse area occupied by canals ($\mu\text{m}^2/\text{mm}^2$), and the frequency of canals (N/mm^2).

Before inoculation, several dendrometric and morphological traits were measured: initial height (IH, cm), previous year height (PH, cm), height increment ($\text{HI} = \text{IH} - \text{PH}$, cm), root-collar diameter (D, mm), and the number of main branches (BN).

2.4. Susceptibility Groups

Pine species were grouped according to their susceptibility to the PWN based on the symptomatology and mortality observed by Menéndez-Gutierrez et al. [11]. Two susceptibility groups were defined: (i) low-susceptible group, which includes *P. canariensis*, *P. halepensis*, *P. pinea* and *P. taeda*, and (ii) high-susceptible group, which comprises *P. pinaster*, *P. radiata* and *P. sylvestris*.

2.5. Statistical Analysis

Constitutive anatomical traits were analyzed by a general linear model (GLM), with susceptibility groups and species (nested to susceptibility group) as fixed factors. Least square means (LSMeans) were estimated when significant differences among susceptibility groups or species were detected ($p < 0.05$). Prior to the analysis, we log10-transformed all variables with the exception of CsX, Xg and Cg to meet normality and homoscedasticity assumptions. The effect of PWN inoculation was analyzed by a GLM considering suscep-

tibility group, species (nested to susceptibility group) and treatment (PWN inoculation and water inoculation) and their interactions. All factors were considered fixed. Prior to the analyses, we log₁₀-transformed all the cortical variables to meet normality and homoscedasticity assumptions. Posthoc LSD tests were applied to compare mean values within factors when any significant effect was detected ($p < 0.05$). Correlation analysis was performed by Spearman's rank correlation coefficient between the percentage of seedlings per species with nematode presence in stem, roots and total seedling (%Ns, %Nr, %Nt), the LSMeans per species of the morphological traits (IH, PH, HL, D and BN) and the LSMeans per species of the constitutive anatomical variables in cortex and xylem (CaC, CfC, CsC, Cg, CaX, CfX, CsX and Xg) ($N = 7$). All statistical analyses were performed using the SAS 9.2 statistical package (SAS Institute, Inc., Cary, NC, USA).

3. Results

3.1. Constitutive Anatomical Traits

A significant variation was found among the two pine susceptibility groups for Xg, CsC and CfC ($p < 0.001$), and also for CsX ($p < 0.05$) (Table 1; Figure S1). The high-susceptible group showed higher Xg and CsC, and lower CfC and CsX than the low-susceptible group. At the species level, all constitutive anatomical traits showed differences among pine species ($p < 0.05$), except for CaC. Two of the three pine species from the high-susceptible group (*P. pinaster* and *P. radiata*) presented higher Xg than those from the low-susceptible group. Furthermore, the high-susceptible species *P. radiata* and *P. sylvestris* showed the highest values of CsC and the lowest values of CfC (Table 1; Figures 2 and S1). The high-susceptible *P. pinaster* also presented high CsC values, but its CfC values were similar to most of the low-susceptible species. The rest of the anatomical traits, except CaC, showed variability among the different pine species, but without any evident association with PWN susceptibility.

3.2. Induced Anatomical Variations in Response to PWN Inoculation

At day 41 after inoculation, only the high-susceptible species showed foliage disease symptoms (Figure S2). The PWN inoculation mainly affected anatomical traits in the xylem (Table 2). Two of the three xylem variables were significantly affected by the treatment: CfX and CaX ($p < 0.05$). Both variables were significantly higher in PWN-inoculated seedlings than in water-inoculated seedlings (0.58 ± 0.01 vs. 0.54 ± 0.01 , and 4.56 ± 0.01 vs. 4.51 ± 0.01 , respectively).

CsX and CfX showed a significant susceptibility group $\times$ treatment interaction ($p < 0.001$). Thus, in the high-susceptible group, PWN inoculation resulted in smaller and more frequent xylem canals, compared to the water-inoculated ones. In the low-susceptible group, however, PWN inoculation resulted in wider xylem canals but of similar frequency than in water-inoculated seedlings (Figures 3 and 4). At the species level, a similar trend was observed for the highly susceptible *P. pinaster* and *P. radiata*, and for the low-susceptible *P. canariensis* (Figure 5a,c).

In the cortex, CsC and CaC showed significant species $\times$ treatment interactions ($p < 0.05$) (Table 2). PWN inoculation induced the formation of higher values of CsC in *P. pinea* (low-susceptible species). Yet, the opposite trend was observed in the also low-susceptible *P. halepensis* (Figure 5b). Similarly, CaC increased with PWN inoculation in *P. pinea* (as well as in the highly susceptible *P. sylvestris*) while it decreased in *P. halepensis* (Figure 5d).

Table 1. F-values and least square means ($\pm$ s.e.) obtained from the general linear model of anatomical variables in xylem and cortex tissues in control seedlings (CsX and CsC: size of axial canals in xylem and cortex, respectively; CfX and CfC: frequency of axial canals in xylem and cortex, respectively; CaX and CaC: area occupied by canals per mm² in xylem and cortex, respectively; Xg: xylem growth; Cg: cortex growth).

Anatomical Trait	F-Values		LSMeans								
	Suscept. Group	Species (Group)	Susceptibility Group		Species (Susceptibility Group)						
			Low	High	Low-Susceptible					High-Susceptible	
					<i>P. pinea</i>	<i>P. canariensis</i>	<i>P. halepensis</i>	<i>P. taeda</i>	<i>P. pinaster</i>	<i>P. radiata</i>	<i>P. sylvestris</i>
Xylem											
Xg (μm)	50.75 ***	6.14 ***	2563 ± 74 a	3441 ± 163 b	2714 ± 48 a	2379 ± 170 a	2387 ± 141 a	2769 ± 139 a	3595 ± 129 b	3926 ± 152 b	2800 ± 267 a
CsX (μm ²)	4.66 *	8.16 ***	10,350 ± 488 b	9186 ± 619 a	10,574 ± 722 cd	12,599 ± 763 d	8041 ± 185 ab	10,185 ± 878 c	11,043 ± 595 cd	9782 ± 976 bc	6735 ± 507 a
CfX (N/mm ²)	0.08	9.83 ***	3.25 ± 0.25	3.47 ± 0.42	2.13 ± 0.14 a	2.77 ± 0.42 a	4.19 ± 0.43 b	3.90 ± 0.27 b	2.38 ± 0.19 a	2.67 ± 0.51 a	5.36 ± 0.53 b
CaX (μm ² /mm ²)	0.89	2.90 *	32,458 ± 2174	29,724 ± 2522	22,876 ± 2740 a	34,603 ± 4548 b	32,704 ± 2502 ab	39,647 ± 4282 b	26,455 ± 3004 ab	26,179 ± 5271 ab	36,537 ± 3549 b
Cortex											
Cg (μm)	1.67	3.68 *	952.44 ± 46.74	886.10 ± 32.21	1117.4 ± 55.13 b	1071.1 ± 112.6 b	806.91 ± 53.26 a	814.40 ± 50.81 a	879.09 ± 37.95 ab	861.22 ± 77.01 a	918.01 ± 55.72 b
CsC (μm ²)	24.41 ***	3.24 *	10,739 ± 931 a	18,330 ± 1649 b	12,176 ± 1541 bc	7805 ± 1415 a	14,801 ± 1408 cd	8171 ± 1299 ab	15,784 ± 1617 cd	19,102 ± 3567 cd	20,104 ± 3265 d
CfC (N/mm ²)	15.69 ***	4.41 **	1.14 ± 0.1 b	0.77 ± 0.06 a	0.87 ± 0.09 ab	1.15 ± 0.13 bc	0.99 ± 0.23 b	1.53 ± 0.16 c	1.02 ± 0.11 b	0.65 ± 0.07 a	0.63 ± 0.02 a
CaC (μm ² /mm ²)	2.99	1.55	10,946 ± 826	13,651 ± 1304	10,267 ± 807	8663 ± 1654	13,616 ± 2131	11,239 ± 1347	15,810 ± 1763	11,930 ± 2549	13,214 ± 2510

Significance levels: *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$. Different letters indicate significant differences ($p < 0.05$) among susceptibility groups or among *Pinus* species within susceptibility groups.

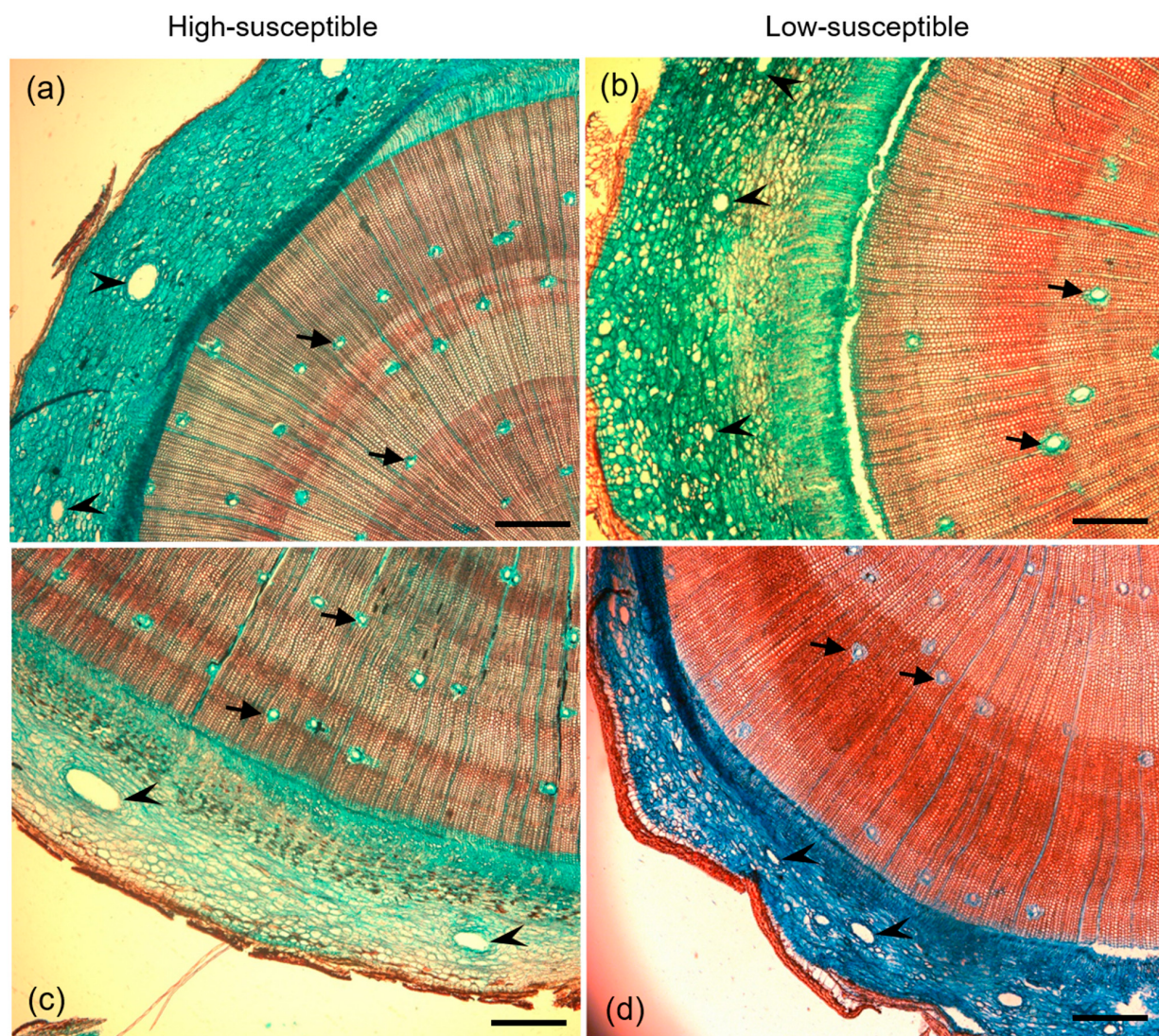


Figure 2. Transverse sections of *Pinus sylvestris* (a), *Pinus canariensis* (b), *Pinus radiata* (c), and *Pinus taeda* (d) seedlings stained with safranin (red) and alcian blue (blue). The images show the constitutive anatomy of high-susceptible (a,c) and low-susceptible (b,d) pine species to the pine wood nematode. Note axial canals in the cortex (arrowheads) and canals in the xylem (arrows). Scale bars = 500 μm .

Table 2. F-values and significance levels obtained from the analysis of variance of anatomical variables. Values represent the effects of susceptibility group (SG; high-susceptible and low-susceptible), species within susceptibility group, and treatment (PWN inoculation and water inoculation) and of their interactions on the studied anatomical traits (canal size, canal frequency and canal area) in xylem and cortical tissues.

Factor	Xylem			Cortex		
	CsX (μm^2)	CfX (N/mm 2)	CaX ($\mu\text{m}^2/\text{mm}^2$)	CsC (μm^2)	CfC (N/mm 2)	CaC ($\mu\text{m}^2/\text{mm}^2$)
SG	0.03	1.23	4.19 *	22.45 ***	21.66 ***	0.03
Species (SG)	26.89 ***	34.23 ***	11.55 ***	11.28 ***	7.73 ***	2.16
Treatment	0.45	5.84 *	5.59 *	0.23	0.28	0.13
SG $\times$ treatment	17.15 ***	12.48 ***	1.52	0.15	1.66	2.19
Species (SG) $\times$ treatment	5.68 ***	1.26	0.21	3.29 **	0.33	3.12 *

Significance levels: *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

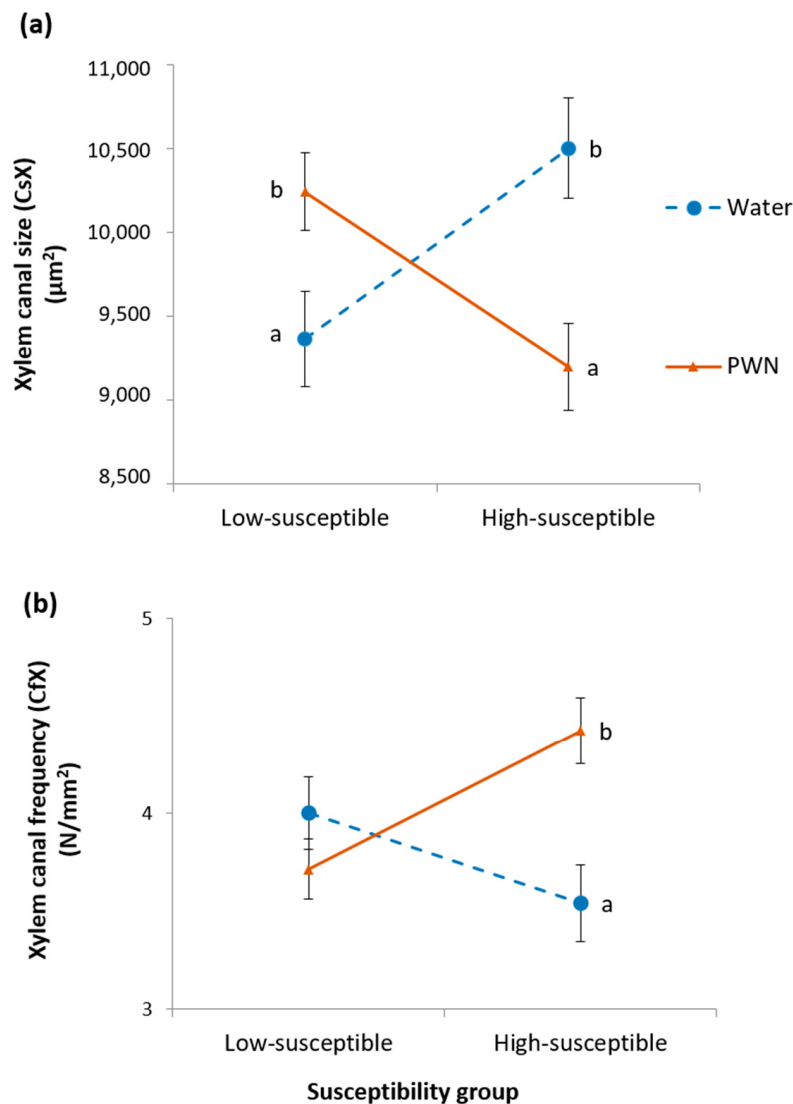


Figure 3. Interaction between the treatment (pine wood nematode and water inoculation) and the susceptibility group (low-susceptible and high-susceptible pine species) in two resin canal traits: (a) xylem canal size (CsX) and (b) xylem canal frequency (CfX). The least square means values (mean $\pm$ SE) are shown. Different letters indicate significant differences ($p < 0.05$) among the studied treatments within each susceptibility group.

3.3. Relationships between Anatomical/Dendrometric Traits and Nematode Colonization

The number of nematodes recovered from inoculated seedlings differed significantly among species ($\chi^2 = 13.895$; $p < 0.0308$), ranging from 0 to 1152.68 *B. xylophilus* individuals per dry gram of wood. The median number of nematodes recovered from the total seedling was higher in the high-susceptible species *P. radiata* and *P. sylvestris* than in the low-susceptible *P. canariensis*, *P. halepensis* and *P. pinea* (Table S2). The percentage of seedlings per species with nematode presence in roots (%Nr) was associated to CsC (Table 3; Figure 6a). The percentage of seedlings per species with nematode presence in the stem (%Ns) was significantly correlated with the plant diameter (D) (Table 3; Figure 6b). Furthermore, the percentage of seedlings with nematode presence in both stem and root tissues (%Nt) was significantly correlated with Xg (Table 3). Regarding relations between constitutive anatomical and dendrometric traits, D was significantly correlated with Xg, and the seedling initial height (IH) with CfC (Table 3).

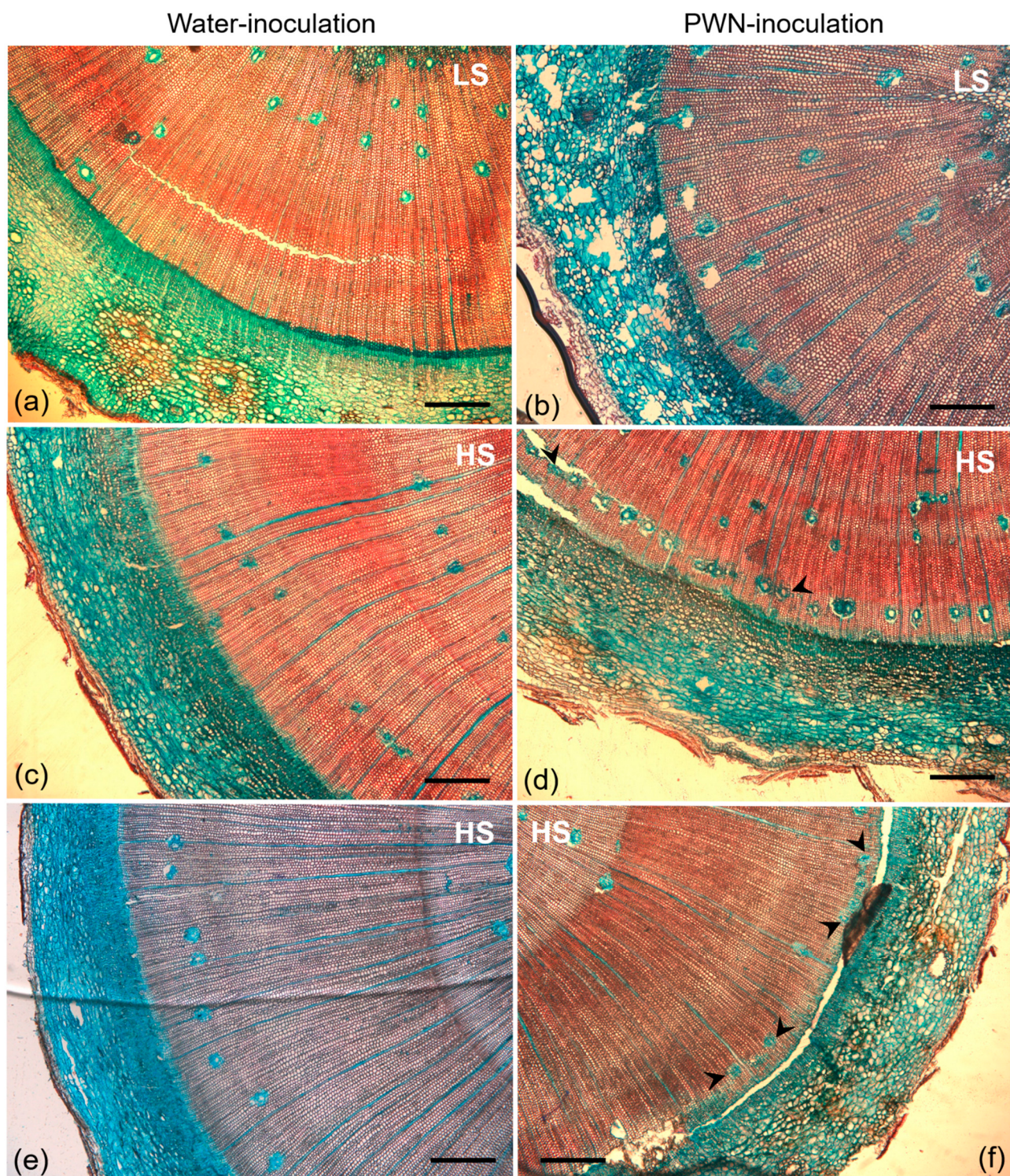
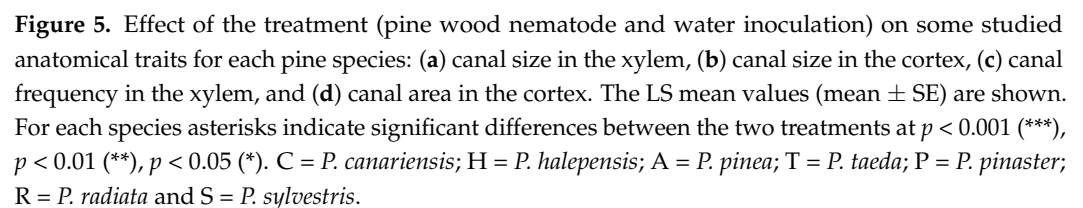


Figure 4. Transverse sections of *Pinus canariensis* (a,b), *Pinus radiata* (c,d), and *Pinus pinaster* (e,f) seedlings 42 days after inoculation with water (a,c,e) or with the pine wood nematode (b,d,f). Note the formation of xylem traumatic resin canals as a response to the pine wood nematode infection (arrowheads). Low-susceptible (LS) and high-susceptible (HS) species to the pine wood nematode are shown. (Scale bar = 500 μ m).

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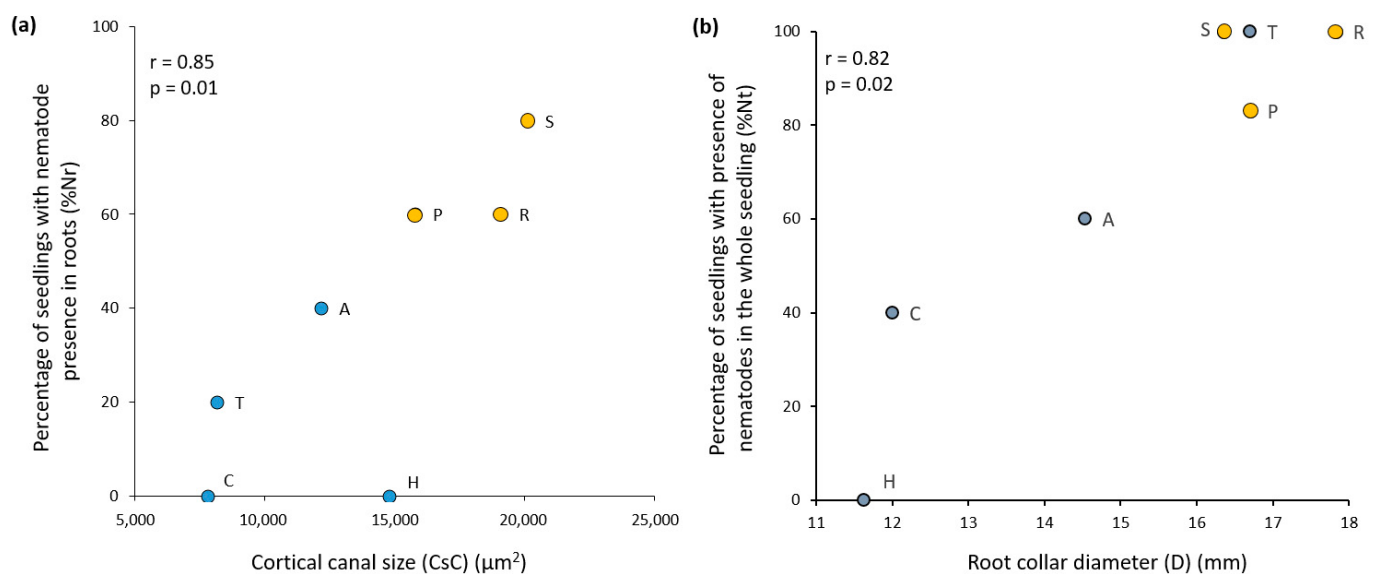


Figure 6. Relationships between two constitutive anatomical traits and the percentage of seedlings with pine wood nematode presence: **(a)** percentage of seedlings with presence of nematodes in roots (%Nr) vs. cortical canal size (CsC); **(b)** percentage of seedlings with presence of nematodes in the total seedling (%Nt) vs. seedling root collar diameter (D). C = *P. canariensis*; H = *P. halepensis*; A = *P. pinea*; T = *P. taeda*; P = *P. pinaster*; R = *P. radiata*; S = *P. sylvestris*. Blue dots = low-susceptible species; orange dots = high-susceptible species.

4. Discussion

Our study showed a high interspecific variability in axial resin canal traits among *Pinus* species. Some of these traits showed marked differences between pine susceptibility groups. However, these differences were not always consistent at the species level. These results generally agree with previous works evidencing that resin canal traits are important for PWN infection success [36,45], but also with other studies suggesting that canal traits do not fully determine the pine species susceptibility [12,37].

4.1. PWN Susceptibility and Constitutive Traits

A relation was observed between the percentage of seedlings with nematode presence, especially in roots, and the radial xylem growth. Possibly, the pine species which allocated more resources to radial growth had limited resources available to differentiation processes, such as constitutive defenses [46,47]. This hypothesis is supported by different studies which focused on the chemical defense system of pine trees [1,11]. Some of them, conducted under greenhouse conditions, showed that *P. pinea* presents slower growth and higher levels of constitutive defenses than *P. pinaster* and *P. radiata* [1]. In our anatomical study, we did not find any clear evidence supporting a higher constitutive defense investment in the low-susceptible tree species. The low-susceptible group presented larger resin canals in the xylem and more resin canals in the cortex than the high-susceptible group, but the total area occupied by resin canals was similar in both groups. Moreover, the high-susceptible species *P. sylvestris* presented lower radial growth than the other two high-susceptible species (*P. pinaster* and *P. radiata*), which is evidence that more complex mechanisms (e.g., chemical composition of defenses, early recognition of the pathogen, or ecophysiological traits) are involved in PWN susceptibility.

The constitutive anatomical differences found between susceptibility groups in the cortex, with the high-susceptible group showing less frequent but wider canals, agree with previous studies [36,37], supporting that cortical canal size is a good predictor of tree susceptibility. Cortical axial resin canals are the main path of PWN migration in seedlings [33,34,45], and wider canals could facilitate this migration. For example, PWN

migration was much faster through cortical than xylem axial resin canals in *Pinus thunbergii*, *P. strobus* and *P. rigida* [38], indicating that xylem canals probably have a secondary role in PWN migration. Furthermore, the average and total lumen area of cortical resin canals of a *P. thunbergii* resistant family were significantly smaller than non-selected (susceptible) *P. thunbergii* families [36]. These authors suggested that the smaller lumen area of cortical canals caused the restriction in PWN migration in resistant *P. thunbergii* individuals. Similarly, nematode migration through the branch segments of different *P. pinaster* provenances was positively correlated with the mean area of the resin canals in the cortex [36]. In agreement with these works, we found a positive correlation at an inter-specific level between the size of cortical resin canals and the percentage of seedlings with PWN presence in roots, supporting the idea that smaller lumen canals in the cortex restrict PWN spread. However, our results do not agree with previous research which showed wider cortical canals in *P. pinea* than in *P. sylvestris* [12]. These authors suggested that the PWN could also use pathways other than canals to migrate from the inoculation point, such as phloem, the cambium, tracheids and the pith, as was also suggested by other authors [3,33].

Regarding the xylem variables, our results agree with previous studies showing that susceptible species, such as *P. sylvestris*, present the smallest axial resin canals in the xylem [12]. In our work, the high-susceptible group of seedlings presented smaller canals in xylem tissues than the low-susceptible group. However, this trait was not correlated with the percentage of seedlings with nematode presence in any examined seedling fraction (root or stem). The size of xylem axial canals does not seem to be a good predictor due to the inconsistency found among pine species of different susceptibility level. Thus, *P. sylvestris*, a high-susceptible species, showed the smallest canal size but was similar to the low-susceptible species *P. halepensis*. On the contrary, *P. pinea*, a low-susceptible species showed wide xylem canals, but *P. pinaster*, a high-susceptible species, showed canals even wider. In sum, constitutive xylem canals cannot be regarded as a cause of susceptibility.

4.2. Anatomical Changes Induced by PWN Inoculation

Pine species show a complex reaction to different types of biotic and abiotic attacks, which operate at different levels (anatomical, chemical and physiological) and at different times [20,21]. The complexity of this defense system should be taken into account when interpreting our results. The formation of traumatic resin canals in response to an external disturbance is an important component of the pine induced resistance [22,48]. In our work, we observed that PWN inoculation induced an increase in the frequency of canals, and in the total area occupied by canals in the xylem. An increase in the axial canal frequency has been observed in response to insect attack, fungal invasion and/or mechanical wounding in *Pinus* species [22,28,46,49]. Furthermore, we found a greater increase in xylem canal frequency after PWN-inoculation in the high-susceptible group, mainly in the fast-growing *P. pinaster* and *P. radiata*. Species with faster growths and lower investments in constitutive defenses might invest more in inducible defenses, as resin canal formation [50]. The induced formation of xylem canals followed the typical pattern of dense concentric series of traumatic canals. This observation agrees with previous studies showing this type of response after PWN inoculation [51].

In spite that the high-susceptible group formed more xylem canals after PWN inoculation, these canals were smaller than those formed by the low-susceptible group. At the species level this was confirmed by the PWN-infected *P. radiata* and *P. pinaster* seedlings which showed a decrease in the xylem canal size together with an increase in canal frequency, while *P. canariensis* showed a significant increase in the average canal size (but no increase in frequency). The formation of more frequent but smaller canals has been also reported as a defense response in *Pinus* spp. in previous studies [49,52]. The presence of more abundant but smaller defensive structures could be a good strategy for developing a dense network of canals to optimize the barrier effect against insects, but possibly limits the structural resin synthesis. Yet, this strategy of induced defense is not a general trend in the genus. For instance, more frequent and larger xylem resin canals were found in

Pinus ponderosa after bark beetle attack [53]. The formation of a denser network of canals might facilitate PWN migration through the xylem, and perhaps a more widespread release of volatiles in the xylem, which seem to be involved in tracheid embolism [35]. This way, changes induced in xylem canals by the PWN could also be the cause of enhanced susceptibility to PWN. It should be noted that the average size of xylem canals would permit, in most cases, the migration of the PWN through them. Thus, the greatest body width of Portuguese *Bursaphelenchus xylophilus* is of $22.3 \pm 1.8 \mu\text{m}$ [54], while the average xylem canal width in *P. sylvestris* (the pine species examined with the smallest xylem canals) was of $46.3 \pm 12.7 \mu\text{m}$ (epithelial and parenchyma cells included), with an average lumen of $28.8 \pm 7.5 \mu\text{m}$.

In general, the effect of the PWN inoculation in the cortical canal traits was low, as no significant effects of the treatment, and the susceptibility group $\times$ treatment interaction were observed. Cortical canals are formed during the primary development of a plant, and stay alive for several years of secondary growth [20]. Several studies showed changes in cortical tissues in response to a wound or an infection, which are described as inducible structural and chemical defenses. However, these responses have not been associated to changes in cortical resin canals traits, but to the ability to provide a physical barrier through the formation of callus tissue that can become lignified, suberized or impregnated with phenolics and terpenes [20,21,55]. This way, even though cortical canals seemed to have an important role in PWN dispersal, they did not experience major changes in their size or frequency in response to PWN inoculation. Although the effect of inoculation on cortical canals was generally low, the effect of inoculation was greater for some pine species than others, as evidenced by the significant pine species $\times$ treatment interaction (see Figure 5b,d).

Other traits not evaluated in our study could be behind the susceptibility of pine trees to PWN. For instance, Pimentel et al. [1] found higher concentrations of tannins and phenols in the phloem and cambium of *P. pinea* compared to *P. pinaster* and *P. radiata*. In turn, the phloem of *P. sylvestris* is scarce in defensive polyphenols but abundant in potentially nutritive compounds such as lipid soluble substances [11]. In *Pinus* species, cortex tissues contain large amounts of phenolics, sclerenchyma and calcium oxalate crystals. These substances create the main defensive barrier during the early stages of stem growth and probably determine, in a stronger way than the anatomical structure, the different susceptibility degree to nematode attack. Pine species produce different amounts of phenolic compounds [11], and there is also some evidence that relative resistance to pathogens might partly be a function of the type of phenolics, nitrogen, lipid-soluble substances or other defense associated substances [1,11,56,57]. These works highlight that further biochemical and physiological research is needed to fully understand susceptibility factors to the PWN.

5. Conclusions

The high-susceptible group of pine species tended to show wider constitutive cortical canals and more xylem canals after infection than the low-susceptible group. The size of constitutive cortical canals could be regarded as one of the causes of susceptibility, influencing the initial migration rate of the PWN. Constitutive xylem canal traits were not clearly associated with susceptibility, although the changes induced after infection in xylem canals might also affect susceptibility. It can be concluded that the anatomy of axial resin canals probably exerts some influence in the interspecific variation of pine susceptibility to the PWN, at least during the seedling stage or in young twigs. In turn, the high-susceptible species tend to show larger radial growths than the low-susceptible species. Yet, these trends were not always straightforward at the species level, most likely due to the role of other chemical or physiological key defensive traits. The results of controlled greenhouse inoculation experiments should be confirmed in the field, as the defense mechanisms of adult trees could be quite different in outdoors conditions; nevertheless, since pine wilt

disease is subjected to strict quarantine measures in Europe, field experiments involving PWN inoculation are not permitted in Spain.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/f14050925/s1>, Table S1. Height and basal diameter of the experimental seedlings (mean $\pm$ SE); Table S2. Median number of nematodes per gram of dry weight recovered from roots (NDR), stem (NDs) and total seedling (NDt) at 42 days after inoculation. Within each column, different letters denote significant differences between pine species (Kruskal-Wallis test, $p < 0.05$, $N = 8$); Figure S1. Constitutive anatomical traits showing differences between susceptibility groups: (a) xylem growth, (b) canal size in xylem, (c) canal size in the cortex, and (d) canal frequency in the cortex. Different letters denote significant differences between pine species ($p < 0.05$). A = *P. pinea*; C = *P. canariensis*; H = *P. halepensis*; T = *P. taeda*; P = *P. pinaster*; R = *P. radiata* and S = *P. sylvestris*; Figure S2. Changes in foliage symptoms shown by high-susceptible species after 3 and 41 days after inoculation (DAI) with the pine wood nematode. No significant changes were observed in low-susceptible species.

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