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The effects of provenance, climate, and chemical defense on the resistance of *Pinus pinaster* Aiton to *Bursaphelenchus xylophilus* (Steiner and Buhner)

Esteban Torres-Sánchez^{1*} , María Menéndez-Gutiérrez¹ , Lucía Villar² and Raquel Díaz¹ 

Abstract

Key message The resistance of *Pinus pinaster* Aiton to pinewood nematode *Bursaphelenchus xylophilus* (Steiner and Buhner) varied among populations from the Iberian Peninsula, with survival rates for inoculated seedlings ranging from 5 to 100%. These differences in resistance were paralleled by differences in some constitutive chemical defenses. Populations from the southeastern Iberian Peninsula displayed higher resistance than northern populations.

Context The presence of the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhner), in Portugal threatens Mediterranean pine species such as *Pinus pinaster* Aiton.

Aims We have focused on assessing the resistance of *P. pinaster* populations to *B. xylophilus*, looking for any relationship between the PWN resistance and some constitutive chemical defenses and geoclimatic parameters.

Methods Two-year-old seedlings from 32 provenances and two seed orchards were evaluated in an experiment of artificial inoculation following a randomized complete block design under greenhouse conditions. We measured growth-related traits, response to *B. xylophilus* inoculations, and constitutive chemical compounds in needles of the evaluated seedlings and compiled geoclimatic data for each population. Mixed models, nonparametric tests, correlations, and PCA were used to analyze the data.

Results Survival, wilting symptoms, morphological traits, and nematode density varied significantly among populations. Lower concentrations of constitutive polyphenols, lipid-soluble substances, and tannins were related to higher PWN resistance. Populations from the southeast of the Iberian Peninsula showed higher survival rates than those from further north. Additionally, we observed that populations to warm, dry climates showed higher resistance to *B. xylophilus* than populations originating from humid, temperate climates.

Conclusion Higher susceptibility to PWN is related to lower growth traits, to lower levels of certain constitutive chemical compounds, and to adaptations to harsher climate.

Keywords Maritime pine, Pinewood nematode, Populations, Constitutive defenses, Geoclimatic pattern

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*Correspondence:

Esteban Torres-Sánchez
esteban.torres.sanchez@xunta.gal

Full list of author information is available at the end of the article



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1 Introduction

Bursaphelenchus xylophilus (Steiner and Buhner) Nickle, the pinewood nematode (PWN), is the agent of pine wilt disease (PWD), a severe disease outside the native region of this North American nematode. It can kill trees in a few weeks or months. The main tree hosts of this pathogen are conifer trees of the genus *Pinus*. Under natural conditions, they are infected through pine sawyer beetles belonging to the genus *Monochamus*, which are vectors of this pathogen (Linit 1988).

The first PWD outbreak was detected in Japan at the beginning of the twentieth century. Since then, the disease has spread through Asian countries, and in 1999, it was detected for the first time in Europe, specifically in the Setubal Peninsula, Portugal (Mota et al. 1999). Later, in 2008, the whole Portuguese territory was considered as demarcated zone (Mota et al. 2009), and since then, several outbreaks have been reported in Spain, some of which still remain active (Junta de Castilla y León 2019; Xunta de Galicia 2019). This situation constitutes a potential threat to the health of European coniferous forests (Evans et al. 1996; Vicente et al. 2012). Recently, several studies have predicted the expansion of the disease from Portugal through Central Spain and into Europe within the next few years (De la Fuente et al. 2018) and the establishment of PWD in Spain (De la Fuente and Saura 2021). The estimated economic losses in Spain due to PWD could reach nearly 12 billion euros for the 2008 to 2030 period if no control measures are efficiently deployed (Soliman et al. 2012).

The present work focuses on *P. pinaster*, which has a circum-Mediterranean distribution area, with fragmented populations spread throughout the Atlantic coast of eastern France, Portugal, and the Mediterranean Basin. Over a third of the forests in Spain are coniferous, and the ample distribution of *P. pinaster* (about 1.4 million hectares, considering both natural and planted forests) makes it an important species for the ecology and economy of the country. Indeed, it is one of the main timber species, amounting to 14.3% of the timber volume of Spanish forests (Bravo et al. 2017). In this country, all-natural *P. pinaster* forests have been classified into 28 provenances according to differences in genetic, phenotypic, and ecological patterns (Alía et al. 1997, 2009). In addition, based on a gene pool study, different geographic areas were defined for *P. pinaster* populations: French, northwest Iberian Peninsula, central and southeast Iberian Peninsula, Italian, and Moroccan groups (Jaramillo-Correa et al. 2015). This differentiation can reflect genetic selection due to diverse climates and environmental conditions (González-Martínez et al. 2002; Lamy et al. 2012, 2011).

Genetic breeding for resistance is one way to manage the impact of PWD. Several breeding programs against *B. xylophilus* reported successful results for different pine species. Japan developed the first breeding programs for *Pinus thunbergii* Parl. and *Pinus densiflora* Sieb. and Zucc. in 1978. Today, the resistant clones obtained from these programs are being used for reforestation in this country (Matsunaga et al. 2016; Kurinobu 2008; Nose and Shiraishi 2008). However, in Europe, breeding programs have been focused on improving traits of economic interest so far. The French breeding program for *P. pinaster* aimed at improved growth and stem forms (Bouffier et al. 2008). Alike, the Galician breeding program for this species was initially developed based on growth traits and stem form (Zas and Merlo 2008). Recently, the selection based on resistance has been evaluated since the potential for breeding PWN-resistant pines was confirmed for *P. pinaster* families of the Galician breeding program (Menéndez-Gutiérrez et al. 2018). In addition, genetic variation in PWN susceptibility has also been demonstrated for other species, such as *Pinus radiata* Don (Menéndez-Gutiérrez et al. 2021).

Growth traits are often a selection criterion in breeding programs. However, the pine trees selected for better growth traits do not have to be necessarily resistant to PWN. For example, Menéndez-Gutiérrez et al. (2018) reported a positive correlation between the growth traits and susceptibility to PWN. Conversely, Carrasquinho et al. (2018) reported a negative relationship between these traits. Other studies have found a positive relation between the number of branches and survival (Hakamata et al. 2013; Menéndez-Gutiérrez et al. 2017b, 2018).

According to Morgenstern (1996), geographic variation in forest species can lead to genetic variation patterns as a consequence of natural selection and other genetic processes. The host response to a disease may vary in populations with different provenances, as was reported for *Pinus halepensis* Mill. provenances against *Gremmeniella abietina* (Lagerb.) Morelet (Romeralo et al. 2016) and *P. pinaster* provenances against PWN (Menéndez-Gutiérrez et al. 2017b). In particular, Menéndez-Gutiérrez et al. (2017b) reported that susceptibility among *P. pinaster* provenances could be positively related to autumn precipitation and mean maximum temperature during summer at the original provenance sites.

Several authors have reported that constitutive chemical compounds may play a major part in defense against PWN (Linit 1988; Stamps and Linit 1998; Rodrigues et al. 2017). At species level, PWN-resistant species showed higher levels of polyphenolic compounds in the phloem (Pimentel et al. 2017a, b) and xylem (Menéndez-Gutiérrez et al. 2017a). In contrast, at provenance level, *P. pinaster* provenances with higher

polyphenolic concentrations in excised branch segments proved to be more susceptible to PWN (Zas et al. 2015). Recently, at family level, Menéndez-Gutiérrez et al. (2021) found higher levels of condensed tannins and soluble carbohydrates in needles from resistant *P. radiata* families to PWN. In addition, it is known that nutrients also regulate metabolic activity associated with susceptibility against diseases and pathogens (Datnoff et al. 2007). Previous studies determined that resistant species such as *P. halepensis* showed higher constitutive content of nitrogen than susceptible species such as *P. radiata* or *Pinus sylvestris*. In contrast, susceptible species showed higher constitutive content of lipid-soluble substances than resistant species (Menéndez-Gutiérrez et al. 2017a, 2021).

In this study, the main objective was to assess the susceptibility of a wide range of *P. pinaster* populations against PWN. To achieve this, we tested seedlings from thirty-two provenances and two seed orchards (one in Galicia, one in France) to the following: (1) identify the variation among *P. pinaster* populations in susceptibility to PWN, (2) explore the relationship between constitutive chemical compounds present in needles and PWN resistance, and (3) determine if there is a geoclimatic cline for susceptibility to PWN.

2 Material and methods

2.1 Plant material and experimental design

Two-year-old *P. pinaster* seedlings from thirty-four populations were evaluated in an inoculation experiment in June 2020. Populations originated from 32 natural stands each from one provenance region. In addition, two seed orchards with two breeding populations were used, HSC26001 in Galicia and HSFRVG006 in France (Table 1). We arranged these populations into six geographic groups based on different gene pools defined by Jaramillo-Correa et al. (2015) through the study of molecular markers related to climate adaptation (Table 1).

The experiment was performed under controlled conditions at the Lourizán Forest Research Center (42° 24' 33" N, 8° 39' 46" W, Pontevedra, Spain). The seedlings were grown in 2-l plastic pots in nursery beds using a mixture of peat moss and vermiculite (9:1v/v) as substrate. The seedlings had a mean height of 100.8 cm at the moment of PWN inoculation. The experiment followed a randomized complete block design with 20 blocks and one tree plot. In five of these blocks, a second seedling of each population was established as control. Twenty seedlings per population were inoculated with *Bursaphelenchus xylophilus* (Steiner and Bührer) and five with distilled water (controls). The duration of this experiment was 133 DAI (days after inoculation). Temperature ranged between 16 and 32 °C, with a mean temperature

of 18.7 °C. Watering by drip irrigation occurred on a weekly basis during the assay.

2.2 Pinewood nematode culture and inoculation procedure

A total of 678 seedlings were inoculated with a local isolate of *B. xylophilus* obtained from As Neves (Pontevedra, Spain) in 2010. Nematodes were cultured on non-sporulating *Botrytis cinerea* Pers. growing on PDA medium at 25 °C. The inoculum was prepared by extracting nematodes using a modified Baermann funnel technique and adjusting the concentration to 4000 nematodes per milliliter in distilled water. A cut was made in the previous year's growth on the stem of all seedlings. Then, a 1-cm-wide strip of gauze was put around the wound, and 300 µl of inoculum was pipetted into the xylem. The gauze was covered by parafilm to prevent the risk of unsuccessful inoculation. Each seedling was inoculated with an average of 1200 nematodes at mixed developmental stages. Control seedlings were inoculated with 300 µl of distilled water using the same method.

2.3 Morphological traits

Prior to inoculation, the following morphological traits were measured: total initial height (H, in cm), height increment from the previous year's growth to the inoculation date (ΔH , in cm), and total number of branches (NB, in number) at the inoculation date.

2.4 Disease development and survival

Wilting symptoms (W) were assessed twice a week until the end of the assay, which occurred when there was no further progress in symptoms. We used a seven-level scale corresponding to the percentage of affected needles from 1 (no external symptoms) to 7 (all needles brown and wilted) (Menéndez-Gutiérrez et al. 2018).

Survival and disease development variables were estimated from the wilting symptoms. Survival (S) was assessed as binary: seedlings with wilting symptoms in levels 1 to 5 were considered alive (1), whereas those in levels 6 or 7 were considered dead (0). Disease development was studied using three variables in populations that had at least five dead seedlings before the end of the experiment. The start of wilting symptoms (SWd) for each seedling was established as the day after inoculation (DAI) when wilting symptoms reached level 3. The DAI on which wilting symptoms reached level 6 was considered the end of wilting symptoms (EWd) for the seedling. The duration of wilting symptoms (DWd) was defined as the difference in days between the start and the end of wilting symptoms.

Table 1 *Pinus pinaster* populations evaluated for resistance to *Bursaphelenchus xylophilus*, along with associated geoclimatic data

Population code	Provenance region/seed orchard	Provenance/seed orchard name	Geographic group ^a	Lat (DD)	Long (DD)	Alt (m)	T (°C)	P (mm)
HSC26001 ^b	HSC-26001	HSC-26001	Atlantic coast of the Iberian Peninsula	43.56	-7.93	289	12.8	1200
HSFRVG006 ^c	HS-FR-VG-006	HS-FR-VG-006	France-Catalonia region	44.20	-1.19	52	13.2	1052
PTES01a	1a	Galician coast	Atlantic coast of the Iberian Peninsula	42.83	-8.54	289	13.6	1580
PTES01b	1b	Galician coastal interior	Atlantic coast of the Iberian Peninsula	42.63	-7.30	605	11.0	1445
PTES02	2	Telero	Central Iberian Peninsula	42.25	-6.08	947	11.0	793
PTES03	3	Oña	Central Iberian Peninsula	42.85	-3.47	782	11.2	836
PTES04	4	Gata-Hurdes	Central Iberian Peninsula	40.29	-6.54	764	13.1	867
PTES05	5	Tiétar	Central Iberian Peninsula	39.97	-6.03	375	15.3	566
PTES06	6	Gredos	Central Iberian Peninsula	40.27	-4.91	877	12.1	515
PTES07	7	Guadarrama	Central Iberian Peninsula	40.59	-4.35	1055	10.3	504
PTES08	8	Meseta Castellana	Central Iberian Peninsula	41.16	-4.68	868	11.7	388
PTES09	9	Soria-Burgos	Central Iberian Peninsula	41.87	-3.07	1156	9.3	505
PTES10	10	Sistema Ibérico Central	Central Iberian Peninsula	41.31	-1.43	954	11.7	428
PTES11	11	Rodenaes de Molina	Southeast Iberian Peninsula	40.96	-2.26	1179	10.8	542
PTES12	12	Serranía de Cuenca	Southeast Iberian Peninsula	39.94	-1.82	1009	11.9	465
PTES13	13	Albarracín	Southeast Iberian Peninsula	40.31	-1.21	1251	11.1	428
PTES14	14	Maestrazgo	Southeast Iberian Peninsula	40.27	-0.73	1041	10.7	447
PTES15	15	Espadán	Southeast Iberian Peninsula	39.84	-0.27	653	15.6	431
PTES16	16	Levante	Southeast Iberian Peninsula	39.09	-0.85	766	13.1	397
PTES17	17	Segura-Alcaraz	Southeast Iberian Peninsula	38.25	-2.66	1111	13.5	443
PTES18	18	Moratalla	Southeast Iberian Peninsula	38.14	-2.01	1130	12.2	459
PTES19	19	Almijara-Nevada	Southeast Iberian Peninsula	36.93	-3.80	1245	13.0	548
PTES20	20	Bermeja	Southeast Iberian Peninsula	36.51	-4.99	572	15.8	680
PTESA	A	Benicasim	Southeast Iberian Peninsula	40.08	-0.01	339	15.7	448
PTESB	B	Pradell	Southeast Iberian Peninsula	40.99	0.86	535	15.5	596
PTESC	C	Litoral Catalán	France-Catalonia region	41.85	2.63	327	12.7	806
PTESD	D	La Safor	Southeast Iberian Peninsula	38.88	-0.24	422	15.8	362
PTESE	E	Fuencaliente	Southeast Iberian Peninsula	38.42	-4.28	1011	14.3	518
PTESF	F	Oria	Morocco-Oria region	37.52	-2.41	1331	12.2	466
PTESG	G	Serranía de Ronda	Southeast Iberian Peninsula	36.66	-5.97	576	17.7	617
PTFR800	PPA 800	Corsica	Corsica	42.23	9.12	100	8.9	919
PTMAATLA	High Atlas	High Atlas	Morocco-Oria region	32.34	-5.22	1000	9.7	319
PTMAATLM	Medium Atlas	Medium Atlas	Morocco-Oria region	33.96	-4.44	1000	15.6	411
PTPORPIV	PNB-RPIV	IV	Atlantic coast of the Iberian Peninsula	40.36	-8.74	55	15.0	884

Lat and *Long* latitude and longitude, in decimal degrees (DD, WGS 84); *Alt* altitude, in m.a.s.l.; *T* annual mean temperature, in °C; *P* annual precipitation, in mm

^a Geographic groups based on Jaramillo-Correa et al. (2015)

^b 1.5 generation seed orchard from Galician breeding program which comes from the PTES01a provenance

^c Second-generation seed orchard from French breeding program in Landes

2.5 Quantification of nematode density

All seedlings from six of the 20 blocks randomly selected were harvested to quantify the nematode density. Every seedling from the selected blocks that reached level 7 was harvested immediately, and their nematode populations

were extracted by a modified Baermann funnel method (Schröder et al. 2009). At the end of the experiment, all seedlings from these blocks that were still alive were harvested. In total, 203 seedlings were harvested and divided into the following: roots and stems. The nematodes were

extracted and counted using a stereomicroscope (Olympus Co., Ltd., Tokyo, Japan). Dry weight (105 °C, 48 h) was determined, and nematode density (per gram of dry weight) was estimated for stem (NDs), roots (NDR), and total seedling (NDt).

2.6 Chemical compounds

Prior to inoculation, needle samples from every seedling of 12 blocks randomly selected were collected and immediately frozen at −20 °C. Some months later, after the end of the experiment, survival data was analyzed, and the 5 most and 5 least susceptible populations were selected for the chemical compound analysis. Then, the 12 needle samples from each population were arranged into four major samples, each one composed by samples coming from three blocks of the inoculation experiment. All frozen needles from these ten populations were cleaned with distilled water, cut into small parts, lyophilized for 72 h, and crushed into a homogeneous powder.

Based on previous works (Datnoff et al. 2007; Zas et al. 2015; Menéndez-Gutiérrez et al. 2017a, 2021; Pimentel et al. 2017a), the following chemical compounds were selected to be quantified: nitrogen (N; %), lipid-soluble substances (Lip; mg·g^{−1} of dry tissue), total polyphenols (Pol; mg·g^{−1} of dry tissue), condensed tannins (Tan; mg·g^{−1} of dry tissue), soluble monosaccharides (Mon; mg·g^{−1} of dry tissue), and starch (Sta; mg·g^{−1} of dry tissue).

Nitrogen was analyzed using the Kjeldahl method as in Menéndez-Gutiérrez et al. (2018). Liposoluble substances were identified through gravimetry, with petroleum ether as the specific solvent, which was removed from the sample with a rotary vacuum evaporator (Heidolph Instruments GmbH and Co. KG, Schwabach, Germany). From the defatted sample, condensed tannins and total polyphenols were extracted by refluxing in Soxhlet, with methanol-water (50:50 v/v) for 5 min. KHSO₃ was added to prevent oxidation of phenolic compounds during extraction, and methanol was removed by a rotary vacuum evaporator (Heidolph Instruments GmbH and Co. KG, Schwabach, Germany). Condensed tannins were determined colorimetrically with H₂SO₄/vanillin reagent (Scalbert et al. 1989), and a Perkin Elmer Lambda 35 UV-Vis spectrometer (Perkin Elmer, Norwalk, CT, USA) was used to measure the samples at 500 nm. Folin-Ciocalteu reagent was used to determine total polyphenols, and the samples were measured at 750 nm with the spectrophotometer. The results were expressed as milligram of catechin equivalent per gram of dry tissue (mg/g). To prevent other substances from interfering, the substances/isolates were measured with and without polyvinylpyrrolidone (Domínguez 1987). The soluble monosaccharides were extracted in

ULTRA-TURRAX (EtOH dilution (80%) and distilled water (20 percent) and then centrifuged. The ethanol and soluble carbohydrates were removed using the Dubois method (rotary evaporator), and the extracts were analyzed as glucose. The same method was applied to analyze starch. All analyses were hydrolyzed with H₂SO₄ 5N [MM3] [TSE4] [MM5] and analyzed colorimetrically as soluble carbohydrates. The results were expressed in mg glucose g^{−1} lyophilized tissue.

2.7 Bioclimatic parameters

Using the central coordinates of every provenance region (Table 1), bioclimatic data for three decades (1970–2000) were obtained from WorldClim (<https://www.worldclim.org/data/worldclim21.html>). More than 25 parameters were applied, and those more highly correlated with survival were selected: June precipitation (P6, mm); annual precipitation (P, mm); mean minimal temperature in August (T8, °C); annual mean temperature (Tm, °C); mean temperature of warmest quarter (Ts, °C); maximum temperature of warmest month (Tmax, °C); and Martonne aridity index (MAR), estimated as follows:

$$MAR = \frac{P}{Tm + 10} \quad (1)$$

and Rivas-Martínez thermicity index (RV), calculated as follows:

$$RV = 10 \times (Tm + Tmax + Tmin) \quad (2)$$

where *Tmin* is the minimum temperature of the coldest month of each population (°C).

2.8 Statistical analysis

Morphological traits (H, AH, and NB) were analyzed using a mixed model where the population was considered as a random effect. Disease development variables (SWd, EWd, DWd, and W) were analyzed with a mixed model where both the population and the block were considered as random effects. For survival (S), analysis was performed using a binomial generalized mixed model with block and population as random effects. Additionally, so as to evaluate the existence of variation in susceptibility to PWN among geographic groups and populations within these groups, survival was reanalyzed considering the geographic group as a fixed effect and the population nested within geographic group and block as random effects. Lastly, differences among populations for each geographic group were evaluated with population and block as random effects.

All analyses were performed with R (version 4.0.3 Vienna, Austria; R Core Team 2020). For analysis of morphological traits and disease development variables, the *lmer* function from *lme4* package (Bates et al. 2015) was

used. When needed, the variables were transformed to achieve normality and homoscedasticity assumptions. For survival analysis, *glmer* logit function from the *lme4* package was used. The Tukey test was performed to find significant differences among geographic groups. Finally, predicted survival for each population was estimated with the inverse logit function in each model.

In all analyses, the significance of random factors was determined using the likelihood ratio test (*LRT*):

$$LRT = -2 \times (LLR_{reduced} - LLR_{full}) \quad (3)$$

where the *LLR_{reduced}* is the restricted log likelihood of the model without the random effect of interest and *LLR_{full}* is the restricted log likelihood of the whole model.

Nonparametric Kruskal-Wallis test was used to search for nematode density differences among populations in the stem, roots, and total seedling.

Spearman's correlation was estimated to measure phenotypic correlation among all variables using the observed values and BLUP correlation using the population BLUP. The BLUPs for survival were obtained from the mixed model analysis where the geographic group was not considered. We then computed residuals from the mixed model analyses for each trait; these residues do not include the main effects. Using these residuals, the Spearman's correlations were calculated again.

All chemical compounds were analyzed by ANOVA using the two resistance classes and the 5 populations nested within each resistance class as fixed factors.

To analyze bioclimatic parameters, a principal component analysis (PCA) was performed. First, Pearson correlations for bioclimatic parameters, latitude, and survival rate were determined. Variables correlated to

survival were chosen for the PCA analysis. Then, a linear regression was performed with survival rate as the dependent variable and principal components (PC) as independent variables, to determine the relation between PC and survival.

3 Results

3.1 Morphological traits

All morphological traits differed significantly among populations (Table 2). HSFVRG006, PTES06, and populations from the Atlantic coast of the Iberian Peninsula showed the highest values for height variables (H and ΔH). Moreover, PTES06 and PTES04 had the highest number of branches. In contrast, Moroccan populations presented the lowest growth and number of branches (data not shown).

3.2 Survival and disease duration

Highly significant differences were found for wilting symptoms and survival among populations (Table 2). Mean survival at the end of the experiment was 44%, and the mean wilting symptom level was 4.9, ranging by population from PTES20 (100% survival and 2.2 for wilting symptoms) to PTESF (6% survival and 6.56 for wilting symptoms). The Galician populations (PTES01a and PTES01b), PTES09, and both seed orchards were the most susceptible to PWN, with the lowest survival values and the highest symptom levels. In contrast, populations from the south of the Iberian Peninsula presented the highest survival and the lowest symptom levels (Fig. 1).

We found highly significant differences among geographic groups ($\chi^2_{LRT} = 25.94$, $p < 0.001$) and populations within groups ($\chi^2_{LRT} = 18.89$, $p < 0.001$) for survival at the end of the experiment. The ratio population

Table 2 Mean values \pm standard deviations (SD), variance components (σ^2), likelihood ratio significance test (χ^2_{LRT})

Variable	Mean \pm SD	Random effect					
		Populations			Blocks		
		χ^2_{LRT}	σ^2	$p > \chi^2$	χ^2_{LRT}	σ^2	$p > \chi^2$
H ^a	100.8 \pm 20.5	524.99	0.62	<0.001	--	--	--
ΔH ^a	44.2 \pm 12.7	96.31	0.16	<0.001	--	--	--
NB	13.0 \pm 4.5	72.92	2.85	<0.001	--	--	--
SWd ^{a,b}	40.6 \pm 16.2	1.52	2.12 $\cdot 10^{-06}$	0.22	18.74	9.04 $\cdot 10^{-06}$	<0.001
DWd ^{a,b}	25.5 \pm 12.0	0.00	0.00	1.00	3.72	0.01	0.0538
EWd ^{a,b}	66.1 \pm 20.5	2.16	7.33 $\cdot 10^{-07}$	0.14	18.07	2.57 $\cdot 10^{-06}$	<0.001
W ^a	4.9 \pm 2.3	66.83	0.09	<0.001	15.08	0.03	<0.001
S	0.44 \pm 0.50	67.23	1.03	<0.001	11.33	0.24	<0.001

H initial height (cm), ΔH height increment (cm), NB number of branches (no.), SWd and EWd start and end of wilting symptoms (days after inoculation, DAI), DWd duration of wilting symptoms (days), W wilting symptoms (scale 1–7), S survival (scale 0–1)

^a Mean values and their standard deviations were calculated from real data, while analysis values were based on their respective transformations

^b Analysis performed only for provenances with five or more dead seedlings at the end of the assay

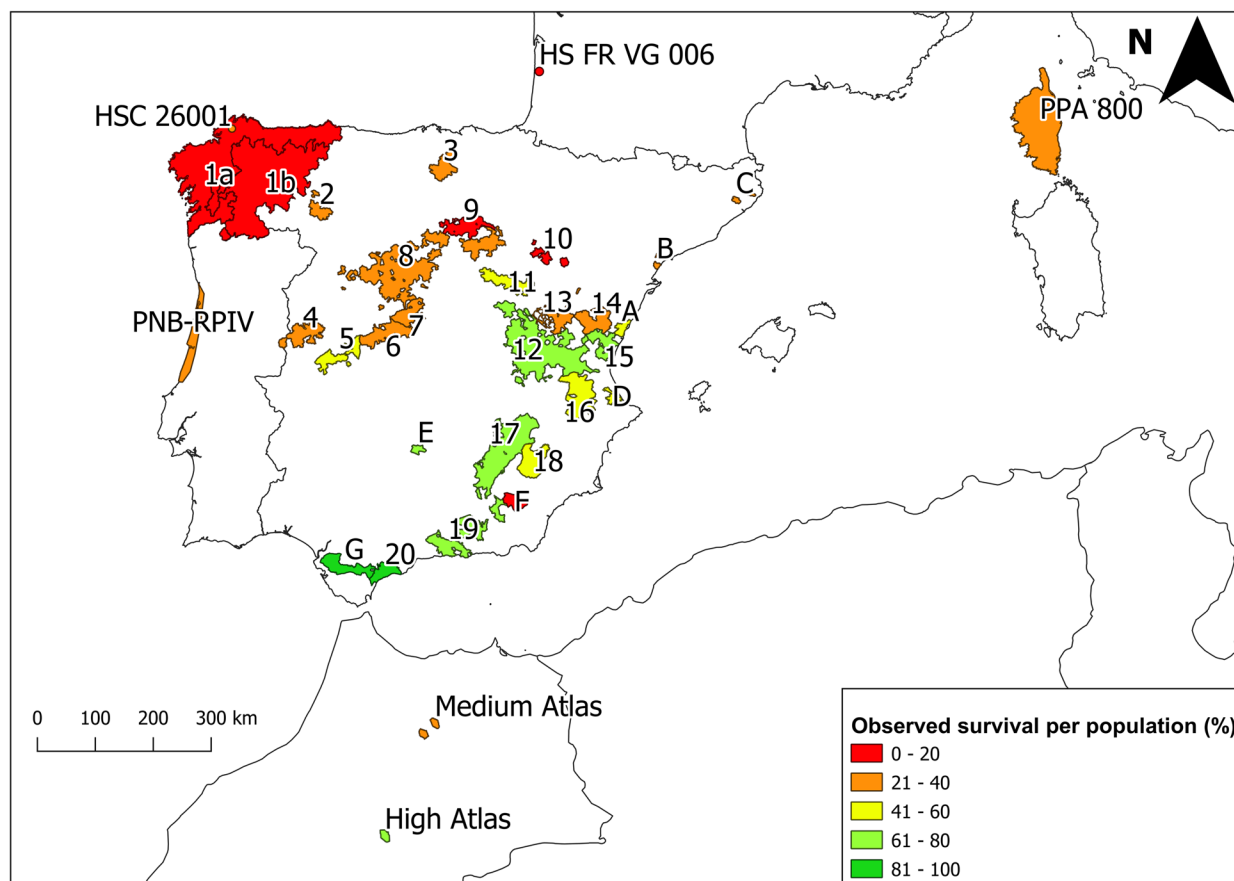


Fig. 1 *Pinus pinaster* populations evaluated in the experiment. The color gradient represents the different intervals of the observed survival rate

variance to residual variance was 0.49 when only population and block were considered, and it decreased to 0.19 when the geographic group was added (data not shown). Hence, a large part of the population variance obtained initially could be explained by the geographic group. The southeast of the Iberian Peninsula was the most resistant geographic group since it showed both the highest best linear unbiased estimator (BLUE) and predicted survival (0.62, Fig. 2). By contrast, the Atlantic coast and Central Iberian Peninsula geographic groups had the lowest predicted survival (0.21 and 0.31, respectively). The predicted survival for the rest of geographic groups was 0.25 for France-Catalonia region, 0.35 for Morocco-Oria region, and 0.39 for Corsica Island. Significant differences among populations within each geographic group were found for all groups, except the Atlantic coast of the Iberian Peninsula populations (Fig. 2).

No differences were found for the start, end, and duration of symptoms for populations (SWd, EWd, and DWd, Table 2). On average, first symptoms started 41 DAI, and seedlings died at 66 DAI.

3.3 Nematode density

Analyses of nematode densities revealed significant differences among populations in roots ($\chi^2=48.33$, $p=0.041$), stem ($\chi^2=55.05$, $p=0.009$), and total seedling ($\chi^2=50.66$, $p=0.025$, Fig. 3). The median number of *B. xylophilus* recovered from the total seedling ranged from 0.1 to 2123.66 nem·gdw⁻¹. At the end of the assay, the vast majority of the surviving seedlings showed mean wilting symptoms below level 3 (92 out of 95) and not more than 22 nem·gdw⁻¹. The only three seedlings that survived with a wilting level of 5 had higher numbers of nematodes (PTES17, 167.9 nem·gdw⁻¹; PTMAATLA, 265.1 nem·gdw⁻¹; and PTES18, 1115.0 nem·gdw⁻¹). No *B. xylophilus* were recovered from control seedlings (Fig. 3).

3.4 Correlations

Phenotypic correlations showed that survival was negatively correlated with all morphological traits, except the number of branches. Nematode densities (NDr, NDs, and NDt) were negatively correlated with survival, and NDs and NDt were also negatively correlated with some

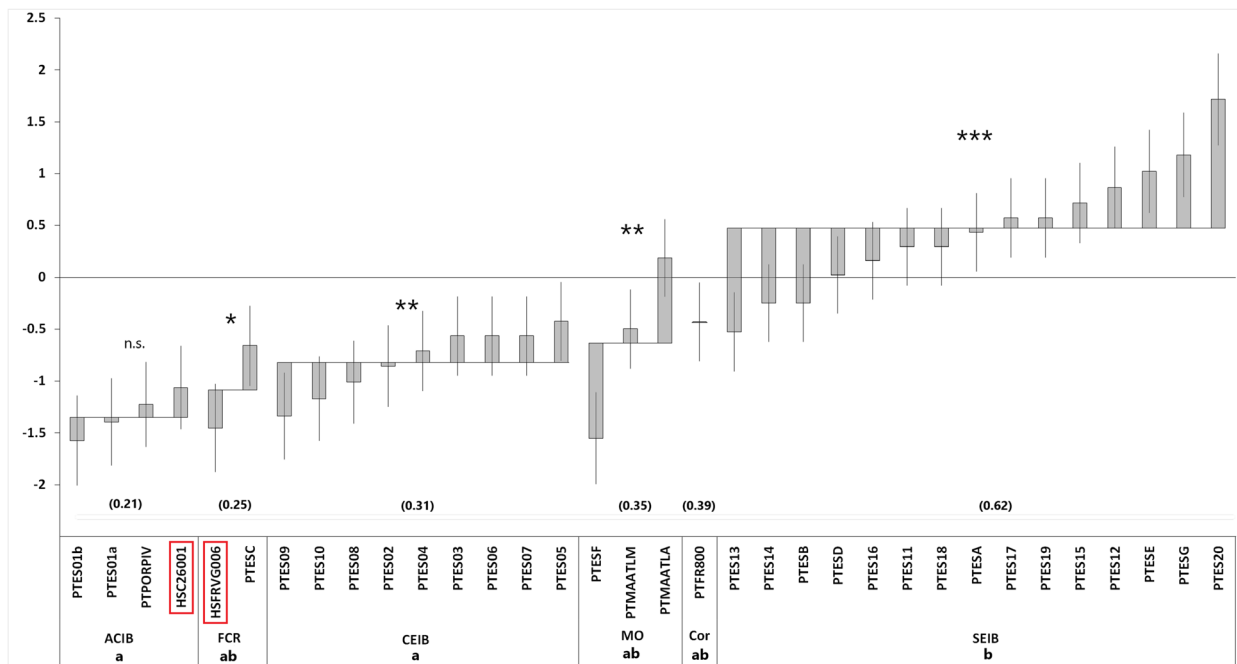


Fig. 2 Survival ranking of populations by geographic group at 133 DAI. Values were calculated from a binomial generalized mixed model with geographic group as a fixed effect and populations within geographic groups and blocks as random effects. The predicted survival for each geographic group is shown in brackets. Solid horizontal line represents best linear unbiased estimates (BLUEs) of each geographic group on the logit scale. Gray bars represent best linear unbiased predictors (BLUPs) of each population on the logit scale. Vertical lines are standard errors of BLUPs. Seed orchards are highlighted with a red rectangle. ACIB, Atlantic coast of the Iberian Peninsula; CEIB, Central Iberian Peninsula; FCR, France-Catalonia region; MO, Morocco-Oria region; Cor, Corsica Island; SEIB, southeast the Iberian Peninsula

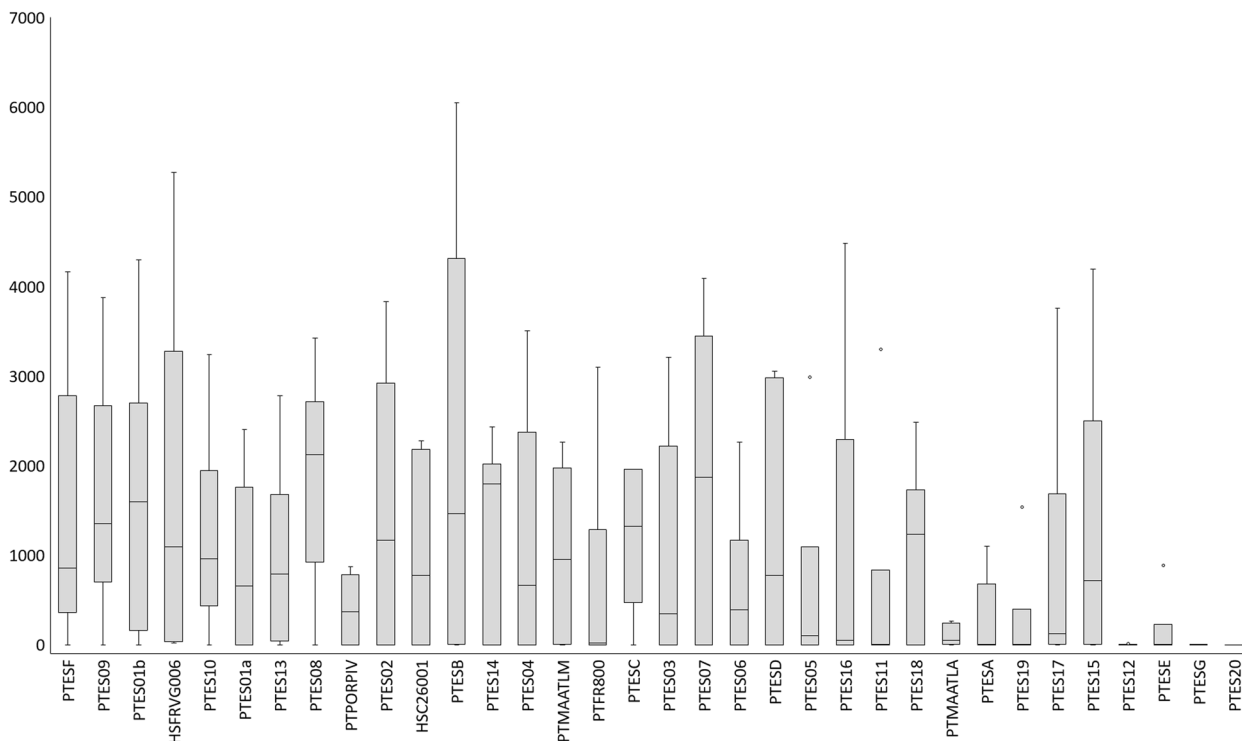


Fig. 3 *Bursaphelenchus xylophilus* per gram of dry wood of the entire plant (roots, stem, and needles) by population. Circles represent outliers, and horizontal line represents median values

disease duration variables (SWd and EWd). BLUP correlations showed that survival was negatively correlated with all morphological traits. SWd was positively correlated with EWd (Table 3). Regarding the residual correlations, we found no significant correlation for any trait except for the number of branches that showed a slight positive correlation with survival (0.08, p -value < 0.05). Thus, there is no environmental effect identified affecting two traits at the same time.

3.5 Chemical compounds

Significant differences between resistant classes were found for constitutive total polyphenols, lipid-soluble substances, and condensed tannins. Moreover, we

observed significant differences in populations within resistant groups for nitrogen, total polyphenols, condensed tannins, and soluble monosaccharides (Table 4). Populations in the resistant class had lower levels of constitutive total polyphenols, lipid-soluble substances, and condensed tannins than those in the susceptible class. The summary statistics for each chemical compound in each resistant class and population was shown in Table 5 in Appendix.

3.6 Relation with bioclimatic variables

Only one principal component (PC) was significant in the PCA regression with survival (PC1; t -value = 4.37; $p \leq 0.001$; Fig. 4). This PC had a positive relation with

Table 3 Spearman’s correlation matrix of BLUPs (above the diagonal) and phenotypic (below the diagonal) coefficients between pairs of traits

	<i>W</i> ^a	<i>S</i>	<i>H</i> ^a	ΔH ^a	<i>NB</i>	<i>SWd</i> ^{a,b}	<i>Ewd</i> ^{a,b}	<i>DWd</i> ^{a,b}	<i>NDR</i> ^c	<i>NDs</i> ^c	<i>NDt</i> ^c
<i>W</i> ^a		-0.98***	0.37*	0.38*	0.39*				-	-	-
<i>S</i>	-0.93***		-0.36*	-0.34*	-0.39*	-	-	-	-	-	-
<i>H</i> ^a	0.12**	-0.13**		0.78***	0.56***				-	-	-
ΔH ^a		-0.08*	0.76***		0.35*				-	-	-
<i>NB</i>			0.23***						-	-	-
<i>SWd</i> ^{a,b}	-0.50***	-					0.80***		-	-	-
<i>Ewd</i> ^{a,b}	-0.55***	-			0.11*	0.80***			-	-	-
<i>DWd</i> ^{a,b}	-0.23***	-					0.59***		-	-	-
<i>NDR</i> ^c	0.85***	-0.84***								-	-
<i>NDs</i> ^c	0.87***	-0.85***				-0.23*	-0.26**		0.80***		-
<i>NDt</i> ^c	0.87***	-0.85***				-0.20*	-0.25*		0.87***	0.97***	

NDR nematode density in roots, *NDs* nematode density in stem, *NDt* nematode density in the total seedling, *H* initial height (cm), ΔH height increment (cm), *NB* number of branches (no.), *SWd* and *Ewd* start and end of wilting symptoms (days after inoculation, DAI), *DWd* duration of wilting symptoms (days), *W* wilting symptoms (scale 1–7), *S* survival (scale 0–1)

Levels of significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

^a BLUP correlations were performed with BLUPs obtained only from the analysis where populations and blocks were considered, $N = 34$

^b Correlations between the variables of disease development and survival (*S*) were not calculated

^c BLUP correlations for nematode density were excluded because we did not obtain BLUPs for their analyses

Table 4 Results of ANOVA, LS Mean values \pm standard deviations (SD) by resistance classes for constitutive chemical compounds, *F* ratios (degrees of freedom as subscript), and p -values are shown

Variable	Resistant classes		Provenances within resistance		Resistant class	Susceptible class
	<i>F</i> _{1,30}	<i>P > F</i>	<i>F</i> _{8,30}	<i>P > F</i>	LS Mean \pm SD	LS Mean \pm SD
N (%)	0.027	0.928	11.452	< 0.001	0.89 \pm 0.18	0.90 \pm 0.11
Pol (mg·g⁻¹)	9.561	< 0.01	4.207	< 0.01	40.97 \pm 9.63	47.49 \pm 7.52
Lip (mg·g⁻¹)	16.741	< 0.001	0.896	0.532	26.93 \pm 5.75	33.42 \pm 4.04
Tan (mg·g⁻¹)	27.234	< 0.001	3.960	< 0.01	29.00 \pm 6.57	38.64 \pm 8.21
Mon (mg·g⁻¹)	0.136	0.715	2.806	0.019	70.36 \pm 9.50	71.38 \pm 10.98
Sta (mg·g⁻¹)	3.774	0.062	2.020	0.078	81.98 \pm 17.97	72.91 \pm 14.41

Bold type indicates significant differences

N nitrogen, *Lip* lipid-soluble substances, *Pol* total polyphenols, *Tan* condensed tannins, *Mon* soluble monosaccharides, *Sta* starch

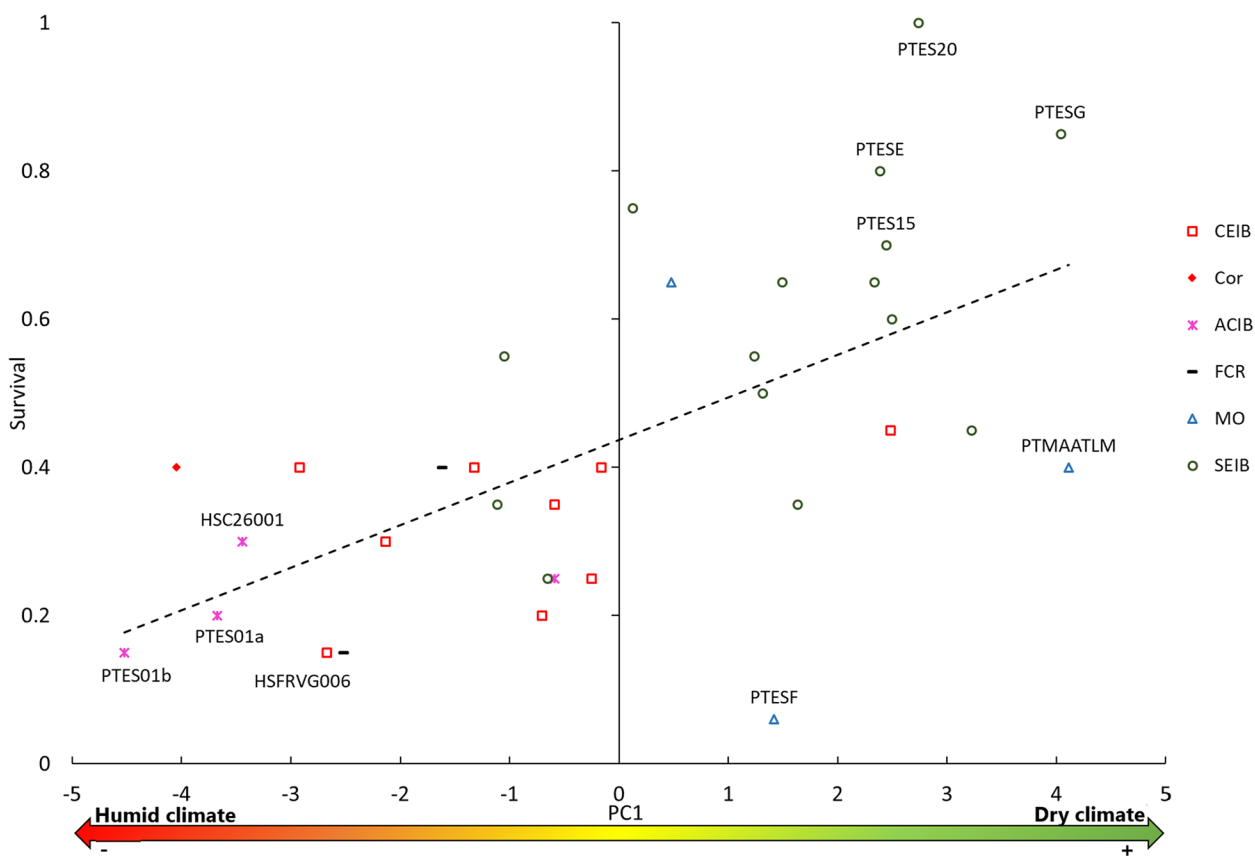


Fig. 4 Principal component analysis defined by one component and represented as a ranking of aridity climate conditions. ACIB, Atlantic coast of the Iberian Peninsula; CEIB, Central Iberian Peninsula; FCR, France-Catalonia region; MO, Morocco-Oria region; Cor, Corsica Island; SEIB, southeast the Iberian Peninsula; dashed black line represents the main survival trend ($R^2=0.37, p\text{-value} < 0.001$)

survival and explained 63.37% of the climate variables. It was positively associated with temperature variables (T_m , T_8 , T_s , T_{max} , and RV) and negatively associated with precipitation and a geographical variable (P , P_6 , MAR , and latitude). Specifically, populations coming from places with high temperatures in summer and low precipitation (especially in summer) had higher survival after nematode inoculation.

4 Discussion

Our study confirms the large variability of *P. pinaster* susceptibility to *B. xylophilus* among populations and also among geographic groups, with the largest resistance in southern Iberian populations. Considering the geographic groups, there were differences among populations within all groups, except the Atlantic coast of the Iberian Peninsula group. The relation found in this study between survival and morphological traits agree with other authors, since survival is negatively related to growth rate and the number of nematodes (Menéndez-Gutiérrez et al. 2017b, 2018). Besides, we have observed an association between PWN resistance and

concentration in needles of some constitutive chemical compounds, such as lipid-soluble substances. Lastly, as other authors reported for other species and diseases (Romeralo et al 2016), we found a clinal pattern in the PWN susceptibility among populations that was related to climate at the population site. Thus, a northwest to southeast gradient of survival was observed in the Iberian Peninsula.

The highly significant differences in resistance to PWN among populations found in this study corroborate the results reported by Menéndez-Gutiérrez et al. (2017b) for six *P. pinaster* provenances. Other pine species have also shown differences among provenances for resistance to PWN, such as *P. densiflora* (Fujimoto et al. 1989; Fuyuan et al. 1996), *Pinus massoniana* Lamb. (Okamura et al. 2010), and *P. thunbergii* (Matsunaga et al. 2016). However, Hopf-Biziks et al. (2016) found no variation in resistance to this nematode among several German *Pinus sylvestris* L. provenances, since all of them displayed a 100% susceptibility at the end of the experiment. Currently, provenance-based variations in resistance to pathogens are commonly found in pine species.

Examples include *P. halepensis*, *Pinus contorta* Douglas, *P. sylvestris*, and *Picea abies* L. to *Gremmeniella abietina* (Hansson 1998; Romeralo et al. 2016), *P. sylvestris* to *Dothistroma septosporum* (Perry et al. 2016), or *Pinus tecunamii* Eguiluz and Perry. and *Pinus patula* Schl. and Cham. to *Fusarium circinatum* Nirenberg and O'Donnell (Hodge and Dvorak 2007).

The large variation found in resistance to PWN among pine populations in Spain is an important finding and should be taken into account to more accurately predict the future impact of PWD there. De la Fuente et al. (2018) and De la Fuente and Saura (2021) developed disease-spread prediction models considering the same level of resistance for all *P. pinaster* populations. Currently, testing the resistance of adult populations in field trials is forbidden in Spain since *B. xylophilus* is a quarantined organism. For this reason, it is difficult to determine whether the resistance observed in greenhouse experiments can be extrapolated to field trials. Nevertheless, different studies reported a similar PWN susceptibility in young seedlings than in adult trees in some pine species (Hopf-Biziks and Schröder 2019; Linit and Tamura 1987; Donald et al. 2003; Daub 2008). Given this situation, introducing the resistance levels of each population in these models could provide us a rough prediction that would help predict where central, regional, and local governments should be more concerned about the spread of PWD.

Our results also highlight the high differentiation among geographic groups for resistance to PWN, even though differences among populations within regions remain significant. Other authors, such as Menéndez-Gutiérrez et al. (2017b), Hurel et al. (2021), and De Miguel et al. (2022), also reported similar results for different pathogens when they grouped provenances into these geographic groups, based on Jaramillo-Correa et al. (2015). Analyzing each geographic group separately, all of them showed significant differences among populations for survival with the exception of the Atlantic coast of the Iberian Peninsula group. Populations within each geographic group share the same evolutionary history (Jaramillo-Correa et al. 2015), but local adaptations within groups can affect the interaction between the population and multiple biotic and abiotic traits (Ramírez-Valiente et al. 2022). Some groups, such as Central Iberian Peninsula, southeast Iberian Peninsula, and Morocco-Oria region, have a great environmental heterogeneity which leads to small-scale local adaptations, as have been highlighted for growth traits (Alía et al. 1997; Archambeau et al. 2021; Hurel et al. 2021; Sugai et al. 2023). In the case of the France-Catalonia region group, the Pyrenees Mountain range is a physical barrier separating both populations, probably resulting in different specific adaptations, likely affecting the

PWN resistance levels. On the contrary, the low environmental heterogeneity of the Atlantic coast of the Iberian Peninsula group (Archambeau et al. 2021) could explain the lack of differentiation in PWN resistance among populations of this group.

The significant differences found among populations for nematode density in this study are not in agreement with the results of Menéndez-Gutiérrez et al. (2017b), who found no differences among provenances. However, the number of provenances considered was different between their study and ours. We quantified nematode density in thirty-four populations from 32 provenances, while their study included six provenances. Since we increased the number of populations analyzed, the possibility of finding significant differences also increased. Therefore, the higher southern Iberian populations, which were the most resistant, had the lowest nematode densities. At the end of the assay, our findings corroborated those of Son and Moon (2013); lower symptoms in surviving seedlings were accompanied by lower nematode density, usually below 1 nem-gdw⁻¹. As expected, and as other authors have reported (Menéndez-Gutiérrez et al. 2017b, 2018), nematode density showed a negative correlation with survival and a positive correlation with symptomatology.

Growth traits were negatively correlated to survival, i.e., resistance to PWN. It seems that the lower the growth of a population, the greater its constitutive defenses (Matteo and Voltas 2016). While Carrasquinho et al. (2018) found positive relationships among survival and height in Portuguese half-sib *P. pinaster* families, other authors have demonstrated that greater height implies higher mortality (Menéndez-Gutiérrez et al. 2018). Similarly, the number of branches limits the spread of PWN through the host (Hakamata et al. 2013; Menéndez-Gutiérrez et al. 2017b, 2018). However, we observed that the populations with the lower number of branches had the higher survival. Breeding programs usually consider these traits as selection criteria. In comparison, the Galician seed orchard showed higher survival but lower growth rate than the French one. To improve resistance to PWD in both programs and avoid loss of growth yields, controlled cross-breeding with the most PWN-resistant populations may provide interesting options for breeding programs. Likewise, although the disease resistance was not considered in the Australian breeding program for *P. pinaster*, this program has included several provenances since its inception to improve several growth traits (Butcher 2007).

Despite the fact that PWN does not directly affect needles, constitutive defenses were studied in this tissue as a proxy, since several studies have already related the constitutive chemical compounds in needles with those in phloem and xylem (Wainhouse et al. 2000; Moreira et al.

2012; Alonso Santos et al. 2013, 2021). In addition, some authors already reported a significant relation between the susceptibility to PWN and the chemical composition in needles in *P. massoniana* (Zou et al. 2000) and *P. radiata* (Menéndez-Gutiérrez et al. 2021). The high concentration of total polyphenols, tannins, and lipid-soluble substances found in needles of susceptible populations is in agreement with the results reported by Zas et al. (2015) where the provenances with higher concentrations of total polyphenolic compounds had a higher incidence of nematode migration through branches in *in vitro* experiments. Conversely, even though it has not been studied in our work, it would be possible that resistant populations might have developed higher levels of induced defenses, since studies performed on coniferous species suggest that lower levels of constitutive defense in needles are related to a higher response of induced defenses (Moreira et al. 2014). Besides, the period of time between the attack and the induction of defenses is known to be a key factor against some diseases (Ganthaler et al. 2017). The relation between the level of total polyphenols and tannin content to PWN resistance is different at the species and provenance level. Our results suggest that resistant *P. pinaster* provenances have lower levels of these compounds than susceptible provenances, while resistant pine species show higher levels than susceptible pine species (Menéndez-Gutiérrez et al. 2017a; Pimentel et al. 2017b, a; Canas et al. 2021). Moreover, other authors have highlighted the important role of specific phenolic compounds as constitutive defenses in other coniferous species (Nunes da Silva et al. 2013; Ganthaler et al. 2017). Regarding the rest of chemical compounds, we did not find differences among resistance classes for nitrogen, monosaccharides, and starch. However, Alonso Santos et al. (2021), working at species level, reported higher levels of nitrogen and starch in needles of resistant pine species.

Even though differences were found in polyphenolic compounds and tannins concentrations, the wide variation observed among populations within resistance groups and the small number of populations represented make it difficult to establish for detecting resistant populations based on levels of these chemical compounds. Clearer results were obtained for liposoluble substances, and their content in needles could be a useful approach to discard the most susceptible populations. Nevertheless, since populations other than those analyzed can display different behaviors, these results must be considered cautiously. Further analysis of chemical compounds should be done in order to advance in this field.

In general, we observed that *P. pinaster* susceptibility varied according to the geographical origin of each

population, as other studies have reported for other species (Hansson 1998; Hodge and Dvorak 2007; López-Goldar et al. 2015; Menéndez-Gutiérrez et al. 2017b; Romeralo et al. 2016). Such differences in susceptibility to PWN may be due to how the different populations have adapted to their respective climates, as occurred with *P. sylvestris* provenances and resistance to *D. septosporum* (Perry et al. 2016). Indeed, Menéndez-Gutiérrez et al. (2017b) suggested that susceptibility could be related to the autumn precipitation and mean maximum temperature during summer at the original provenance sites. Different temperatures and precipitations among the different locations of each population can generate a significant differentiation in terms of adaptive traits (Correia et al. 2014). For example, *P. pinaster* response to drought stress varies depending on the origin of the population (Corcuera et al. 2012; Gaspar et al. 2013).

Populations originating from arid climates showed higher survival and lower growth traits than those populations originating from humid and temperate climates. The resource availability hypothesis supports our results since stressful environments with low-resource availability favor plants with higher levels of defenses and lower growth rates. Conversely, plants adapted to low-stress environments with ample resource availability tend to prioritize growth over defense mechanisms, resulting in lower disease resistance (Coley et al. 1985; Endara and Coley 2011). We suggest that adaptations to more arid origins may be associated with greater resistance to PWD. Future research lines could be developed to identify those possible adaptations to aridity, whether chemical or physical, may be associated with resistance to PWN. This is especially interesting in the context of climate change and rising temperatures.

5 Conclusion

The variation in resistance to PWN found in populations of *P. pinaster* was high. In addition, part of this variation can be explained by geographic groups. Both the height traits and number of branches are negatively correlated to resistance to PWN. Some constitutive chemical defenses found in this work, such as the constitutive level of lipid-soluble substances, could be a helpful reference for selecting the putatively most resistant *P. pinaster* populations. The adaptations to the climate of origin of each population affect the resistance to PWN. In addition, populations adapted to harsher, arid climates are more resistant than populations adapted to climates with less water stress and milder temperatures.

Appendix

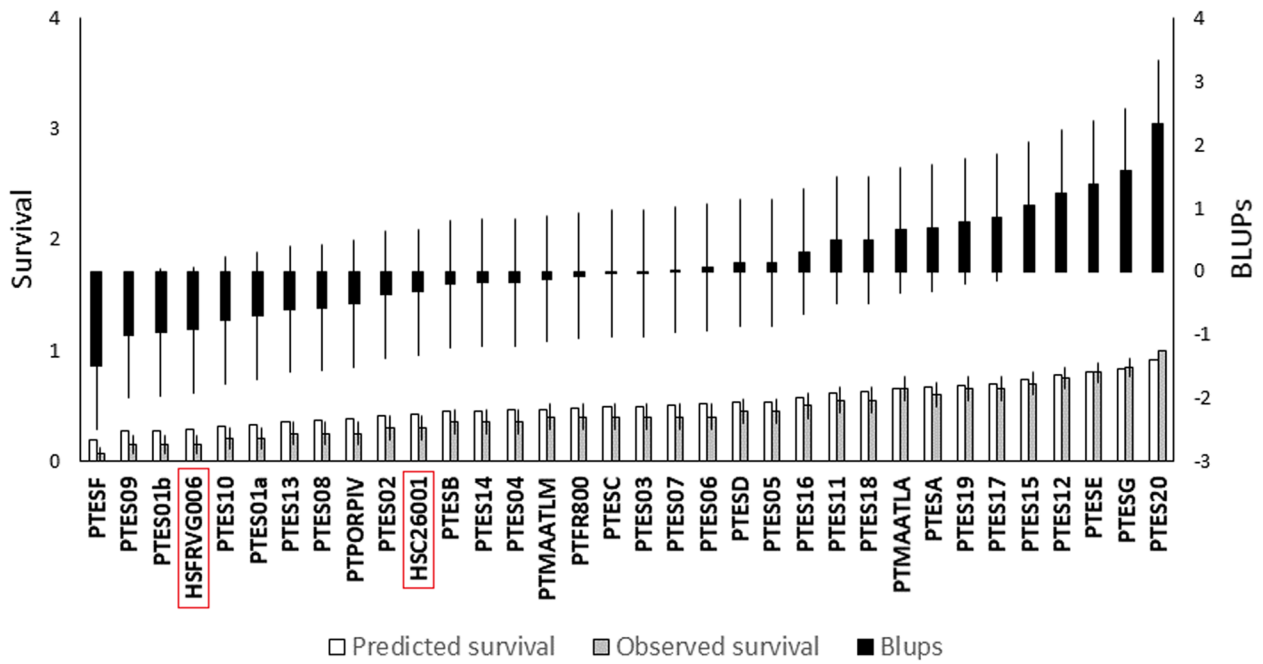


Fig. 5 Black columns represent BLUPS (on the logit scale, right axis), white columns show predicted survival (scale 0–1, left axis), and gray columns indicate observed survival (scale 0–1, left axis of the graphic) for each population. Values were calculated from a binomial generalized mixed model for survival at 133 DAI with population and block as random effects. Vertical lines are standard errors of each variable

Table 5 Statistical summary for chemical compounds in needles by resistance classes

Resistant class	Population	Sta (mg·g ⁻¹)					Lip (mg·g ⁻¹)					Mon (mg·g ⁻¹)					N (%)					Pol (mg·g ⁻¹)					Tan (mg·g ⁻¹)				
		μ	± SD	Min	Max	μ	± SD	Min	Max	μ	± SD	Min	Max	μ	± SD	Min	Max	μ	± SD	Min	Max	μ	± SD	Min	Max	μ	± SD	Min	Max		
R	PTES12	73.04	± 6.84	65	81.69	28.4	± 9.2	17.2	37.6	69.8	± 3.9	64.8	73.2	1.1	± 0.1	1.05	1.23	44.2	± 10.1	36.2	58.1	30.9	± 7.3	25.5	41.5						
	PTES15	86.94	± 9.91	77.8	100.9	30.4	± 2.7	28.4	34.4	75.2	± 6.58	70.1	84.8	1	± 0.1	0.94	1.08	39.2	± 5.79	34.5	47.5	26.9	± 4.9	23.7	34.1						
	PTES20	83.55	± 13.5	72.4	103.2	21.7	± 5.2	17.4	27.7	76.8	± 9.68	70.3	91.2	0.8	± 0.1	0.69	0.88	32	± 9.73	21.5	40.3	23.5	± 5.7	17.9	30.2						
	PTESE	100.4	± 27.3	68	134.3	27.6	± 4.1	22.6	31.7	70.5	± 3.89	65.7	74.9	0.7	± 0.1	0.58	0.78	52.2	± 4.08	48.3	58	36.6	± 4.9	31	42.8						
	PTESG	66.01	± 6.35	60.5	74.31	26.6	± 4.3	23.9	32.9	59.6	± 12.7	46.3	72.3	0.9	± 0.1	0.77	0.91	37.3	± 5.34	32	44.3	27.1	± 2.6	24.2	30.4						
S	PTES01a	77.51	± 13.7	59.4	92.03	33.8	± 2	31	35.7	79.6	± 4.99	75.1	84.6	0.8	± 0.1	0.76	0.87	45.1	± 5.21	39.3	51.4	37	± 5.2	30.3	42.9						
	PTES01b	71.59	± 15.2	49.1	82.28	32.1	± 3.6	28.1	36.9	66.9	± 16.4	42.4	77.2	0.8	± 0.1	0.8	0.9	45.1	± 9.62	36.8	55.1	36.6	± 9.1	29.4	49.5						
	PTES09	78.83	± 18.4	64.4	103.8	34.5	± 5.1	27.3	39.4	76.5	± 1.43	75.4	78.6	0.9	± 0.2	0.71	1.08	50.2	± 4.49	43.6	53.6	45	± 4.1	39.3	48.5						
	PTES10	74.69	± 9.93	61.5	84.42	33	± 5	27.2	39.5	73.8	± 7.84	63.6	82.3	0.9	± 0.1	0.86	1.01	56.2	± 2.12	54.2	58.6	45.1	± 7.3	36	51.5						
	PTESF	61.91	± 14.7	50.6	83.49	33.7	± 5.5	27.5	40.5	60.2	± 8.84	50.5	67.9	1	± 0.1	0.92	1.1	40.8	± 5.1	34.9	46.3	29.5	± 4.5	25.5	35.1						

(R resistant class, S susceptible class) and population. μ mean, ±SD standard deviations, Min minimum observed value, Max maximum observed value, N nitrogen, Lip lipid-soluble substances, Pol total polyphenols, Tan condensed tannins, Mon soluble monosaccharides, Sta starchAcknowledgements

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Code availability

The custom code and/or software application generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Conceptualization, RD; methodology, RD; investigation, ETS and LV; data curation, ETS and LV; formal analysis, ETS; writing — original draft preparation, ETS; writing — review and editing, ETS, MMG, LV, and RD; funding acquisition, MMG and RD; and supervision, RD. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. The custom code and/or software application generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations**Ethics approval and consent to participate**

Not applicable.

Consent for publication

All authors gave their informed consent to this publication and its content.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Centro de Investigación Forestal de Lourizán, Ctra. de Marín, km. 4, Pontevedra 36153, Spain. ²Fundación Arume, Camino Valdeiribas, 93C, Santiago de Compostela 15707, Spain.

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