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Variation in pinewood nematode susceptibility among *Pinus pinaster* Ait. provenances from the Iberian Peninsula and France

María Menéndez-Gutiérrez[1] (iD) · Margarita Alonso[1] · Gabriel Toval[1] · Raquel Díaz[1,2]

Abstract

· **Key message** ***Pinus pinaster*** **Ait. susceptibility to pinewood nematode significantly differed among provenances, and the two Atlantic provenances of the Iberian Peninsula being the most affected. However, significant provenance × environment interaction was found. Provenance susceptibility was related to basal diameter, number of branches and oleoresin flow, and some climatic parameters.**

· **Context** The pinewood nematode *Bursaphelenchus xylophilus*, native to North America, is an important pest affecting pine forests throughout Eurasia. In Europe, it has been detected in Portugal and Spain and is primarily associated with *Pinus pinaster*, an important Mediterranean tree species.

· **Aims** We have investigated the differences in susceptibility among several *P. pinaster* provenances in the Iberian Peninsula and France, as well as their relationship to certain growth traits and physiological parameters.

· **Methods** Three independent inoculation tests were performed on 3 to 4-year-old trees, followed by assessment of growth traits and physiological variables, along with time course destructive sampling for nematode quantification.

· **Results** The results showed significant differences among provenances for almost all growth traits, wilting, and mortality, though a significant provenance × environment interaction was also detected. Two Atlantic provenances, Noroeste-Litoral and Leiria, displayed the largest susceptibility to pinewood nematode. Changes in susceptibility to *B. xylophilus* between experiments were influenced by temperature and seasonality. Autumn precipitation and mean maximum temperature during summer at the original provenance sites could be related to provenance susceptibility.

· **Conclusion** Noroeste-Litoral and Leiria were the most disease-affected provenances. This study emphasizes the need for further research on how tree growth stage influences susceptibility and on the possibility of cross-breeding among provenances.

Keywords Pine wilt disease (PWD) · Chlorophyll fluorescence · Oleoresin flow · Water potential · Growth traits · Nematode density

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1 Introduction

Pine wilt disease (PWD) is caused by the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle. It is transmitted by *Monochamus* spp. (Coleoptera: Cerambycidae) into healthy trees during maturation feeding or into decaying trees through oviposition (Linit 1988). This native North American pathogen became an important threat over a century ago in Japan, where it was first described in 1905 (Yano 1913). It subsequently spread to other Asian countries (China, Korea, and Taiwan), devastating native *Pinus thunbergii* Parl., *Pinus densiflora* Sieb. & Zucc., *Pinus massoniana* Lamb., and *Pinus koraiensis* Sieb. & Zucc pine forests. In Europe, pinewood nematode (PWN) is regulated as a quarantine pest; it was first detected in the Setubal Peninsula, Portugal, in 1999 (Mota et al. 1999) and has since affected all of continental Portugal and Madeira (Fonseca et al. 2012). In Spain, three out of the five outbreaks that have occurred to date are still active (Abelleira et al. 2011; Zamora et al. 2015; Xunta de Galicia 2016). Currently, pine wilt disease is considered one of the most important pine diseases worldwide (Webster and Mota 2008). It causes browning of needles, accompanied by early reduction of oleoresin flow (Mamiya 1983) and water conduction dysfunction due to tracheid cavitation and embolism (Utsuzawa et al. 2005). When this occurs, photosynthesis shuts down, water potential and transpiration decrease, and the tree dies within a few months or even weeks (Fukuda 1997; Ikeda and Kiyohara 1995).

Tree decline after PWN infection can be evaluated through different indicators of tree physiological and functional status. Chlorophyll fluorescence is used as an early indicator of damage to the photosynthetic apparatus (Melakeberhan et al. 1991; Ali and Honermeier 2013). Oleoresin flow is also widely used for early detection of PWD, since decreased resin production is the first detectable internal symptom after nematode infection (Ikeda and Kiyohara 1995; Futai 2003). Water potential provides another variable for determining the physiological state of trees by measuring the water status of the tree as pinewood nematodes damage the water-conducting pathway (Melakeberhan et al. 1991; Fukuda 1997).

Pine species vary a great deal in their response to PWN infection. *P. taeda* L. and *P. strobus* L. (Mamiya 1983), as well as *P. halepensis* Mill. and *P. canariensis* Chr. Sm ex DC. (Menéndez-Gutiérrez et al. 2017b), are highly tolerant. In contrast, *P. sylvestris* L. (Hopf-Biziks et al. 2016), *P. thunbergii*, *P. densiflora*, and *P. luchuensis* Mayr. (Mamiya 1983) show extreme susceptibility, while others like *P. radiata* D. Don and *P. pinaster* Ait. are moderately susceptible to PWN (Nunes da Silva et al. 2015; Menéndez-Gutiérrez et al. 2017b). Maritime pine (*P. pinaster*) is native to forests of the western Mediterranean basin, where it has high ecological and economic impact. This species presents fragmented distribution and high genetic variation among populations in studies using molecular markers and quantitative traits (González-Martínez et al. 2002; Eveno et al. 2008; Prada et al. 2016). The highest genetic differentiations among provenances were found in morphological and adaptive traits (Danjon 1994; Alía et al. 1995; Correia et al. 2004), which are explained to a certain extent by natural selection for frost and drought adaptation (Danjon 1994; Correia et al. 2010).

Concerning intraspecific variation in relation to resistance against pathogens, some maritime pine provenances from the Western Mediterranean were reported to be resistant to the scale insect *Matsucoccus feytaudi* Duc. (Di Matteo and Voltas 2016). Genetic intrapopulation variation was also observed in *P. pinaster* resistance to *Hylobius abietis* L., *Dioryctria sylvestrella* Ratz., and *Fusarium circinatum* Nirenberg and O'Donnell (Kleinhentz et al. 1998; Zas et al. 2005; Vivas et al. 2012). As far as we know, there is little information on the susceptibility of European pines to *B. xylophilus* at provenance level. Hopf-Biziks et al. (2016) found no variation among *P. sylvestris* provenances, as all suffered 100% mortality. However, *P. pinaster* is known to have higher genetic variation among populations than *P. sylvestris* (Eveno et al. 2008). Using an in vitro bioassay, Zas et al. (2015) reported differences in migration ability among *P. pinaster* provenances. This could indicate differences in susceptibility, since nematode migration and multiplication ability seem to be involved in PWD development (Ichihara et al. 2000; Son and Moon 2013). These results would have to be confirmed by a tree inoculation test.

In the present work, we sought to determine the differences in susceptibility to *B. xylophilus* among *P. pinaster* provenances. Several Mediterranean provenances were selected from central (Sierra de Gredos), north-central (Montaña de Soria-Burgos), and southeastern Spain (Sierra de Oria), as well as Atlantic provenances from Portugal (Leiria), southwestern France (Mimizan), and northwestern Spain (Noroeste-Litoral). We specifically aimed to (1) determine variation in susceptibility to PWN among these *P. pinaster* provenances; (2) detect significant relationships between PWD and certain growth and physiological traits, in order to identify traits related to tolerance (pre-inoculation traits) and other traits that would improve our understanding of the disease and might facilitate early detection (physiological traits measured after inoculation); and (3) explore local adaptation that might be related to *P. pinaster* tolerance to *B. xylophilus* by analyzing correlations between disease traits and geoclimatic data from the place of origin.

2 Materials and methods

2.1 Plant material and experimental design

We tested six *P. pinaster* provenances: Noroeste-Litoral (NOR), Montaña Soria-Burgos (SBUR), Sierra de Gredos

(GRE) and Sierra de Oria (ORI) in Spain, Leiria (LEI) in Portugal and Mimizan (MIM) in France. These were chosen in an attempt to represent most of the latitudinal distribution range and different climatic conditions in which this species naturally occurs. NOR, MIM, and LEI are characterized by a moist, temperate climate and low altitude; GRE and ORI have low summer precipitation and high summer maximum temperatures, whereas SBUR has very low minimum temperatures in winter. These provenances can be grouped into four major genetic groups according to Jaramillo et al. (2015); NOR and LEI are in the same genetic group (Atlantic Iberian Peninsula), and SBUR and GRE are together in another one (central Spain) (Table 1).

We performed three inoculation tests, one in 2014 and two in 2015, under controlled greenhouse conditions. All experiments were arranged in a randomized complete block design with four blocks and two treatments (inoculated, control). The plant material was the same and belonged to the same seed lot in the three experiments. Seeds were grown in 4-l plastic pots in the nursery beds of the Lourizán Forest Research Center (Xunta de Galicia, 42° 24′ 35″ N 8° 00′ 12″ W, Pontevedra, Spain). The soil used was a mix of peat moss and vermiculite (9:1 v/v), and trees were watered twice a week according to their demand.

In experiment 1 (June 2014), we used 3-year-old trees, nine trees per block and provenance, eight of which were inoculated with *B. xylophilus* and one with distilled water, to serve as the control. A total of 216 trees were inoculated. Daily mean greenhouse temperature was 22.5 °C, and temperature oscillated between 18.1 and 26.9 °C. The duration of this experiment was 58 DAI (days after inoculation), which was shorter than planned due to an electrical failure in the greenhouse.

In experiment 2 (April 2015), we tested 4-year-old trees, using the same number and arrangement as in experiment 1. Daily mean greenhouse temperature was 25.1 °C, and temperature oscillated between 24.0 and 26.2 °C. The duration of this experiment was 104 DAI.

In experiment 3 (July 2015), we used 4-year-old trees, inoculating three trees with *B. xylophilus* and one control tree with distilled water, per provenance and block. A total of 96 trees were inoculated in this experiment. Daily mean greenhouse temperature was 22.7 °C, and temperature oscillated between 20.7 and 24.6 °C. The duration of the experiment was 136 DAI.

2.2 Pinewood nematode culture and inoculation procedure

We used the *B. xylophilus* isolate extracted from a *P. pinaster* tree in As Neves (Pontevedra) in 2010 for inoculation in the three experiments. The isolate was maintained on a fungal mat of non-sporulating *Botrytis cinerea* Pers., previously cultured on potato dextrose agar (PDA) medium at 25 °C and 100% relative humidity in the dark. We prepared a 300 μl inoculum

Table 1　Geographic and climatic parameters of *P. pinaster* provenance origins. Climatic data were generated from the ClimateEU v4.63 software package

Parameter	Provenances					
	Noroeste-Litoral (NOR)	Leiria (LEI)	Mimizan (MIM)	Oria (ORI)	Soria-Burgos (SBUR)	Gredos (GRE)
Genetic groups[a]	Atlantic Iberian Peninsula		Atlantic France	Southeastern Spain	Central Spain	
Country	Spain	Portugal	France	Spain	Spain	Spain
Latitude	42° 49′ N	39° 47′ N	44° 80′ N	37° 31′ N	41° 50′ N	40° 11′ N
Longitude	8° 27′ W	8° 57′ W	1° 18′ W	2° 21′ W	3° 30′ W	5° 60′ W
Altitude (m)	150	20	37	1123	1096	753
Mean annual temperature (°C)	14.9	16.1	14.1	13.5	10.7	14
Mean warmest month temperature (°C)	21.8	20.9	21.3	23.4	20.4	24.3
Continentality index (°C)	12.2	9.7	13.7	17.7	18.1	19.1
Autumn precipitation (mm)	445	298	402	147	160	467
Mean annual precipitation (mm)	1313	810	1211	495	510	121
Mean summer precipitation (mm)	333	149	464	147	197	322
No. of frost-free days	362	365	346	319	279	31
Mean summer maximum temperature (°C)	24	23.7	25.4	29.2	26.5	23.3
Mean autumn minimum temperature (°C)	11.9	14	10.2	8.7	5.8	8.8
Mean summer temperature (°C)	20.1	20.1	20.5	22.2	19.4	153

[a] Genetic grouping according to Jaramillo-Correa et al. (2015)

suspension of *B. xylophilus* at mixed developmental stages, using the Baermann funnel technique to extract the nematodes and then adjusting the concentration with distilled water. In experiment 1, we used an inoculum density of 1200 nematodes per tree, whereas in experiments 2 and 3, the inoculum dose was 600 nematodes per tree. Appropriate inoculum density was determined in a previous experiment, where different *B. xylophilus* inoculum concentrations were tested on *P. pinaster* seedlings (Abelleira et al. 2013).

For tree inoculation, we made a wound in the previous year's growth of the main stem down to the xylem surface, which we covered with a 1-cm-wide strip of gauze bandage. Using Parafilm® to form a funnel around the wound, we saturated the bandage with the nematode suspension, or with distilled water in the case of control trees. The parafilm funnel was then closed to avoid tissue desiccation (Online resource 1).

2.3 Pre-inoculation measurements

To study inherent differences among provenances, some variables were assessed prior to inoculation. We measured growth traits for all trees in experiment 1, including number of branches, basal diameter, total height at the inoculation date, height to the end of the previous year's growth, and growth increment from the previous year's growth to the inoculation date. Similarly, we also measured the physiological variables of chlorophyll fluorescence, oleoresin flow, and water potential on four trees per provenance. All these physiological variables were measured prior to inoculation in experiment 2. Each physiological variable measurement procedure is described below.

2.4 Plant damage variables

We assessed the wilting symptoms in each experiment twice a week from the outbreak of external wilting symptoms to the end of the assay, when no further symptom evolution was observed. Wilting was assessed using a seven-level scale based on the percentage of needle discoloration, from 1 (no external symptoms) to 7 (all needles brown and wilted) (Menéndez-Gutiérrez et al. 2017b). We established level 3 as the onset (start) of the disease, since at this level, the symptoms were definitely attributable to PWD, and level 6 as the end, when there was no possibility for the tree to recover.

Mortality and disease evolution variables—the start, end, and duration of wilting symptoms—were determined by periodic wilting assessments. Mortality was assessed in the three experiments and disease evolution variables in experiments 2 and 3, because the duration of experiment 1 was too short to estimate these variables. Disease duration was calculated as the difference between the end and start of wilting symptoms. Disease evolution variables were measured in number of days.

We used a binary variable to assess mortality: 1 indicated that the tree was regarded as dead (wilting symptom level 6 or 7), and 0 indicated that it was alive (levels 1 to 5).

Oleoresin flow was assessed in experiments 1 and 2 at sampling date intervals of 10 to 14 days. We measured four inoculated and four control trees per provenance, one per block, on four sampling dates in each experiment. To evaluate oleoresin flow, we induced resin exudation by piercing the bark with a drawing pin, 5 cm below the inoculation point (Futai 2003). After 24 h, we evaluated this variable by measuring the length of the resin drop. Oleoresin flow values of control trees did not vary significantly among provenances per assessment date, so we estimated oleoresin flow ratio as the oleoresin flow value of each inoculated tree at each date divided by the average oleoresin flow values of all control trees per date.

2.5 Nematode quantification

Nematodes in plant tissues were quantified as indicators of plant susceptibility. In the three experiments, nematodes were extracted separately from the roots and aerial fractions of the trees using the Baermann funnel technique and then quantified under a stereomicroscope (Olympus Co., Ltd., Tokyo, Japan). To express nematode density in the number of nematodes per gram of dry weight, we dried samples at 105 °C for 48 h to obtain a standard determined dry weight of each fraction.

In experiments 1 and 2, we sampled eight inoculated trees per provenance, at 20 and 47 DAI. In experiment 2, nematode extraction was also performed on 12 inoculated and 4 control trees at the end of the experiment (104 DAI). In experiment 3, nematode extraction was performed on eight inoculated and four control trees at the end of the experiment (136 DAI).

2.6 Chlorophyll fluorescence and water potential

We measured chlorophyll fluorescence and water potential as proxies of plant stress. After inoculation, we measured chlorophyll fluorescence in experiments 2 and 3 at intervals of approximately 10 and 20 days, respectively. This variable was assessed on the same trees that were used to measure oleoresin flow throughout each experiment: four inoculated and four control trees per provenance, one per block. There were four sampling dates for each experiment, with the exception of chlorophyll fluorescence in experiment 3, which was assessed on seven dates.

To evaluate chlorophyll fluorescence, we collected three homogeneous needle samples per tree within a short period of time to ensure uniform conditions. After a 30-min dark acclimation period, we used a photosynthesis yield analyzer (MINI-PAM, Walz, Effeltrich, Germany) to measure chlorophyll fluorescence. Needles were exposed to far-red illumination for a few seconds, after which the PSII reaction centers

opened and minimum (F_0) chlorophyll fluorescence was measured. Subsequently, a saturating pulse of light was applied to close PSII reaction centers, and maximum chlorophyll fluorescence (F_m) was measured (Maxwell and Johnson 2000). We then calculated the maximum quantum yield of PSII photochemistry, an estimate of the PSII maximum photosynthetic efficiency, as the ratio Fv/Fm (Genty et al. 1989), where the variable chlorophyll (Fv) is the difference between the maximum (F_m) and minimum (F_0) chlorophyll fluorescence values.

We measured the shoot water potential of needles using the Scholander pressure chamber (Soil Moisture Equipment Corp., Santa Bárbara, CA, USA) to evaluate changes in tree water status after *B. xylophilus* inoculation. Pre-dawn and midday needle water potential were assessed in experiments 1 (22, 44 DAI) and 2 (22, 30 DAI) on each date. We collected 1-year-old needles from the same four inoculated and four control trees per provenance that were used for measuring the other physiological variables.

2.7 Climate data

We estimated the climatic variables (Table 1) with the ClimateEU v4.63 software package (Wang et al. 2006), using historical data for the 1991 to 2009 period. We studied 32 geoclimatic variables, which included altitude (ALT), mean annual temperature (MAT), mean warmest month temperature (MWMT), mean coldest month temperature (MCMT), continentality index (CI = MWMT − MCMT), mean annual precipitation (MAP), mean summer precipitation measured from May to September (MSP), number of frost-free days (NFFD), and other precipitation and temperature seasonal variables.

2.8 Data analysis

After normal score transformation, we analyzed wilting symptoms and mortality by repeated measures ANOVA, using the SAS MIXED procedure (SAS Institute Inc., Cary, NC, 2014). The first analysis included treatment (inoculated or control), experiment, and their interaction as fixed factors. A second analysis was performed using only data from the inoculated plants. This analysis included provenance, experiment, and their interactive effect as fixed factors and date nested to experiment and the interaction provenance × date nested to experiment as random factors. The same analysis was performed using the major genetics groups instead of provenance.

Water potential, chlorophyll fluorescence, and the oleoresin flow ratio were also analyzed by repeated measures ANOVA, but all factors were fixed in the analysis of these variables.

We applied one-way ANOVA to analyze disease duration variables (start, end, and duration of wilting symptoms).

These variables were log transformed, and mean nocturnal and diurnal temperatures were taken into account as covariates, due to the high influence of temperature on PWD expression. For the start and end of wilting variables, we used the mean nocturnal and diurnal temperatures as covariates and calculated them individually for each tree as mean temperatures from the inoculation date to the start or the end of wilting, respectively. For the wilting duration variable, the covariates were calculated as the mean nocturnal and diurnal temperatures from the start to the end of wilting for each tree. Provenance, experiment, and their interaction were considered as fixed factors.

One-way ANOVA was also applied to analyze growth traits assessed prior to inoculation (number of branches, basal diameter at inoculation date, total tree height at inoculation date, tree height until the end of the previous year's growth, growth increment from the previous year's growth to the inoculation date, i.e., the difference between the two tree heights described above) and pre-inoculation values of physiological variables (chlorophyll fluorescence, oleoresin flow, pre-dawn, and midday water potentials). Only provenance was treated as a fixed factor in the model. Least square means (LS means) were estimated for all variables.

Differences in nematode density (stem, root, and total tree) were determined using a non-parametric analysis of variance, the Kruskal-Wallis test, for each experiment. Considering provenance as a fixed factor, we performed the Mann-Whitney test to compare provenances when significant differences were found.

Spearman correlations among provenances (genetic correlations, provenances, LS means) and among individuals (phenotypic correlations) were obtained to study the relationships between growth traits, tree physiological state variables, disease-related variables, and origin parameters. All statistical analyses were done with SAS System software (SAS Institute Inc., Cary, NC, 2014).

3 Results

3.1 Pre-inoculation measurements

All growth trait variables presented significant differences among provenances ($F_{5, 228} \geq 7.88$, $p < 0.0001$, Table 2). LEI and NOR provenances stood out for their lower growth in diameter and number of branches, while GRE and ORI had higher diameter growth and number of branches. MIM had the highest tree height growth during the three first years, and ORI had the lowest. However, LEI presented the highest growth increment from the previous year to the inoculation date, followed by NOR, whereas SBUR and GRE had the lowest growth increment. The Atlantic LEI, MIM, and NOR

Table 2 Provenance effect and least square means values (LS means) ± standard deviations (SD) of the variables measured before inoculation (growth traits and physiological variables). Degrees of freedom (DF), F values, and significance level are presented. Within each row, significant differences ($p < 0.05$) are designated by different letters

Variable	DF	Provenance F	Provenance $p < F$	LS means ± SD Gredos	Leiria	Mimizan	Oria	Soria-Burgos	Noroeste-Litoral
Growth traits									
Basal diameter (mm)	5, 228	7.88	<0.0001	15.78±0.30 a	13.4±0.30 c	14.02±0.30 bc	14.74±0.30 bc	14.10±0.30 bc	13.86±0.30 c
Tree height until previous year's growth (cm)	5, 228	22.49	<0.0001	54.29±1.48 b	55.92±1.48 b	63.66±1.48 a	42.55±1.48 c	58.37±1.48 b	57.32±1.48 b
Total height (cm)	5, 228	35.61	<0.0001	88.74±1.45 c	101.63±1.45 a	100.76±1.45 a	79.82±1.45 d	86.50±1.45 c	96.05±1.45 b
Growth from the previous year growth until inoculation date (cm)	5, 228	16.72	<0.0001	34.45±1.40 c	45.71±1.40 a	37.11±1.40 bc	37.26±1.40 bc	28.13±1.40 d	38.74±1.40 b
Number of branches	5, 228	19.18	<0.0001	16.24±0.69 a	9.45±0.69 c	14.32±0.69 ab	16.11±0.69 a	13.18±0.69 b	9.55±0.69 c
Physiological variables									
Chlorophyll fluorescence (Fv/fm)[a]	5, 18	0.3	0.9092	0.82±0.04	0.81±0.04	0.79±0.04	0.83±0.04	0.78±0.04	0.77±0.04
Oleoresin flow (mm)	5, 39	2.47	0.0499	104.61±22.28 a	32.16±22.28 bc	38.97±22.28 bc	34.07±22.28 bc	92.88±22.28 ab	20.21±22.28 c
Water potential (MPa)	5, 72	0.88	0.4973	−0.30±0.02	−0.33±0.02	−0.35±0.02	−0.33±0.02	−0.35±0.02	−0.33±0.02

[a] Fv/fm: maximum potential PSII efficiency

provenances had the highest tree height at inoculation date, while ORI, the driest provenance site, had the lowest.

Among physiological variables, oleoresin flow was the only parameter that showed marginal differences among provenances ($F_{5, 39} = 2.47$, $p = 0.0499$; Table 2): GRE, followed by SBUR, stood out for their abundant constitutive oleoresin flow, and NOR for its notably low value.

3.2 Plant damage variables

None of the control trees died or showed disease symptoms. We found significant differences between treatments (control versus inoculated) for mortality ($F_{1, 50} = 98.88$, $p < 0.0001$; data not shown) and wilting symptom development ($F_{1, 49} = 77.06$, $p < 0.0001$; data not shown), but there was no interaction effect between treatment and the other factors on mortality or on wilting symptoms.

Looking only at *B. xylophilus*-inoculated trees, we found differences among experiments ($p < 0.0001$), provenances ($p < 0.02$), and for experiment × provenance interaction ($p < 0.0001$), for wilting symptom development and mortality (Table 3). When analyzing mortality by genetic groups, we also found an experiment × provenance interaction effect for wilting symptom development ($F_{6, 147} = 2.96$, $p = 0.0092$; data not shown) and mortality ($F_{6, 147} = 3.34$, $p = 0.0041$; data not shown).

In experiment 1, mortality incidence was just starting when the experiment was interrupted (55 DAI). All provenances had higher mortality in experiment 2 than in the other experiments, and the highest incidence of mortality occurred mainly between 23 and 41 DAI in this experiment. In experiment 3, tree mortality started later and progressed more steadily, occurring mostly from 69 to 116 DAI (Fig. 1). In experiment 2, only MIM presented significantly lower mortality than the other provenances (49 versus 67.4% of average, Fig. 2b), Atlantic France genetic group showed significantly lower mortality than the other groups. In experiment 3, ORI had the lowest mortality, followed by SBUR and GRE, while NOR showed the highest mortality (Fig. 2c). In this experiment, southeastern and central Spain genetic groups showed significantly lower mortality.

In experiment 1, NOR and SBUR were the most wilt-affected provenances when the experiment was unexpectedly terminated at an early date (Fig. 2d). In experiment 2, NOR was the most wilt-affected provenance and NOR, LEI, and ORI trees were significantly more wilt-affected than MIM (Fig. 2e). Atlantic France and central Spain were the least wilt-affected genetic groups. In experiment 3, NOR showed significantly more wilting symptoms than all other provenances, except LEI (Fig. 2f). The Atlantic Iberian Peninsula group was the most affected in this experiment.

All the disease evolution variables were influenced by mean diurnal and nocturnal temperatures, except duration of

Table 3 Results of mixed model analysis for the indicator of plant physiological stress and plant damage. F ratios and significance levels of the effects considered for the different variables measured after *B. xylophilus* inoculation

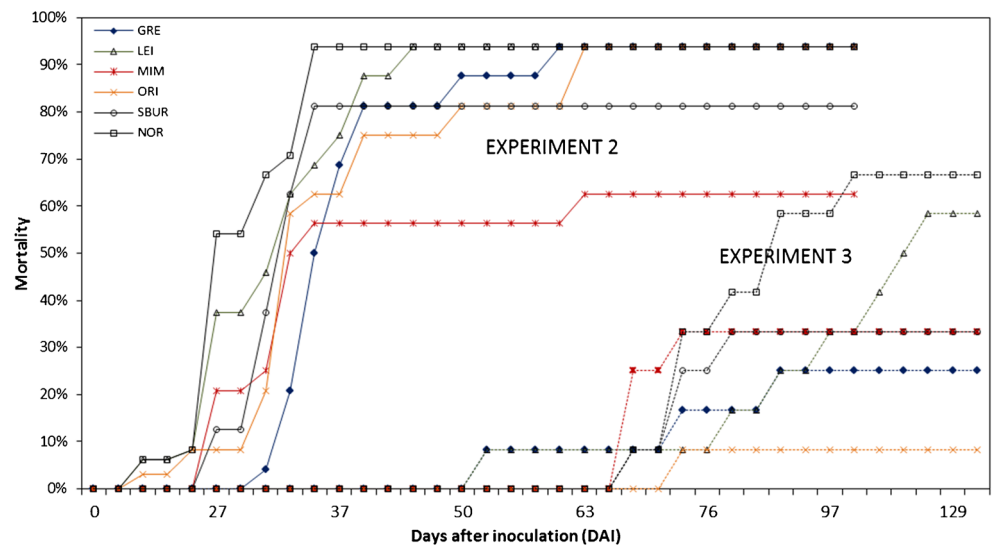| Variables	Experiment			Provenance			Provenance × experiment			Provenance × DAI (exp)			DAI (exp)		
	DF	F	$p > F$	DF	F	$p > F$	DF	F	$p > F$	DF	F	$p > F$	DF	F	$p > F$
Wilting	2, 49	7.58	< 0.0001	5, 245	2.14	0.0224	10, 245	30.15	< 0.0001		7.17[b]	< 0.0001		4.85[b]	< 0.0001
Mortality	2, 49	5.50	< 0.0001	5, 245	2.16	0.0206	10, 245	27.89	< 0.0001		7.45[b]	< 0.0001		4.90[b]	< 0.0001
Oleoresin flow ratio	1, 36	20.39	< 0.0001	5, 36	1.38	0.2559	5, 36	1.08	0.3878	25, 90	0.95	0.5376	5, 90	5.90	< 0.0001
Water potential (MPa)	1, 36	58.76	< 0.0001	5, 36	1.37	0.2591	5, 36	1.36	0.2606	10, 36	1.97	0.0067	2, 36	43.30	< 0.0001
Chlorophyll fluorescence (Fv/fm)[a]	1, 36	35.22	< 0.0001	5, 36	0.91	0.4850	5, 36	2.61	0.0413	40, 144	1.33	0.1144	8, 144	21.75	< 0.0001

DAI (exp) date nested to experiment

[a] Fv/fm: maximum potential PSII efficiency

[b] Z values of random effects

wilting symptoms, which was only affected by mean diurnal temperatures (Table 4). This was also the only variable that did not vary significantly among experiments. Wilting symptoms started and ended an average of 23 and 32 DAI, respectively, in experiment 2, and an average of 40 and 76 DAI, respectively, in experiment 3. However, only the end of wilting symptoms showed significant differences among provenances ($F_{5, 123} = 2.55$, $p < 0.03$).

Oleoresin flow ratio differed significantly between experiments ($F_{1, 36} = 3.22$, $p < 0.0001$) and assessment dates ($F_{5, 90} = 5.90$, $p < 0.0001$), but not among provenances. There was no interaction effect on this trait (Table 3). Oleoresin flow ratio was significantly different among provenances 56 DAI in experiment 2 ($F_{5, 36} = 3.22$, $p < 0.017$; data not shown). ORI and NOR showed lower oleoresin flow ratio values than MIM, LEI, and SBUR (Fig. 3c). Oleoresin flow values for control trees showed no significant differences among dates, although they slightly increased during the experiment.

3.3 Nematodes

In experiment 2, the total number of nematodes was significantly higher than in the other experiments ($\chi^2 = 75.47$, $p < 0.0001$, data not shown; Fig. 4). We found no significant differences among provenances in the number of nematodes in the roots, stem, or total tree at any date, except 47 DAI in experiment 1, when nematode median numbers were significantly higher in NOR, SBUR, and ORI (779, 19, and 6 *B. xylophilu*s per gram of stem dry weight, respectively) than in the other provenances (0, 1, and 1 *B. xylophilu*s per gram of stem dry weight for MIM, GRE, and LEI, respectively) (Fig. 4).

At the end of experiment 2 (104 DAI), ORI presented the highest median number of nematodes (1677 *B. xylophilu*s per gram of stem dry weight) and MIM the lowest (535 *B. xylophilu*s per gram of stem dry weight) (Fig. 4). At the end of experiment 3 (136 DAI), NOR had the highest median number of nematodes (242 *B. xylophilu*s per gram of stem dry
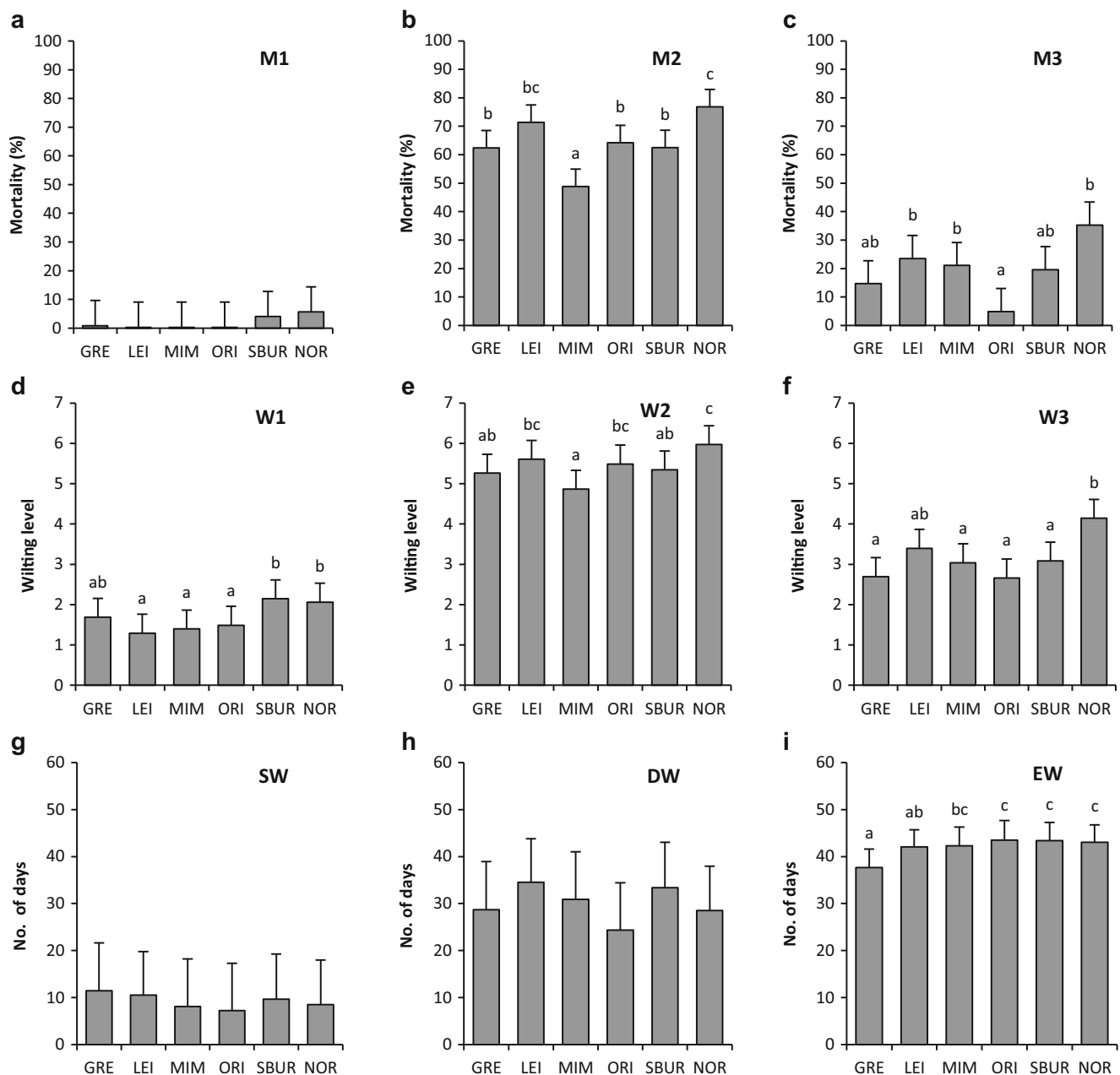
Fig. 1 Mortality rate for *P. pinaster* provenances in days after *Bursaphelenchus xylophilus* inoculation until the end of the experiment in experiments 2 and 3. Provenances: Sierra de Gredos (GRE), Leiria (LEI), Mimizan (MIM), Sierra de Oria (ORI), Montaña de Soria-Burgos (SBUR), and Noroeste-Litoral (NOR)

Fig. 2 Mortality and wilting symptoms (LS means ± SD) of different provenances of *Pinus pinaster* after an inoculation with *Bursaphelenchus xylophilus*. **a** Mortality in experiment 1 (M1). **b** Mortality in experiment 2 (M2). **c** Mortality in experiment 3 (M3). **d** Wilting symptoms in experiment 1 (W1). **e** Wilting symptoms in experiment 2 (W2). **f** Wilting symptoms in experiment 3 (W3). **g** Start of wilting symptoms in number of days (SW). **h** Duration of wilting symptoms in number of days (DW). **i** End of wilting symptoms in number of days (EW). Provenances: Sierra de Gredos (GRE), Leiria (LEI), Mimizan (MIM), Sierra de Oria (ORI), Montaña de Soria-Burgos (SBUR), and Noroeste-Litoral (NOR)

weight), while GRE, ORI, and MIM had very low median numbers (0, 1, 2, respectively) (Fig. 4).

Notably, in experiment 2, GRE, MIM, and ORI died with a higher median total number of nematodes per gram of dry wood than LEI, NOR, and SBUR (data not shown). Nematodes migrated from the inoculation site to the roots in at least one tree per provenance and date, except for GRE and MIM 20 DAI in experiment 1.

3.4 Chlorophyll fluorescence and water potential

After inoculation, there were significant differences in chlorophyll fluorescence among experiments and assessment dates, as well as a significant interaction effect between provenance and experiment (Table 3). In experiment 2, there was no significant difference among provenances (Fig. 3a). In experiment 3, ORI, MIM, and NOR presented significantly lower

Table 4   Results of mixed model analysis for disease duration variables. F ratios and associated p value

Effect	Start of wilting symptoms			Duration of wilting symptoms			End of wilting symptoms		
	DF	F	$p > F$	DF	F	$p > F$	DF	F	$p > F$
Experiment	1, 121	10.45	0.0016	1, 122	0	0.9795	1, 123	66.75	< 0.0001
Provenance	5, 121	0.55	0.7353	5, 122	0.16	0.9766	5, 123	2.55	0.0311
Provenance × experiment	5, 121	1.06	0.3848	5, 122	0.34	0.8888	5, 123	1.12	5, 123
Nocturnal mean temperature	1, 121	42.92	< 0.0001	1, 122	0.49	0.4858	1, 123	2.88	< 0.0001
Diurnal mean temperature	1, 121	14.48	0.0002	1, 122	27.86	< 0.0001	1, 123	657.73	< 0.0001

chlorophyll fluorescence values than the other provenances (Fig. 3b).

Water potential differed significantly between experiments ($F_{1, 36} = 58.76$, $p < 0.0001$) and assessment dates ($F_{2, 36} = 43.3$, $p < 0.0001$), but not among provenances, and there was no interaction effect between provenance and experiment or between provenance and date (Table 3). For all assessment dates, water potential values were much lower in experiment 2 than in the others. Pre-dawn and midday water potential values were lower in LEI and NOR, and greater in MIM (Fig. 3d), but no statistical differences were found in the analysis of all water potential data. Statistical results for both water potential ratios were practically the same, so we only presented the pre-dawn water potential in Table 3. The provenances

only varied significantly 30 DAI in experiment 2 between a group formed by LEI, NOR, and SBUR—concurrent with the most wilt-affected provenances at this date—and another group composed of GRE, ORI, and MIM.

3.5 Phenotypic correlations

In experiment 1, at phenotypic level, there was a significant negative correlation of total height at the inoculation date with wilting symptoms at the end of the experiment and number of branches (Online resource 2).

Water potential, oleoresin flow, and nematode density followed the same correlation tendency in all experiments (Online resource 2). Water potential values were positively

Fig. 3 Physiological parameters (LS means ± SD) of different provenances of *Pinus pinaster* after an inoculation with *Bursaphelenchus xylophilus*. **a** Chlorophyll fluorescence in experiment 2 (CF2). **b** Chlorophyll fluorescence in experiment 3 (CF3). **c** Oleoresin flow ratio for all experiments (OF). **d** Water potential for all experiments (WP). Provenances: Sierra de Gredos (GRE), Leiria (LEI), Mimizan (MIM), Sierra de Oria (ORI), Montaña de Soria-Burgos (SBUR), and Noroeste-Litoral (NOR)
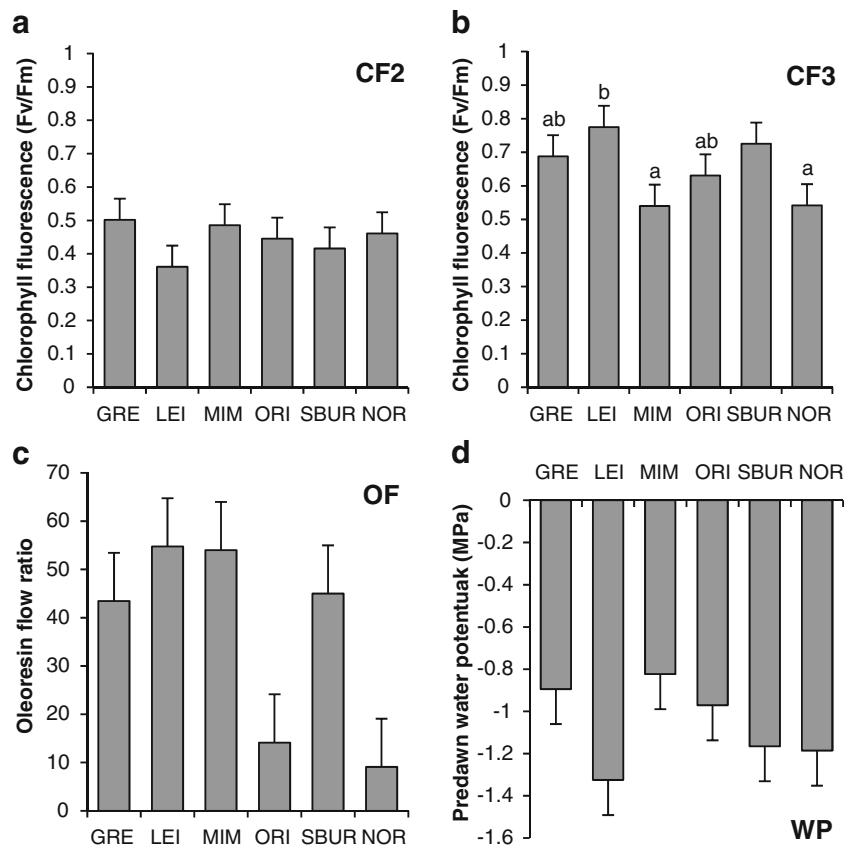
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Fig. 4 Nematode density per
P. pinaster provenance in the
three experiments. Box-whisker
plot, median (line), 25–75%
quartiles of values (box), min–
max span of values (whisker).
Nematode extraction dates:
experiment 1, 47 days after
inoculation; experiment 2,
104 days after inoculation;
experiment 3, 136 days after
inoculation. Provenances: Sierra
de Gredos (GRE), Leiria (LEI),
Mimizan (MIM), Sierra de Oria
(ORI), Montaña de Soria-Burgos
(SBUR), and Noroeste-Litoral
(NOR)
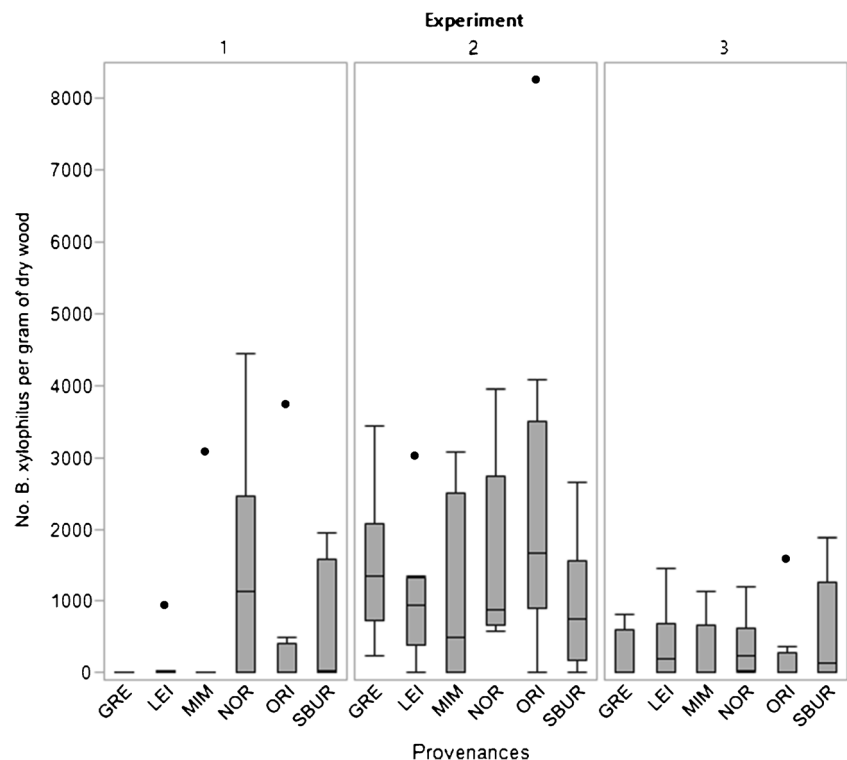
related to wilting and mortality when they were assessed on a similar date; oleoresin flow measured on different dates was strongly correlated with mortality and wilting on the same date, especially the last measurement dates, and the number of nematodes (stem, roots, and total) was also correlated with mortality and wilting symptoms on almost all quantification dates. The strongest correlations occurred with stem tissue at the end of the assay in experiment 3.

3.6 Genetic correlations between traits

Provenances with greater basal diameter and higher number of branches had significantly lower mortality and wilting symptoms in experiment 3 and lower chlorophyll fluorescence in experiment 2 (Table 5).

The provenances with greater constitutive oleoresin exudation showed significantly lower growth increment from the previous year's growth to the inoculation date, but also lower mortality and wilting symptoms, as well as marginally greater chlorophyll fluorescence in experiment 2. There was also a significant correlation between mortality and wilting in each experiment.

3.7 Genetic correlations with geoclimatic parameters

We found significant relationships indicating that mortality and wilting were affected by autumn precipitation and mean summer maximum temperature at the location of origin in experiment 3: provenances with higher damage and mortality

corresponded to places with lower mean summer maximum temperatures and higher autumn precipitation (Table 5). Chlorophyll fluorescence in experiment 2 was also significant and positively correlated with mean summer maximum temperature.

The correlations observed between growth traits and geoclimatic data of origin showed that provenances with greater total height came from lower altitudes and had higher mean autumn minimum temperatures. However, provenances coming from areas with higher mean summer temperatures and continentality presented the highest basal diameter. The number of branches was also strongly correlated with both climatic parameters and mean summer maximum temperature.

4 Discussion

Our results showed greater susceptibility to PWN in two of the Atlantic provenances, Noroeste-Litoral and Leiria, which composed the Atlantic Iberian Peninsula genetic group. We also found experiment × provenance interaction in susceptibility to *B. xylophilus* and differentiation among provenances for constitutive oleoresin exudation and growth traits. No clear geoclimatic pattern was observed, but the most wilt-affected pine provenances were from locations of origin characterized by high autumn precipitation and low mean summer maximum temperatures.

Table 5 Spearman correlation matrix among growth traits, tree physiological stress variables, disease-related variables, and altitude and climatic parameters at the site of origin in six *Pinus pinaster* provenances. $N = 6$

	Growth traits			Stress variables		Disease-related variables				Altitude	Climatic variables				
	Total height (m)	Growth from the previous year's growth until inoculation date (cm)	Number of branches	Pre-inoculation oleoresin flow (cm)	Chlorophyll fluorescence ratio exp. 2	Mortality exp. 2	Mortality exp. 3	Wilting exp. 2	Wilting exp. 3	Altitude (m)	Continentality index	Mean summer maximum temperature (°C)	Mean autumn minimum temperature (°C)	Mean summer temperature (°C)	Autumn precipitation (mm)
Basal diameter (mm)	-0.77†		0.94**	0.77†	-0.83*		-0.89*		-0.83*	0.77†	0.94**	0.81*	-0.77†		-0.77†
Total height (cm)							0.77†			-0.90*		-0.77†	0.89*		
Growth from the previous year's growth until inoculation date (cm)				-0.89*							-0.89*		0.83*		
Number of branches					-0.94**				-0.89*		0.83*	0.94**		0.81*	
Pre-inoculation oleoresin flow (cm)					-0.77†	-0.83*		-0.83*			0.89*	0.77†			
Chlorophyll fluorescence ratio exp. 2						-0.83*		0.83*	0.77†		-0.77†	-0.83*		-0.75†	
Mortality exp. 2								1.00***							
Mortality exp. 3									0.94**		-0.77†	-0.89*	0.77†		
Wilting exp. 2															0.94**
Wilting exp. 3												-0.83*		-0.75†	0.83*
Altitude (m)													-0.89*		
Continentality index												0.94*	-0.83*		

***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; †$p < 0.10$

We found significant differences among provenances for several growth traits and physiological parameters as expected, based on what is known about intraspecific maritime pine variation on adaptive traits (González-Martínez et al. 2002; Zas et al. 2005; Prada et al. 2014). Trees from the most susceptible provenances, Noroeste-Litoral and Leiria, had fewer branches, smaller basal diameter, and greater height growth, indicating greater competitive ability. At the phenotypic level, we only found a significant negative relationship between total height and wilting at the onset of wilting symptoms, since it was detected 57 DAI in experiment 1. This suggests that disease development could start earlier in provenances with taller trees. Significant relationships for growth traits were found at both the phenotypic and genetic level in previous experiments on *P. pinaster* families, where the tallest families and those with fewer branches were the most susceptible (Menéndez-Gutiérrez et al. 2017a). Yamanobe (2009) hypothesized that more branches and wider diameter would act as resistance factors, pointing out the greater potential of wider trees to maintain xylem and phloem transport. The author supported the idea of higher susceptibility in trees with fewer branches with other studies indicating that joints with abundant branches might physically or chemically affect PWN migration (Kawaguchi 2006; Kuroda 2008). With respect to tree height, negative correlations between growth potential and the expression of constitutive or induced defenses (i.e., phenolic compounds, density of resin canals, resin production) have been reported by several authors (Huot et al. 2014; Moreira et al. 2015; Desprez-Loustau et al. 2016), indicating the likelihood of a trade-off between height growth and the production of chemical defenses. In line with the growth differentiation balance hypothesis (Herms and Mattson 1992), we found lower oleoresin production in provenances with the fastest-growing trees, which were also the most susceptible to PWN. Oleoresins are recognized as important chemical defenses in conifer species. They can repel or physically entrap organisms and may have antifungal properties (Phillips and Croteau 1999; West et al. 2016).

The Atlantic *P. pinaster* provenances of Noroeste-Litoral, Leiria, and Mimizan correspond to areas with mild climatic conditions, while the Sierra de Oria and Sierra de Gredos provenances come from areas with less favorable environmental conditions, summer drought, and greater temperature oscillations (high maximum temperatures in summer and low minimum temperatures in winter). In such conditions, growth is limited and trees invest more resources in constitutive defenses (Herms and Mattson 1992; Moreira et al. 2014). Geoclimatic variation among *P. pinaster* provenances has been reported by other authors for other pathogens, such as *M. feytaudi* in *P. pinaster* (Burban et al. 1999) or

Gremmeniella abietina Lagerb. in *P. halepensis* (Romeralo et al. 2016). However, we did not find a clear climatic pattern since Mimizan was one of the least PWD-affected provenances and climatic characteristics at the location of origin are similar to those of the Noroeste-Litoral location of origin, which was the most affected provenance. Similarly, other provenance studies on susceptibility to PWN did not report any clear geoclimatic pattern (Zas et al. 2015; Hopf-Biziks et al. 2016). The correlations observed between height traits and climatic data of origin showed that provenances from milder climates, i.e., Atlantic areas, have a higher competitive ability. This corroborates other studies, such as Prada et al. (2016).

Hopf-Biziks et al. (2016) reported 100% mortality in experiments to test the susceptibility of German *P. sylvestris* provenances to PWN. However, our results showed variation in susceptibility, with significant differences in wilting symptoms and mortality among *P. pinaster* provenances. Our clearest finding was that the Noroeste-Litoral and Leiria provenances, which belong to the Atlantic Iberian Peninsula genetic group, were the most wilt-affected provenances. Zas et al. (2015) observed greater resistance for the Noroeste-Litoral provenance than for Leiria, but their results were expressed as nematode migration ability through stem sections, which may explain the difference with respect to our observations. The central Spain genetic group was stable across experiments and less susceptible than the Atlantic Iberian Peninsula group. However, provenance mortality ranges and tolerance rankings changed from one experiment to another: Mimizan was the least susceptible in experiment 2, whereas Sierra de Oria showed the lowest mortality rate in experiment 3. Similar tendencies have been observed by other authors, suggesting that environmental factors greatly influence tree response to PWN (Toda and Kurinobu 2001; Miyashita and Watanabe 2015). The Sierra de Oria and Sierra de Gredos provenances, which correspond to southeastern and central Spain groups, respectively, experienced the most drastic tolerance ranking change between experiments. Surprisingly, both provenances had 93.75% mortality in experiment 2, whereas in experiment 3, mortality was 8.33% for Sierra de Oria and 25% for Sierra de Gredos. Mean diurnal and nocturnal temperatures were higher in experiment 2 than in experiment 3. Given that these temperatures significantly influenced the start, end, and duration of wilting symptoms, temperatures during the experiments might be involved in the differences between experiments. Nocturnal temperature seems to have greater influence, leading to very high mortalities. However, though temperatures account for part of the variation, the significant differences that still exist between experiments point to the involvement of other factors. Our results agree with those of the PHRAME project (2007), suggesting an intermediate effect of temperature on tree physiology and nematode populations. Our observations also support

the results of the REPHRAME project (2015), indicating the strong influence of temperature on PWD expression. In this project, a simple model was developed using mean summer temperature to predict the probability of PWD. Locations with mean summer temperatures between 19.31 and 20 °C are at risk of PWD expression, and locations over 20 °C are at high risk of PWD. In the species studied, maximum mortality was reached when mean temperature was over 20 °C.

Previous results could also be explained by seasonal changes in susceptibility. Experiment 2 took place in April, at the beginning of the growing season, while experiment 3 started in mid-July, when summer drought tends to stop growth in the Mediterranean provenances. This leads us to think that a relation between the growth stage and susceptibility to PWN could exist. Panesar and Sutherland (1989) performed several inoculation tests under the same climatic conditions but different inoculation months, and reported higher seedling mortality rates for March inoculations. A seasonal pattern was even observed for *B. xylophilus* multiplication in *P. densiflora* and *P. pinaster* branch sections (Matsunaga and Togashi 2009; Menéndez-Gutiérrez et al. 2017c) due to the environmental conditions of the trees prior to the experiment.

Among the physiological variables assessed after inoculation, only chlorophyll fluorescence showed significant variation among provenances. However, the results we obtained, especially for experiment 3, do not concur with the results for mortality or wilting because the samples measured did not represent average provenance behavior. A larger sample size is needed to solve this problem, and others related to physiological variables, as the low sample number made it impossible to detect significant differences in oleoresin flow ratio and water potential values.

As in other studies, the nematode numbers were correlated with mortality and wilting at the phenotypic level (Oku et al. 1989; Menéndez-Gutiérrez et al. 2017a). We obtained higher numbers of *B. xylophilus* in the stem than in the roots on all quantification dates, but found no significant differences among provenances. However, we found different population densities among the trees that died: Leiria, Sergude, and Soria-Burgos seemed to require fewer nematodes to provoke irreversible damage. These results disagree with other studies (Futai 1980; Daub 2008), which observed greater nematode invasion in the roots than in the stem, due to its lower water content and similar nematode densities in dead trees. However, findings from those studies support the idea that a threshold nematode population density must be reached to induce irreversible wilt.

In light of known *P. pinaster* susceptibility and the presence of the pinewood nematode in Portugal and northwestern Spain, breeding programs were initiated with their respective origin provenances in order to select the families that were most tolerant to *B. xylophilus* (Ribeiro et al. 2012; Menéndez-Gutiérrez et al. 2017a). In earlier inoculation tests

on tree families in the Galician breeding program in northwestern Spain (Noroeste-Litoral provenance), variation in susceptibility to PWN was detected among families, some of them presenting notably higher tolerance (Menéndez-Gutiérrez et al. 2017a). Since some *P. pinaster* provenances seem less susceptible than Noroeste-Litoral and Leiria, the creation of new cultivars through cross-breeding for resistance to PWD presents an interesting possibility. Some authors have reported on intraspecific cross-breeding among resistant trees of susceptible species, such as *P. densiflora* and *P. thunbergii*, and interspecific crossing with resistant species, such as *Pinus tabulaeformis* × *P. densiflora* and *Pinus mugo* × *P. thunbergii* (see Zhao et al. 2008).

5 Conclusion

Differences in mortality and disease evolution were found among provenances at early ages. The most susceptible provenances were Noroeste-Litoral from coastal Galicia, and the Portuguese provenance of Leiria. The susceptibility of the other provenances could not be clearly determined, due to the genotype × environment interaction that was observed.

Based on the results obtained in this work, we conclude that the experiments should be repeated in diverse growth stages and seasons for more precise genetic characterization and confirmation of the lower susceptibility of certain provenances.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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