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Assessing Genetic Variation in Resistance to Pinewood Nematode (*Bursaphelenchus xylophilus*) in *Pinus radiata* D. Don Half-Sib Families

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Abstract: Full understanding and control of pine wilt disease (PWD) is a work in progress and breeding for disease resistance constitutes an essential management strategy for reducing its impact, as evidenced by advanced breeding programs in countries such as Japan. Since *Pinus radiata* is one of the most commercially relevant species in northern Spain, we designed a study to assess genetic variation in susceptibility to this pathogen using 44 *P. radiata* half-sib families from the Galician breeding program. Three *Bursaphelenchus xylophilus* (pinewood nematode, PWN) inoculation experiments were performed to evaluate disease-related variables, estimate genetic parameters, and study sources of genotype by environment interaction (G × E). We also looked at differences in the constitutive chemical compounds of susceptible and non-susceptible individuals. The results showed great variation in PWN susceptibility, with survival rates for *P. radiata* families ranging from 0% to 90%. In addition, heritability estimates (h_i^2 = 0.43, h_f^2 = 0.72) and genetic gain (>26% selecting 50% of the families) were both moderately high for survival. Significant differences in several constitutive chemical compounds were found between susceptible and non-susceptible seedlings in the two susceptibility groups considered. These results confirm the potential of breeding to obtain *P. radiata* genotypes that are resistant to pine wilt disease and open possibilities for mitigating its future impact on *P. radiata* stands.

Keywords: heritability; tree breeding; disease resistance; pine wilt disease; genotype by environment interaction; genetic gain

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1. Introduction

Monterey pine, *Pinus radiata* D. Don, is a tree species native to very limited areas of the California coast in the United States and Baja California in Mexico [1]. However, as one of the most widely planted tree species in the world, it is cultivated in Australia, New Zealand, Chile, South Africa, and southwest Europe. In the latter region, plantations are mainly located in northern Spain, specifically in the Basque Country and Galicia, which contain 47.6% and 33.3% of the total area covered by *P. radiata* in Spain, respectively. In 2019, 2.2 million m^3 of this species were harvested in the Basque Country and 1.8 million m^3 in Galicia, representing 95.2% and 46.2% of the total conifer harvest volume in each region, respectively [2].

Tree species are under increasing threat worldwide from diseases and insect pests, many of which are non-native. One health menace to *P. radiata* is *Bursaphelenchus xylophilus* (pinewood nematode, PWN), the organism that causes pine wilt disease (PWD). Although *P. radiata* is only weakly affected by PWN in its native area [3], mortality in planted areas was reported in the outbreak that occurred in Sancti-Spiritus (Salamanca, Spain) [4]. In previous experiments under greenhouse conditions, this species experienced mortality rates of 40–95% following exposure to the pinewood nematode [5].

With the increasing temperatures and droughts that accompany global warming, *P. radiata* stands in northern Spain—where average summer temperatures reach 20 °C—will be at high risk of being affected by PWD [6]. The spread of PWN also threatens countries such as Australia and New Zealand, where this pine species constitutes a significant component of the forest industry [7]. Careful monitoring of coniferous forests and strict inspections of wood trade are ongoing in these countries to prevent the establishment of this pest.

Since the first report of *B. xylophilus* being introduced in Japan in 1905 [8], this pathogen has spread widely and has been reported in China [9], Korea [10], and Taiwan [11]. In Europe, PWD was first reported in Portugal [12], where the pathogen continues to cause irreparable damage to forest ecosystems and severe economic losses [13]. Spain declared the first outbreak in 2008 [14], and five other foci have been declared to date [4,15], two of which are currently eradicated.

All countries affected by PWD have dedicated significant effort to stopping it, but none have been able to fully control the disease. Management measures, such as aerial spraying of insecticides, removing dead or infected trees, and stand management, have only slowed down the spread of the disease. However, combining some of these measures with the deployment of PWN-resistant genotypes might be an important strategy for mitigating the impact of PWD [16]. Breeding for resistance to PWD has been successfully developed in Asian countries. The first PWN resistance breeding program began in Japan in 1978 with the selection of surviving trees within severely PWD-damaged stands of susceptible *Pinus thunbergii* Parl. and *Pinus densiflora* Siebold & Zucc [17,18]. More recently, the potential for breeding PWN-resistant *Pinus pinaster* Ait. individuals has also been confirmed [19,20] and PWN resistance has been included as a selection trait in the Galician *P. pinaster* breeding program [20].

Genetic variation in susceptibility to PWN has not been broadly studied for *P. radiata*, though some studies have shown variation in resistance to other pests, such as the pine aphid *Essigella californica* Essig [21], and to diseases such as dothistroma needle blight [22] or *Cyclaneusma* needle cast [23]. In fact, resistance to *Dothistroma pini* Hulbary is an important selection trait in genetic breeding programs for *P. radiata* in New Zealand and Australia.

One of the difficulties that forest breeders must address in the implementation of efficient breeding programs are genotype-by-environment interactions (G × E). The expression of resistance may differ among genotypes across a range of environments due to G × E, making changes in genotype ranking a primary concern for tree breeders. Consequently, the detection of G × E is relevant for estimating the expected genetic gain in any breeding program [24,25].

Although breeding forest tree species for resistance is a long-term solution, it is also a long-term process. Genomic selection using molecular markers such as single nucleotide polymorphisms (SNPs) could shorten breeding cycles [26,27] while greater command of the *Pinus* defences involved in resistance to *B. xylophilus* may prove useful for selecting resistant *Pinus* genotypes. Chemical compounds in pines constitute a major defence against a wide range of pests and diseases. Accordingly, different authors have demonstrated the significant role that various chemical compounds play in resistance to PWN. Phytoalexins, for instance, appear to have nematocidal action [28] while concentrations of total phenolics and condensed tannins seem to be related to PWN resistance [29–31]. Other studies point to the importance of nutrients in PWD development [5,31].

The main purpose of this paper was to determine whether the *P. radiata* breeding population in the Galician breeding program has enough genetic variation in PWN resistance to reduce disease impact through selection and breeding. Specifically, we aimed to (i) estimate genetic parameters, (ii) explore the genotype by environment interaction, and (iii) study constitutive chemical differences between susceptible and resistant *P. radiata* seedlings.

2. Materials and Methods

2.1. Plant Material and Experimental Design

Three experiments were performed to test half-sib families from the *P. radiata* genetic improvement program begun in Galicia (NW Spain) in the 1990s. Seeds for the three experiments were collected from a progeny trial located in A Coruña (Spain). They were grown in nursery beds at the Lourizán Forest Research Center (Xunta de Galicia, 42°24′35″ N 8°00′12″ W, Pontevedra, Spain) in two-litre plastic pots using a mixed substrate of peat moss and vermiculite (9:1 *v*/*v*.).

Three-year-old seedlings from 44 *P. radiata* open-pollinated families were evaluated; 41 in Experiment 1 of Supplementary Materials (Figure S1), 44 in Experiment 2, and 40 in Experiment 3. Each family was evaluated in at least two out of the three experiments (Table 1).

Table 1. Main features of the *P. radiata* inoculation experiments.

	Experiment 1	Experiment 2	Experiment 3
Age	3	3	3
Inoculation date	April 2019	April 2020	July 2019
Experiment duration (days)	83	83	83
Average temperature (°C)	25.3	22.3	23.8
Average temperature (night-day) (°C)	22.4–28.2	19.0–26.7	19.6–28.0
Height (cm)	121.69 ± 1.1	122.05 ± 1.04	112.22 ± 1.13
Diameter (mm)	19.25 ± 0.2	19.32 ± 0.2	18.44 ± 0.23
No. branches	11.47 ± 0.29	11.47 ± 0.28	14.67 ± 0.34
Survival	0.20 ± 0.02	0.50± 0.02	0.26 ± 0.02
Wilting	6.39 ± 0.06	5.52 ± 0.07	6.07 ± 0.08
No. families tested	41	44	40
No. seedlings inoculated per family	10 BX + 3 H_2O	10 BX + 3 H_2O	10 BX + 3 H_2O
No. blocks	10	10	10
Total No. of seedlings	533	572	520

BX: inoculated with *Bursaphelenchus xylophilus*; H_2O: inoculated with distilled water.

All experiments followed a randomized complete block design with 40–44 families, ten blocks, and one-tree plots. For each experiment, we inoculated ten seedlings per family with *B. xylophilus* and three with distilled water as controls. The three blocks including control seedlings were randomly selected. Seedlings in Experiments 1 and 2 were inoculated in April, while those in Experiment 3 were inoculated in July.

2.2. Pinewood Nematode Culture and Inoculation Procedure

Seedlings in the three experiments were inoculated using *B. xylophilus* which had been isolated from the outbreak that occurred in As Neves (Pontevedra, NW Spain) in 2010 [14].

Nematodes were reared using a non-sporulating form of *Botrytis cinerea* Pers. fungus cultured on PDA medium at 25 °C. The day before inoculation, nematodes were extracted by a modified Baermann funnel technique and adjusted to 2000 nematodes per ml in distilled water.

For seedling inoculation, a wound was made in the previous year's growth of the stem and a 1 cm-wide strip of gauze bandage was placed around the wound. Nematode suspension was pipetted onto the gauze bandage and the inoculation site was sealed with Parafilm® to avoid desiccation. Control seedlings were inoculated with 300 µL of distilled water while inoculated seedlings were administered a 300 µL suspension of distilled water containing 600 *B. xylophilus* nematodes at mixed developmental stages.

2.3. Pre-Inoculation Variables

In the three experiments, growth variables were assessed for all seedlings prior to the inoculation date. We measured height to the previous year's growth (HPY), growth increment from the previous year's growth to the inoculation date (IH), seedling height

at the inoculation date (H), basal diameter at the inoculation date (D), and number of principal branches (NB).

2.4. Wilting Symptom Assessment and Survival

We assessed wilting symptoms in all experiments twice a week from the onset of external wilting symptoms until no symptom evolution was observed. Wilting symptoms were assessed using a seven-level scale based on the percentage of discoloured needles, ranging from 1 (no external symptoms) to 7 (all needles brown and wilted) [5].

Survival and disease evolution variables were estimated from the wilting symptom assessments. Survival was determined as a binary variable: 1, dead seedling (wilting symptom Level 6 or 7), and 0, alive (Levels 1–5). For disease duration variables, Level 3 was established as the start of wilting symptoms and Level 6 as the end of wilting symptoms. Disease duration was estimated as the difference between the end and the start of wilting symptoms. All disease evolution variables were expressed in number of days.

2.5. Nematode Quantification

In the three experiments, nematodes were extracted from the stem in two control and eight inoculated seedlings per family in a subset of twelve randomly selected families. The same twelve families were used for nematode quantification in all three experiments.

Nematodes were extracted using the modified Baermann funnel technique and then quantified under a stereomicroscope (Olympus Co., Ltd., Tokyo, Japan). Stem samples were dried at 105 °C for 48 h to express nematode density as the number of nematodes per gram of dry stem weight.

2.6. Chemical Compounds Analysis

Needles were collected from all seedlings prior to *B. xylophilus* inoculation in each experiment. These samples were immediately frozen at −20 °C until all experiments concluded. At the end of each experiment, the 25 most and 25 least susceptible seedlings were selected and needles from these individuals were arranged into a total of 10 samples per experiment. Five samples per experiment and susceptibility group (resistant and susceptible) were composed of needles from five seedlings. The samples of the resistant individuals belonged to 30 families and the susceptible to 39. Needles from the selected seedlings were then lyophilised before blending to make the samples.

Chemical analyses were performed on the samples to determine water content and levels of lipid-soluble substances, total polyphenols, condensed tannins, macronutrients (N, P, K, Ca, and Mg), and micronutrients (Fe and Mn), as described by Menéndez-Gutiérrez et al. [5].

Soluble carbohydrates and starch concentrations were analysed as per Chow and Landhausser [32] and DuBois et al. [33], with some adjustments. Soluble carbohydrates were extracted with aqueous ethanol (EtOH:H_2O) (80:20) (v/v) using an ultra turrax, followed by centrifugation. A rotatory evaporator was used for ethanol removal and soluble carbohydrates in the extract were analysed as glucose, following the Dubois method [33]. Non-structural storage carbohydrates (starch) were hydrolysed with H_2SO_4 5N and analysed colorimetrically as soluble carbohydrates. The results were expressed in mg glucose g−1 lyophilized tissue.

2.7. Statistical Analysis

Joint analyses of the three experiments were performed for all variables. Survival, wilting symptoms, and disease duration variables (SW, DW, EW) were analysed using the following general linear mixed model:

$$X_{ijk} = \mu + \mathrm{E_i} + F_j + B_k(E_i) + E_i \times F_j + \varepsilon_{ijk} \quad (1)$$

where X_{ijk} is survival, wilting symptom or any of the disease duration variables of individual seedlings, μ is the overall mean, E_i is the fixed effect of the *ith* experiment, F_j is the

random effect of the *jth* family, $B_k(E_i)$ is the random effect of the *kth* block within the *ith* experiment, $E_i \times F_j$ is the random interaction between the *ith* experiment and the *jth* family, and and ε_{ijk} is the residual random error.

For survival, the analysis was also performed including diameter (the trait most correlated to survival in the present work) as a covariate, to determine whether differences in susceptibility were merely due to differences in diameter or if other causes were involved.

Pre-inoculation variables (HPY, IH, H, D, NB) were analysed using the model described above but excluding the block effect.

The SAS System MIXED procedure was applied to fit the model for wilting symptoms, disease duration and pre-inoculation variables. However, binary survival data (0/1) were analysed using the SAS System GLIMMIX procedure of presuming a binary distribution and a logit link function. Accordingly, the function used to estimate predicted survival (p) was:

$$\eta_{ijk} = ln\left(\frac{p}{(1-p)}\right) = \mu + E_i + F_j + B_k(E_i) + E_i \times F_j + \varepsilon_{ijk} \quad (2)$$

where η_{ijk} is the link function $g(p)$, μ the conditional mean, and $\ln [p/(1-p)]$ the odds of survival.

The significance of random factors was tested by a likelihood ratio test, which was determined by the difference in two times the log-likelihood of the models, including and excluding the assessed random factor. The likelihood ratio was distributed as a one-tailed χ^2 with one degree of freedom [34].

Individual (h_i^2) and family (h_f^2) heritabilities were estimated as follows from the joint analyses:

$$h_i^2 = \frac{\sigma_A^2}{\sigma_f^2 + \sigma_{fe}^2 + \sigma_\varepsilon^2} \quad (3)$$

$$h_f^2 = \frac{\sigma_f^2}{\sigma_f^2 + \frac{\sigma_{fe}^2}{NB} + \frac{\sigma_\varepsilon^2}{NBE}} \quad (4)$$

where ${\sigma_A}^2$ is the additive variance, assuming true half-sib families was calculated as ${\sigma_A}^2 = 4{\sigma_f}^2$; ${\sigma_f}^2$, the family variance component; ${\sigma_{fe}}^2$, the family variance component by experiment interaction; ${\sigma\varepsilon}^2$, the residual variance; N, the harmonic mean number of seedlings per plot; B, the number of blocks; and E, the number of experiments. Standard errors were calculated as in Wright [35].

For binomial variable survival, the residual variance was established as $\pi^2/3$, the variance on the underlying scale for logit link function [36].

The predicted genetic gain in percentage from the across site family selection was estimated as the average best linear unbiased predictors (BLUPs) of the selected less susceptible families divided by the population mean, multiplied by 100 and the individual heritability [37].

Chemical compound analyses were carried out using the SAS MIXED procedure, including the experiment, the susceptibility group (susceptible or non-susceptible seedlings), and their interactive effect as fixed factors.

Likelihood-based analyses were performed to thoroughly examine genotype by environment interaction (G × E) causes for survival and wilting symptoms, according to Yang [38] and De la Mata and Zas [39]. Initially, a full model was fitted using an unstructured family covariance structure in the SAS PROC MIXED procedure, considering the experiment as a fixed factor and the family and block within the experiments as random factors. Then, reduced models with specific constraints to the family covariance structures were fitted and compared to the full model. The following sources of G × E were tested: homogeneity of family variance across experiments, perfect family correlation between all experiment pairs, and homogeneity of family covariance across experiment pairs. These three hypotheses were evaluated by comparing the restricted log-likelihood ratio obtained

for the full model to the likelihood ratio of the reduced covariance model. Under the null hypothesis (i.e., full covariance model is not different from the reduced covariance model), the log-likelihood ratio is distributed as χ^2 with degrees of freedom given by the difference in the number of covariance parameters that defined the full model and the reduced model [34].

Differences in nematode density in the stem at the end of the experiments were studied using the Kruskal–Wallis non-parametric analysis of variance. These analyses were performed separately for each experiment and then jointly. The Mann–Whitney U test was performed to compare families when significant differences were found.

Pearson correlation coefficients between all traits were calculated for individual-seedling values of the three experiments (phenotypic correlations) and for family breeding values from joint analyses (genetic correlations) using the CORR procedure in SAS. Spearman correlations were only used when nematode densities were analysed.

SAS System Software (SAS Institute Inc., Cary, NC, USA, 2014) was used to perform all statistical analyses.

3. Results

3.1. Pre-Inoculation Variables

All *P. radiata* seedling variables measured prior to *B. xylophilus* inoculation (H, IH, HPY, D, NB) showed significant differences among experiments ($p < 0.05$), families ($p < 0.0001$), and family × experiment interaction ($p < 0.015$) (Table 2).

Table 2. Overall means and standard error (SD); Variance components (σ^2) and likelihood ratio significance test (χ^2_{LRT}) for random effects; F ratios and significance levels for the fixed effect, using mixed-model analysis to calculate heritability in *P. radiata* families inoculated with *Bursaphelenchus xylophilus*. Height (H), Height to the previous year's growth (HPY), growth increment from the previous year's growth to the inoculation date (IH), Diameter (D), Number of principal branches (NB), Wilting symptoms (W), Survival rate (S), Start (SW), End (EW) and Duration (DW) of wilting symptoms of dead seedlings.

Variables	Mean ± SD	Experiment		Family			Experiment × Family			Heritability	
		$F_{2,79}$ *	$p > F$	σ^2	χ^2_{LRT}	$p > \chi^2$	σ^2	χ^2_{LRT}	$p > \chi^2$	h_i^2	h_f^2
H	118.93 ± 0.64	11.79	<0.0001	0.068 ± 0.020	29.1	<0.0001	0.039 ± 0.010	44.4	<0.0001	0.77 ± 0.10	0.76 ± 0.21
HPY	102.89 ± 0.65	3.25	0.044	0.033 ± 0.009	30.2	<0.0001	0.015 ± 0.005	22.4	<0.0001	0.67 ± 0.09	0.77 ± 0.20
IH	16.06 ± 0.23	26.03	<0.0001	0.031 ± 0.011	13.8	<0.0001	0.018 ± 0.009	5.9	0.015	0.29 ± 0.06	0.63 ± 0.12
D	19.03 ± 0.12	3.75	0.0278	0.016 ± 0.005	20.2	<0.0001	0.012 ± 0.004	36.4	<0.0001	0.57 ± 0.08	0.70 ± 0.18
NB	12.45 ± 0.18	19.62	<0.0001	0.058 ± 0.018	21.5	< 0.0001	0.031 ± 0.011	16.4	<0.0001	0.51 ± 0.08	0.72 ± 0.17
W	5.98 ± 0.04	10.24	0.0005	0.137 ± 0.048	15.9	< 0.0001	0.045 ± 0.035	2.2	0.138	0.29 ± 0.06	0.66 ± 0.11
S	0.33 ± 0.01	37.64	<0.0001	0.414 ± 0.163	12.3	0.0004	0.161 ± 0.124	2.4	0.119	0.43 ± 0.07	0.72 ± 0.15
SW	33.04 ± 0.28	42.72	<0.0001	2.301 ± 1.528	2.8	0.0943	3.895 ± 1.898	7	0.0082	0.18 ± 0.04	0.45 ± 0.09
EW	55.31 ± 0.45	33.37	<0.0001	8.978 ± 4.552	5.3	0.0213	6.739 ± 4.473	3.3	0.0693	0.26 ± 0.05	0.59 ± 0.16
DW [a]	22.35 ± 0.37	44.31	<0.0001	9.278 ± 3.187	14.7	0.0001	-	-	-	0.38 ± 0.07	0.76 ± 0.14

* $F_{2,27}$ for SW, EW and DW, $F_{2,79}$ for the rest variables. [a] Experiment × Family was excluded from the analysis for this variable.

Seedlings from the experiments inoculated in April (Experiments 1 and 2) presented significantly greater height, growth increment from the previous year's growth to the inoculation date, height to the previous year's growth, and basal diameter compared to seedlings inoculated in July (Experiment 3). Conversely, the number of principal branches was greater in Experiment 3 (data not shown).

Individual narrow-sense heritability for these traits was high in all cases ($h_i^2 > 0.50$), except for IH ($h_i^2 = 0.29$). Family heritability estimates were also high, with heritability ranging from 0.63 (IH) to 0.77 (HPY) (Table 2).

3.2. Disease-Related Variables

Wilting symptoms and survival differed significantly among *P. radiata* families, but also between experiments and among blocks. In contrast, this analysis detected no differences in the G × E interaction for both traits (Table 2). Differences among *P. radiata* families for both variables remained highly significant when diameter was included as covariate in the

previous analyses ($p < 0.01$, data not shown), indicating that other genetically controlled factors might be involved in genetic susceptibility to PWN.

In Experiment 2, 50% of the seedlings survived *B. xylophilus*, compared to 20% in Experiment 1 and 26% in Experiment 3. In those two experiments, the mean diurnal and nocturnal temperatures were higher than in Experiment 2 (Table 1). Despite the low *P. radiata* survival rates, great variation in survival rates was observed among families. The observed family survival rates ranged from 0% to 90 % in Experiment 2 and from 0% and 70% in the other two experiments. One group of families stood out as having reasonably high predicted survival rate (over 65%, a range oscillating from 24% to 75%) and fewer wilting symptoms in all the experiments (Figure 1).

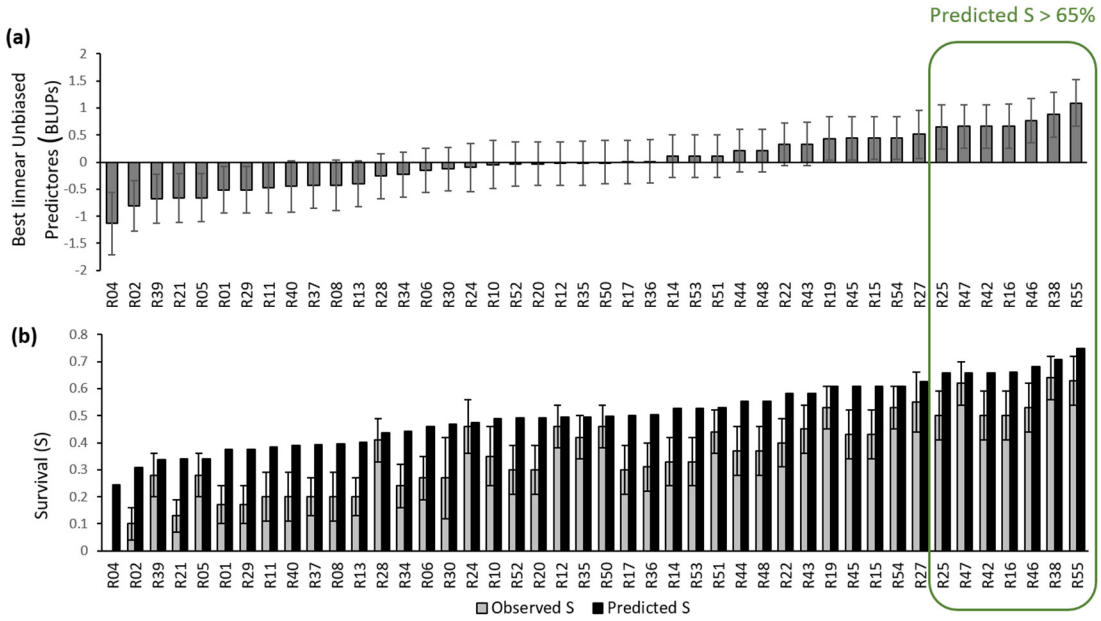

Figure 1. (**a**) BLUPs survival ranking for the 44 *Pinus radiata* families (**b**) Predicted and observed survival 83 days after inoculation. Error bars are standard errors.

Significant differences among families were also observed for the end and duration of wilting symptoms, though not for the start of symptoms. The G × E interaction was only significant for the disease duration variable SW (Table 2).

On average, wilting symptoms started 33 days after inoculation (DAI) and ended 55 DAI, so the average duration of the disease was 22 days. However, all these variables differed significantly among experiments (Table 2).

Both individual and family heritability estimates were especially high for survival and all pre-inoculation variables (H, HPY, D, NB) except height increment, which showed lower values (Table 2). Wilting symptoms and disease duration variable (SW, EW, DW) values were moderately high for both individual and family heritabilities (Table 2).

The value for predicted genetic gain for survival was high. By selecting the 50% of the families with the highest survival, we obtained a genetic gain of up to 26.4%. As the number of families selected decreased, this value grew to almost 52.7% for the seven families that were finally selected (Figure 2).

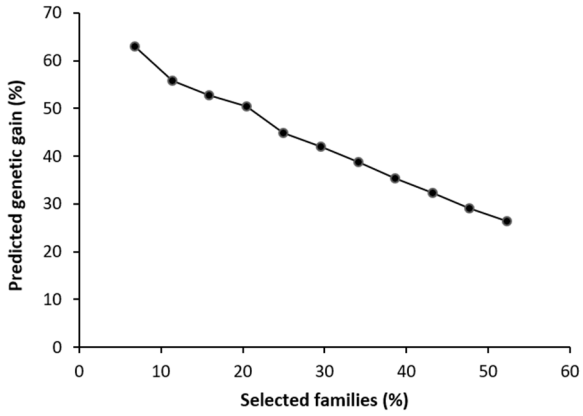

Figure 2. Predicted genetic gain in survival of *Bursaphelenchus xylophilus* in relation to the number of *Pinus radiata* families selected.

3.3. Genotype by Environment Interaction

The G × E interaction was not significant for wilting symptoms or survival in the conventional mixed model analyses (Table 2). However, more specific G × E analyses involving various sources of interaction showed a significant G × E effect (Table 3) that was not explained by any of the sources studied. In fact, one of the key implications of G × E in a breeding program, the family ranking changes, was not significant (Table 3). This interaction did not seem relevant in the end, since the ratio family variance component to G × E variance for these traits did not exceed the interaction relevance threshold proposed by Shelbourne [40] ($\sigma_f^2/\sigma_{fe}^2 < 2$). The values for survival and wilting symptoms were 0.39 and 0.29, respectively (data not shown).

Table 3. Likelihood ratio tests from analyses of different sources of genotype by environment interaction (G × E) across the three experiments. Chi-squared values (χ^2) and significance of the sources of G × E are estimated by comparing the likelihood ratio of the full and reduced models. Degrees of freedom (df) are the difference between the number of (co)variance parameters estimated in the full and reduced models.

Null Hypotheses	df	Wilting		Mortality	
		χ^2	$p > \chi^2$	χ^2	$p > \chi^2$
No family by environment interaction	5	20.3	0.001	16	0.007
Homogeneity of family variance across experiments	4	2.3	0.681	4.2	0.241
Perfect family correlation between all experiment pairs	3	5.3	0.151	3.8	0.283
Homogeneity of family covariance across experiment pairs	2	5.2	0.074	4.4	0.111

3.4. Nematodes

The number of nematodes sampled from inoculated stems differed significantly among the three experiments (Kruskal–Wallis $\chi^2 > 10.12$, $p < 0.0064$; data not shown). The median number of nematodes was higher in Experiment 1 than in the other experiments, but we only found significant differences among families in Experiment 3 (Kruskal–Wallis $\chi^2 > 23.87$, $p < 0.0324$; data not shown) when data were analysed separately for each experiment.

In the joint analyses, the number of nematodes differed significantly among families (Kruskal–Wallis $\chi^2 > 34.01$, $p < 0.0021$; Figure 3, data not shown).

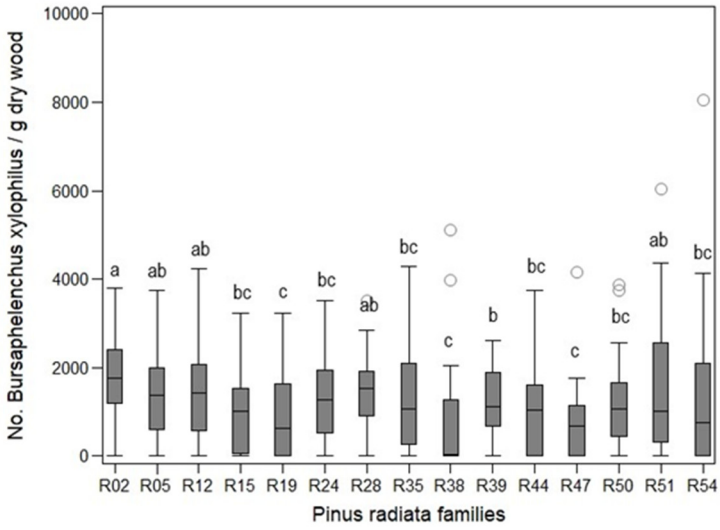

Figure 3. Number of *Bursaphelenchus xylophilus* per dry gram of wood extracted from stem of *Pinus radiata* families. Box-whisker plot, Line (median), Box (25%–75% quartiles of values), Whisker (min-max span of values), Circles (outliers).

At the end of the assays, the number of nematodes recovered from the stems of seedlings at wilting levels 1–4 ranged from 0 to 69.50 *B. xylophilus* per dry gram of wood. In seedlings at wilting levels 6 and 7, the number ranged from 40.73 to 8059.16 *B. xylophilus* per dry gram of wood.

We did not recover any nematodes from the control seedlings.

3.5. Correlations

Survival and wilting symptoms presented a markedly strong negative correlation at phenotypic and genetic levels. Hence, they had relationships with the same traits but opposite signs (Table 4). At the phenotypic level, all pre-inoculation traits except IH were correlated positively with wilting symptoms and negatively with survival, especially NB and D. Similarly, nematode density was also strongly correlated with both traits. Disease duration variables (SW, EW, DW) had a negative relationship with wilting (Table 4).

The same relationships were observed at the genetic level, except for all height traits, which were not significantly correlated with survival or wilting symptoms (Table 4).

Table 4. Pearson correlation matrix of phenotypic values of the three experiments (below the diagonal) and genotypic values from joint analyses of the three experiments (above the diagonal) coefficients between pairs of variables. Height (H), Height to the previous year's growth (HPY), Growth increment from the previous year's growth to the inoculation date (IH), Diameter (D), Number of principal branches (NB), Wilting symptoms (W), Survival rate (S), Start (SW), End (EW) and Duration (DW) of wilting symptoms in dead seedlings, Number of *B. xylophilus* in the stem per gram of dry weight (ND).

	H	HPY	IH	D	NB	W	S	SW	EW	DW
H		0.97 ***		0.62 ***	0.37 *					
HPY	0.94 ***			0.55 ***	0.30 *					0.37 *
IH	0.12 ***	−0.23 ***							−0.30 *	−0.35 *
D	0.58 ***	0.59 ***			0.75 ***	0.45 **	−0.38 *	−0.29 *		
NB	0.32 ***	0.33 ***		0.48 ***		0.44 **	−0.39 **	−0.32 *		
W	0.16 ***	0.17 ***		0.21 ***	0.19 ***		−0.94 ***	−0.40 **	−0.54 ***	−0.36 *
S	−0.097 ***	−0.112 ***		−0.14 ***	−0.15 ***	−0.89 ***		0.39 **	0.58 ***	0.41 **
SW			0.11 **		−0.19 ***	−0.25 ***	-		0.48 ***	
EW	0.11 **	0.12 ***		0.07 *		−0.57 ***	-	0.56 ***		0.82 ***
DW	0.15 ***	0.18 ***	−0.11 **	0.13 ***	0.18 ***	−0.50 ***	-	−0.08 *	0.79 ***	
ND					0.12 *	0.67 ***	−0.70 ***		−0.15 *	−0.26 ***

Significance levels: *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$. Blank spaces indicate non-significant correlations and (-) correlations that cannot be performed.

3.6. Chemical Compounds

The constitutive macronutrients N, P, and K and the micronutrient Mn, along with condensed tannins and soluble carbohydrates, showed significant differences among susceptibility groups. In addition, we observed a significant interaction effect between susceptibility group and experiment for Mn and lipid-soluble substances (Table 5). The group comprised of resistant individuals had the highest concentration of condensed tannins and soluble carbohydrates but was lower in N, P, and K contents (Figure 4). We only found significant differences for lipid-soluble substances between the resistant and susceptible groups in Experiment 3, where concentrations of this chemical were much greater in the resistant group. Mn concentrations were also significantly higher for the resistant individuals in Experiment 2 (data not shown).

Table 5. Results of mixed-model analysis of the constitutive chemical compounds in needles. F ratios (degrees of freedom are shown as a subscript, F factor, error) and associated *p* values are shown. WC: Water content, N: Nitrogen, P: Phosphorus, K: Potassium, Ca: Calcium, Mg: Magnesium, Fe: Iron, Mn: Manganese, LS: Lipid-soluble substances, POL: Total polyphenols, TAN: Condensed tannins, CAR: Soluble carbohydrates STA: Starch. Significant *p*-values ($p \leq 0.05$) are shown in bold.

Variables	Susceptibility Group		Experiment		Susceptibility Group × Experiment	
	$F_{1, 25}$	$p > F$	$F_{2, 25}$	$p > F$	$F_{2, 25}$	$p > F$
WC (%)	2.03	0.1662	4.13	**0.0282**	0.2	0.8175
N (%)	13.95	**0.001**	22.21	**<0.0001**	0.43	0.6577
P (%)	16.84	**0.0004**	2.87	0.0753	0.06	0.9447
K (%)	6.43	**0.0178**	6.58	**0.005**	0.36	0.6995
Ca (%)	3.38	0.0781	2.21	0.1302	0.94	0.4039
Mg (%)	0.04	0.8344	1.5	0.2421	0.49	0.6197
Fe (ppm)	0.13	0.7173	6.53	**0.0052**	1.24	0.3064
Mn (ppm)	7.27	**0.0124**	0.25	0.7795	4.47	**0.0219**
LS ($mg \cdot g^{-1}$)	0.21	0.6476	22.81	**<0.0001**	5.93	**0.0078**
POL ($mg \cdot g^{-1}$)	1.07	0.3115	1.3	0.2902	1.13	0.3383
TAN ($mg \cdot g^{-1}$)	5.14	**0.0323**	1.44	0.2548	0.01	0.9883
CAR ($mg \cdot g^{-1}$)	5.08	**0.0333**	7	**0.0038**	1.91	0.1686
STA ($mg \cdot g^{-1}$)	1.13	0.2971	2.46	0.1058	1.15	0.3334

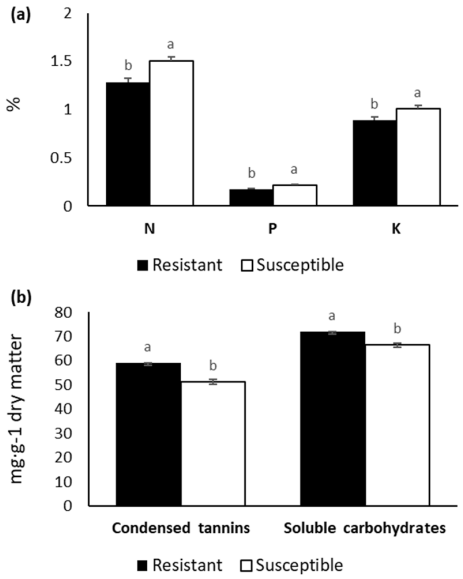

Figure 4. Constitutive concentration of the macronutrients N, P, K (**a**); condensed tannins and soluble carbohydrates (**b**) in *Pinus radiata* needles from resistant and susceptible groups.

4. Discussion

Our results revealed significant genetic variation with a broad range of susceptibility to pinewood nematode among *P. radiata* half-sib families from the Galician breeding program. The moderately high heritability estimates and genetic gain obtained from these experiments confirm the potential of this species for breeding PWD-resistant trees to mitigate future damage to *P. radiata* plantations.

Resistance breeding has proven successful in controlling diverse pests and pathogens that affect forest tree species [41–43]. In PWD resistance breeding, numerous resistant *P. densiflora* and *P. thunbergii* clones have been identified in Japan since 1972, with respective survival rates that are 40% and 16% higher than the non-selected population [44]. These data justify the need to continue advancing this research line as part of the strategy to control the disease. Resistance breeding programs are also underway for other susceptible species, though they are in earlier stages than the Japanese program. A PWD resistance breeding program for *P. massoniana* was initiated in China in 2001 with the selection of 1201 resistant *P. massoniana* individuals [45]. On the Iberian Peninsula, significant genetic variation in susceptibility to PWD was found among half-sib families of *P. pinaster*, prompting both Portugal and Spain (Galicia in particular) to start improvement programs [19,20]. Six families from the Galician *P. pinaster* breeding program (NW Spain) have been registered as parents of families resistant to PWD in the National Catalogue of Base Materials [46] and are currently ready to be commercialized.

Australia and New Zealand, where *P. radiata* has a great economic importance, have prioritized breeding for resistance to diverse foliar diseases, especially *Dothisthoma* needle blight and *Cyclaneusma* needle cast, which have been studied and included as selection criteria for several decades [47]. The variation in resistance to these diseases has a strong additive genetic basis [22,23].

The results of this study also justify starting a PWD resistance breeding line for *P. radiata* as part of the Galician breeding program for this species. As in the previous examples, significant genetic variation in susceptibility to PWD was observed, along with reasonably high heritability estimates, indicating that resistance is inherited and mainly due to additive genetic effects. The individual narrow-sense heritability estimate for survival was similar to the maximum heritability estimates reported for open-pollinated *P. thunbergii* families [18] and *P. pinaster* families, which were selected from Portuguese areas severely affected by PWD and studied under controlled conditions [19]. However, the heritability values obtained for *P. pinaster* families from the Galician breeding population (NW Spain) for mortality were considerably lower [20].

In this study, the isolate used for screening was obtained from a dead Maritime pine from the outbreak that occurred in As Neves (Galicia, NW Spain). Many authors have emphasized the importance of using the most virulent isolate for selecting the most resistant genotypes in breeding programs [48]. Accordingly, we chose the isolate for this assay based on a previous study of isolates of diverse origins to determine the most virulent isolate for *P. radiata* [49].

Many factors seem to affect variation in PWN susceptibility, including the physicochemical characteristics of the host and environmental factors, such as temperature and drought. In this study, we observed a significant genetic and phenotypic relationship between growth traits and diseased-related variables, in which vigorously growing families tended to have higher mortality and develop more wilting symptoms. This relationship was also reported for *P. pinaster* families from the Galician breeding population: trees from the most susceptible families also had greater height growth [20]. The same has been reported for other pests [50,51] and points to a growth–defence trade-off, though other studies found no relation or a negative correlation between growth and mortality [52,53].

Temperature is also known to be widely associated to PWD development, as illustrated by the differential survival rate obtained across experiments. We observed that the highest survival rate occurred in the experiment subjected to the lowest day and night temperatures. Differences in temperature were even greater when we only considered temperature during

the three first weeks after inoculation. This finding is in accordance with a previous study that described the great relevance of early cumulative temperatures in PWD development, especially in resistant *P. thunbergii* families [54]. Another work demonstrated the heavy influence of high nocturnal temperature on tree mortality after PWN inoculation and the likely contribution of other factors to PWD development [53].

While our results suggested a low level of G × E, we have to take into consideration that we studied G × E under greenhouse conditions where the sources of variation are less pronounced. In a study by Matsunaga et al. [55] of six *P. thunbergii* families at three sites with dissimilar climates, only a low proportion of the total variance was explained by site x family interaction and no changes were observed in the resistance family ranking. Similarly, Suontama [23] did not find a significant G × E interaction that affected *P. radiata* resistance to Cyclaneusma needle cast.

Genetic variation in secondary metabolites has been reported for conifers [56]. For this reason, we focused on certain constitutive chemicals in *P. radiata* that may also influence disease development in resistant families. Given the rapid multiplication and spread of the nematode throughout the tree, we hypothesized that the defence system could not inhibit PNW infection unless defence metabolites such as terpenoids or tannin-like substances were already present in the tree or produced in the early stage of the disease [57]. Condensed tannins are phenolic compounds with great defensive capacity. Indeed, these rather potent antibiotics act to protect against insect herbivores [58,59]. In the present work, we observed higher levels of constitutive condensed tannins in resistant individuals. Our findings concur with those of Ohyama et al. [60], who reported a higher proanthocyanidin (condensed tannin) content in pine-wilt resistant *P. thunbergii* and *P. densiflora* clones than in susceptible pines. Resistant individuals also had larger amounts of soluble carbohydrates. These substances are involved in various stress responses and are related to important changes in the balance of reactive oxygen species (ROS) [61], which are known to be released in response to PWN infection [62]. However, this relationship requires more thorough study since soluble sugar can also contribute to ROS scavenging [61].

Plant nutrients can also play an important role in disease resistance by enhancing disease resistant mechanisms. We found higher constitutive levels of Mn in resistant individuals in two of the experiments. This compound plays an important role in the biosynthesis of some defensive substances, such as lignin and phenol. It is also involved in photosynthesis and many other tree functions [63]. By the same principles, the highly PWN-susceptible species *P. sylvestris* was found to have about ten times less Mn than other, less susceptible pine species [5]. Conversely, we observed higher levels of N, P, and K in the susceptible group than in the resistant one. The effect of N and P on disease resistance seems inconsistent and sometimes depends on the plant pathogen [63]. For instance, one study indicated higher levels of constitutive N in the phloem of *P. sylvestris*, which is highly susceptible to PWN, than in other less susceptible species [31], while another study reported much lower amounts of N in the xylem of *P. sylvestris* than in other species [5]. Though we found lower levels of K in the resistant individuals, fertilization with K seems to reduce disease incidence in some crops by promoting the synthesis of various compounds involved in disease resistance [63].

Neither field-based assessments nor field selection of PWN-resistant candidate trees in affected areas were possible in Spain due to EU quarantine organism restrictions, since the disease is currently found in isolated foci and has not spread over the entire territory. Given the impossibility of validating our findings in adult trees in the field, the results must be considered cautiously. However, the use of two- or three-year-old seedlings for testing resistance against pinewood nematode is broadly accepted. In Japan, the evaluation in seedlings has been carried out for many years, obtaining resistant candidates which are being planted. Additionally, Maehara et al. [64] used *P. thunbergii* adult trees, obtaining similar results to those of Kanzaki et al. [65] who used three-year-old seedlings.

Experiences with managing pine wilt disease in affected countries have shown that conventional disease control measures are insufficient and must be complemented with

strategies such as breeding. This work confirms that breeding PWN-resistant trees through selection is possible for *P. radiata*. Our results confirm the existence of genetic variation among *P. radiata* families, with high heritability estimates and a moderately high genetic gain for survival. These findings imply that resistance to PWD can be included as a new selection factor in the *P. radiata* breeding program and survival seems to be the best trait to assess in operational breeding. Furthermore, this work provides evidence indicating the importance of the constitutive chemistry of *P. radiata* seedlings in host resistance to PWN.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f12111474/s1, Figure S1: *Pinus radiata* experiment one month after inoculation with *Bursaphelenchus xylophilus*.

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