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Genetic variation in susceptibility to pine wilt disease of maritime pine (*Pinus pinaster* Aiton) half-sib families

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Abstract

· *Key message* This paper presents a greenhouse study for assessing the genetic variation in maritime pine (*Pinus pinaster* Aiton) in response to pinewood nematode (*Bursaphelenchus xylophilus* (Steiner et Buhren) Nickle), which is a causal agent of pine wilt disease. Fifteen out of 96 half-sib families were selected as less susceptible. This experiment is an important first step for creating a resistance breeding program.
· *Context* Pine wilt disease is caused by the pinewood nematode (*Bursaphelenchus xylophilus* (Steiner et Buhren) Nickle), a quarantine pest, and is a concern to maritime pine (*Pinus pinaster*) in Portugal due to its economic, environmental, and social impacts. This disease is regarded as a major threat to European forests.
· *Aims* This paper aimed to evaluate the genetic variation in maritime pine families that were inoculated with pinewood nematode, identify the most resistant families, and establish the guidelines for a resistance improvement program.
· *Methods* Two-year-old half-sib progenies obtained from 96 plus trees were inoculated. The plants were monitored for survival on four different dates. The statistical analysis followed the mixed model theory.
· *Results* Genetic variability of the susceptibility to pine wilt disease was observed. At 157 days after inoculation, the 15 highest genetic ranking families out of 96 were selected, having a predicted survival mean of 15.6% instead of 11.0% on average for the all 96 families.
· *Conclusion* This study allows for the implementation of an improvement program to help control pine wilt disease.

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Contribution of the co-authors I. Carrasquinho designed the experiment, and coordinated the field work, the greenhouse inoculation trial, and the data collection; I. Carrasquinho and E. Gonçalves performed statistical and quantitative genetic analysis and wrote the manuscript; A. Lisboa collected data in the greenhouse trial; ML Inácio participated in the experimental inoculation tests by rearing and providing the nematodes' suspension and collaborated in the analysis of the results; all authors gave final approval for publication.

This article is part of the topical collection on *Mediterranean pines*

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1 Introduction

Maritime pine (*Pinus pinaster* Aiton) is a widespread thermophilous conifer original from the western Mediterranean basin. The species occurs in the Iberian Peninsula, South France, West Italy, the western Mediterranean Isles, North Morocco, Algeria, and Tunisia (Viñas et al. 2016). In Portugal, maritime pine is the third most important native forest species, occupying approximately 22.6% of the Portuguese forest area. This species is widely planted for timber and has a recognized economic importance, providing approximately 77% of Portuguese forest employment. In 2016, the value chain constituted 3.6% of total Portuguese exports, corresponding to 36.6% of the forest-based sector (Centro PINUS 2015). According to the most recent National Forest Inventory, the total area of this species has decreased by 27% (ICNF 2013). In addition to fire, this decrease may be related to pine wilt disease (PWD) control measures, including not only phytosanitary cuts but also the logging of apparently healthy stands to prevent the spread of the disease.

Pine wilt disease is caused by *Bursaphelenchus xylophilus*, the pinewood nematode (PWN), and is vectored by cerambycid beetles of the genus *Monochamus*. This disease is a widespread threat to conifer forests and causes wilting and tree death in vast areas throughout the world. In the early 1970s, the PWN was identified in Japan as the causal agent of this disease, but the first incidence of PWD was reported in 1905 in Japanese red pine (*Pinus densiflora*) (Futai 2008). In 1999, PWD was recorded in southern Europe (in Portugal) and was associated with maritime pine (Mota et al. 1999). Human activities involving the movement of wood products may be the single most important factor in the spread of PWN (Mota and Vieira 2008; Telford et al. 2015; Ennos 2015). PWD spread from Japan to the neighboring East Asian countries of China and Korea in 1982 and 1988, respectively. After the discovery of PWD in wood chips imported from Canada and the USA, the European Plant Protection Organization (EPPO) listed the PWN and the *Monochamus* beetle vectors as A1 quarantine pests in 1986 (Sutherland 2008).

Traditionally, disease management in the forest has relied on "good" silvicultural practices and preventive actions based on the knowledge of risk factors (Desprez-Loustau et al. 2016). However, once destructive, non-native pathogens or insects have become successfully established, they can become permanent residents of native forest ecosystems. Dramatic changes in tree density and possible changes in genotypes can occur, such as the rapid elimination of most host individuals from the landscape (Ennos 2015). In this scenario, management activities to slow the spread of PWD in native forests can only be partially effective. Nevertheless, even in susceptible host species, there are always rare resistant individuals that can form the basis of a resistance breeding program for developing populations of genetically diverse and resistant trees (Sniezko 2006; Sniezko and Koch 2017). Intensive artificial selection programs may be necessary to generate populations with quantitative resistance that can be used to accelerate the recovery of populations, as natural selection of the very low proportion of genotypes with high quantitative resistance may be insufficient to allow the recovery of populations over short time scales (Ennos 2015). Several authors indicate that if the goal is to develop sources of resistant material for reforestation, the development of a breeding program should be the research priority (Telford et al. 2015; Sniezko and Koch 2017).

When breeding for resistance, classical selection and breeding methods have been applied to address diverse groups of pathogens (Carson and Carson 1989; Burdon 2001; Sniezko 2006; McKinney et al. 2014; Sniezko et al. 2014). Concerning PWD, the first breeding program for *Pinus densiflora* resistance began in western Japan in 1978. Later, other programs were also carried out in other parts of Japan and China for *P. thunbergii* and *P. massoniana*. In all these programs, the adopted breeding strategy was based on identifying apparently healthy trees (candidate trees) from heavily infested stands and creating resistant trees by making crosses (Nose and Shiraishi 2008). Grafted ramets and open-pollinated progenies obtained from the candidate trees were subjected to artificial inoculation tests with PWN to determine the resistance level of the breeding population. Resistant individuals were identified based on survival. During this first genetic selection program, which ran from 1978 to 1984, 92 and 16 resistant red pine and Japanese black pine (*P. thunbergii*) individuals were genetically selected, respectively (Toda and Kurinobu 2002). Now, 35 Japanese red pine and 46 black pine seed orchards have been established with 246 and 183 resistant varieties, respectively (FFPRI 2017). For operational plantings, progenies obtained from those seed orchards have become widely used as resistant seedlings (Mori et al. 2008).

In Portugal, a mass selection program for maritime pine was initiated in 2009. Approximately 500 apparently healthy trees were identified in an area with one of the highest incidence rates of the disease, and they were monitored monthly over the course of 1 year for PWD symptoms. After that, 457 trees were selected as plus trees constituting a reference population for future genetic evaluation studies concerning resistance to PWD (Ribeiro et al. 2012). In Spain, as PWN was detected in small and localized areas, and the affected stands were clear-cut to comply with an EU Directive, Menéndez-Gutiérrez et al. (2018) screened 2 and 3-year-old half-sib plants from 91 maritime pine families belonging to the breeding population for productivity and stem straightness by evaluating susceptibility to PWD. For these authors, who found genetic variability in mortality to PWD after artificial inoculation, the best long-term solution for protecting a pine forest against pine wilt disease lies in the selection and breeding for tolerance.

In plant nematology and in plant health in general, resistance is commonly defined as the ability of a host to suppress nematode development and reproduction, whereas tolerance describes the sensitivity of a host to parasitism or amount of damage sustained (Boerma and Hussey 1992; Woodcock et al. 2018). Methodologies for the determination of susceptibility to PWD based on the observation of external symptoms in artificially inoculated plants do not permit the distinction of resistance from tolerance. In the current study, "resistance" was used to refer to the ability of an individual host tree to defend against or withstand attack by the invading organism with an associated and measurable increase in fitness, thus includes both resistance (*sensu stricto*) and tolerance components.

The main objectives of this study were to evaluate the genetic variation in maritime pine for susceptibility to PWD to identify the most resistant families and establish the guidelines for a tree resistance improvement program. To achieve these objectives, an open-pollinated progeny trial was established in a greenhouse using 2-year-old half-sib plants that were artificially inoculated with PWN. The response variable was survival and vigor effects were used as covariates to focus on specific response mechanisms to the nematode.

2 Materials and methods

2.1 Plant material

Seeds were obtained from a random sample of 96 trees from the reference population for PWD resistance from "Herdade da Comporta" in southern Portugal. This reference population includes 457 trees resulting from a mass selection program initiated in 2009 in a maritime pine forest area that had the highest disease incidence in Portugal; this area was left for 10 years without silvicultural practices to control the disease. The criteria for the selection of these trees were dominance (dominant trees), age (adult trees), diameter at breast height (greater than 20–25 cm), and the absence of disease symptoms (Ribeiro et al. 2012).

In November 2012, seeds were germinated in a greenhouse at "Quinta do Furadouro", and 2 months later, the half-sib progenies were transferred to the nursery. In both places, the families were randomly assigned. An artificial inoculation progeny trial was established in the greenhouse on the Oeiras INIAV campus in September 2014.

2.2 PWN inoculum

Bursaphelenchus xylophilus isolate, Bx013.003, which was used for artificial inoculations, was obtained from an infested field tree exhibiting wilt symptoms and was included in the INIAV nematology collection. The sequence of the ITS region was deposited to the GenBank database (NCBI) under the accession number MF611984.1. Nematodes were kept in pure culture at 25 ± 1 °C on a non-sporulating *Botrytis cinerea* strain grown on barley grains that were previously autoclaved twice and were successively subcultured. Prior to inoculations but before the last subculturing, nematodes were allowed to grow on sterilized wood with the goal of keeping them as close as possible to natural conditions; after which, they were re-established on mycelial mats. Multiplied nematodes were separated from the culture media using the "tray" method (Whitehead and Hemming 1965). The suspension of nematodes was adjusted to a concentration of 1000 nematodes/ml of water.

2.3 Greenhouse inoculation trial

The experimental design layout consisted of a randomized complete block design with four blocks (replications). In each block, 96 half-sib families were represented by 15 seedlings per experimental unit. The four blocks were established to control specific conditions such as plant vigor (differences in height and diameter were found in the nursery), position inside the greenhouse, and inoculation date.

A total of 5760 plants were inoculated with an estimated number of 500 nematodes in the middle part of the current year's shoots. The applied nematode suspension resulted from a previous study in which the 500 μl nematode suspension provided the highest mortality among the four treatments studied (control, 250, 500, and 750 nematodes/ml) in 2-year-old maritime pine seedlings (Ribeiro 2012). The nematodes were applied with a micropipette in a small stem incision made with a sterile scalpel, after which the incision was covered with a cotton plug and Parafilm™ to prevent desiccation.

Due to dilution effects, variation in vigor traits may affect response to nematode inoculation. For this reason, for each of the 22-month-old plants, the height and diameter at the base were measured once and immediately after inoculation to be used as covariates in the subsequent data analysis. For these assessments, a marked scale and a digital micrometer caliper were used, respectively. The measurements and inoculations of the 1440 plants in each block took 1 and 2 days to perform, respectively. The experiment lasted 22 weeks (157 days). During this period, the maximum and minimum air temperature (°C) and relative humidity (%) inside the greenhouse were registered daily (Supplementary data—Fig. 1).

The percentage of the wilting needles: less than 25%; between 25 and 50%; between 50 and 75%; more than 75% was evaluated for each inoculated plant, on four different dates: 27, 38, 56, and 157 days after inoculation (DAI). Survival (π) was evaluated by calculating the proportion of plants with less than 50% of wilting leaves for each experimental unit. This criterion was used as a proxy of survival because it was observed during the experiment that plants with more than 50% of

wilting leaves on the first dates were all dead at the end, whereas the others with less symptoms were dead or alive.

2.4 Statistical analysis of phenotypic data

2.4.1 Evaluation of genetic variability for height and diameter and survival traits among half-sib families

As the objectives were to evaluate genetic survival variability among families and perform genetic selection of the best mother trees under a randomized complete block design, all the analyses were conducted using the mean of the experimental unit. The data analysis was carried out in two steps. The objective of the first was to evaluate the existence of variability in plant height and diameter at inoculation date among families. In the second step, plant height and diameter were included as covariates in the model of survival analysis.

To explore the relation between the mean heights and mean diameters of each family at the time of inoculation and the observed survival data, a Spearman correlation was computed at the family mean level per experimental unit ($n = 384$).

For height and diameter data, the following linear mixed models were fitted:

$$h_{ij} = \mu + b_i + f_j + e_{ij} \tag{1}$$

$$d_{ij} = \mu + b_i + f_j + e_{ij}, \tag{2}$$

for $i = 1, \cdots, 4$ and $j = 1, \cdots, 96$, where h_{ij} represents the mean height in block i for family j, d_{ij} represents the mean diameter in block i for family j; μ represents the overall mean (the intercept); b_i represents the effect of block i; f_j represents the effect of the family j; and e_{ij} is the random error associated with the observations h_{ij} and d_{ij}. The block effects were assumed to be fixed as they were used to account for known factors in the experiment (plant vigor, inoculation date, and position in the greenhouse). The family effects were assumed to be independent and identically distributed (i.i.d.) random variables with normal distributions, expected values of zero and variances σ_{fam}^2. The random errors were assumed to be i.i.d. random variables with normal distributions, expected values of zero and variances of σ_e^2. The family effects and random errors were assumed to be mutually independent random variables.

To analyze survival data, a generalized linear mixed model with a probit link function was fitted because the response variable was the proportion of the living plants per experimental unit. This link function was chosen because the binary outcome (alive or dead) depends on a hidden normal variable (resistance). In addition, this model has advantages in quantitative genetics contexts where Gaussian variance component models have genetic meaning, and the probit link function allows these meanings to remain intact when the response variable of interest is binomial (Stroup 2013).

The probit model is described as follows:

$$\Phi^{-1}\left(\pi_{ij}\right) = \mu + b_i + \beta_1 h_{ij} + \beta_2 d_{ij} + f_j, \tag{3}$$

$i = 1, \cdots, 4$ and $j = 1, \cdots, 96$, where π_{ij} is the probability of survival for family j in block i; $\Phi^{-1}(.)$ denotes the inverse standard normal cumulative distribution function, μ is the intercept, b_i is the effect (considered fixed) of block i, and β_1 and β_2 are the coefficients associated with the height and diameter variables, respectively; h_{ij} and d_{ij} are the mean height and the mean diameter of the trees in block i of family j, respectively; and f_j is the effect (considered random) of family j. The family effects were assumed to be independent and identically distributed normal random variables with expected values of zero and variances σ_{fam}^2. In this model, only the family effects, f_j, are related to the genetic ability to resist to PWN. The parameters β_1 and β_2 allow for the effects of plant height and diameter to be considered in the survival.

For height and diameter data analysis (models 1 and 2), the variance components were estimated by the residual maximum likelihood (REML) method. For survival data analysis (model 3), the variance components were estimated using a pseudo-likelihood method based on the REML method (Littell et al. 2006; Stroup 2013), adding a scale parameter. To test the family genetic variance component ($H_0 : \sigma_{fam}^2 = 0$ vs $H_1 : \sigma_{fam}^2 > 0$), a residual likelihood ratio test was used for height and diameter data analysis. For survival, to construct a valid likelihood ratio test, the reduced and full models were made comparable by basing the likelihoods on the final pseudo-data for the full model (SAS Institute Inc. 2013). The p value of the test was based on a mixture of chi-square distributions. To test the coefficient associated with the height variable ($H_0 : \beta_1 = 0$ vs $H_1 : \beta_1 \neq 0$) and diameter variable ($H_0 : \beta_2 = 0$ vs $H_1 : \beta_2 \neq 0$), a t test was performed.

One of the goals of this study was to select the best mother trees. Therefore, the family means heritability was computed. An approach that defines heritability in more complex settings (as was the case for survival data) was used. This type of method starts with the definition of heritability as the squared correlation between the predicted and true genetic effects, as considered in Oakey et al. (2006) and Gonçalves et al. (2013). In the current study, the vector of random effects is related to the half-sib family genetic effects, and the variance component in the covariance matrix of random effects is the family variance component; therefore, the generalized measure for the heritability of family means (h_f^2) was computed as follows:

$$h_f^2 = \frac{\sum_{j=1}^{a} \left(1 - \frac{\widehat{PEV}_j}{\hat{\sigma}_{fam}^2}\right)}{a-1},$$

where $\widehat{PEV}_j$ is the prediction error variance estimate of the effect for family j ($j = 1, \cdots, a$), a is the total number of families ($a = 96$), and $\hat{\sigma}^2_{fam}$ is the variance component estimate for the family effects.

2.4.2 Genetic selection and prediction of genetic gain

Following the mixed model equations (Henderson 1975), the empirical best linear unbiased estimator (EBLUE) of the fixed effects in the model and the empirical best linear unbiased predictor (EBLUP) of the random effects in the model (that is, the family effects) were obtained (in the probit scale for survival). The EBLUPs of the family survival effects were ranked to identify the families with higher and lower genetic effects on survival. In addition, the predicted survival for family j in block i ($\hat{\pi}_{ij}$) was obtained from the inverse of the probit link function as follows:

$$\hat{\pi}_{ij} = \Phi\left(EBLUE(\mu) + EBLUE(b_i) + EBLUE(\beta_1)h_{ij} + EBLUE(\beta_2)d_{ij} + EBLUP\left(f_j\right)\right).$$

For the jth family, the mean predicted survival percentage across the four blocks was computed:

$$\bar{\hat{\pi}}_{.j} = \left(\frac{\sum_{i=1}^{4}\hat{\pi}_{ij}}{4}\right) \times 100.$$

The genetic selection for resistance was performed based on the top EBLUPs of the family survival effects, and a balance between genetic gain and genetic variability was considered. For that, 15 top families were selected. The genetic selection gain for survival, ΔG, was computed using the mean of the predicted means of the top-ranking selected families ($\bar{\hat{\pi}}_{sel}$) and the predicted overall survival mean ($\bar{\hat{\pi}}_{..}$) as follows:

$$\Delta G\ (\%) = \frac{\bar{\hat{\pi}}_{sel} - \bar{\hat{\pi}}_{..}}{\bar{\hat{\pi}}_{..}} \times 100.$$

Data analysis was performed in SAS version 9.4 (SAS Institute Inc. 2013) using the MIXED and GLIMMIX procedures for the fitting of the linear mixed models and the generalized linear mixed model, respectively.

3 Results

3.1 Genetic variability among half-sib families for vigor traits at inoculation date and phenotypic correlations between vigor traits and survival rates

The phenotypic values observed for height and diameter at inoculation revealed wide ranges of variation (Table 1). More than 80% of this variation was explained by the genetic differences among families, as can be observed from the heritability of family means for both traits. For any usual significance level, family variabilities in height and diameter were observed ($p < 0.0001$). The existence of genetic variability of these traits is also well understood from the differences between the maximum (height 8.717 cm; diameter 0.827 mm) and minimum (height -9.401 cm; diameter -0.702 mm) EBLUPs of the family effects. In general, the top-ranking families in terms of plant height and diameter were the same (Supplementary data 1).

The Spearman correlations between the plant height observed at the inoculation date and survival rate at 27, 38, 56, and 157 DAI (df = 382) were -0.03 ($p = 0.610$), 0.22 ($p < 0.001$), 0.21 ($p < 0.001$), and 0.29 ($p < 0.001$), respectively. On the same dates, the correlations between the diameter and observed survival rate were 0.33 ($p < 0.001$), 0.28 ($p < 0.001$), 0.27 ($p < 0.001$), and 0.44 ($p < 0.001$), respectively. These results show a low to moderate phenotypic correlation between vigor traits at inoculation time and observed survival rate.

3.2 Variability among half-sib families for susceptibility to PWD and genetic selection

The results from the fitting of the probit model (with a Pearson residual variance close to 1, which means that an adequate model was fitted) are presented in Table 2. Significant family variability for susceptibility to PWD was found for all evaluated dates after inoculation ($p < 0.01$). For this trait, the values of the heritability of family means were moderate, and the lowest value was observed at 157 DAI.

The influence of plant height on survival was positive but was only significant at 27 DAI ($p < 0.05$). On the other hand, a significant positive effect of plant diameter on survival was always observed after 27 DAI ($p < 0.05$). The overall predicted family means of survival decreased from 84.98 to 11.04% from 27 to 157 DAI, respectively (Table 2).

The detection of significant family variability of survival allows for genetic selection for resistance to PWD. In Fig. 1, it is possible to identify different family groups among the top-ranking families according to the EBLUPs ranking for survival of the family effects at 157 DAI. If the top 22 out of the 96 studied families were selected as the most resistant ones, which corresponds to a selection proportion of 22.9%, a

Table 1   Results from the fitting of the linear mixed models (models 1 and 2): block effects test and respective p values; family variance estimate ($\hat{\sigma}_{fam}^2$) and respective p values of the hypothesis test for parameter σ_{fam}^2; average prediction error variance estimate ($\widehat{APEV}$) for the family effects; the heritability of family means (h_f^2); and phenotypic overall mean, maximum, and minimum values

Traits	F value block effects (p value)	$\hat{\sigma}_{fam}^2$ (p value)	$\widehat{APEV}$	h_f^2	Phenotypic overall mean	Phenotypic maximum	Phenotypic minimum
Height (cm)	2.24 (0.0843)	16.061 ($p < 0.0001$)	2.0358	0.882	33.79	43.68	23.14
Diameter (mm)	24.7 ($p < 0.0001$)	0.107 ($p < 0.0001$)	0.0219	0.802	4.62	5.65	3.75

predicted genetic selection gain for survival of 34.52% would be obtained. Alternatively, if the 15 top-ranking families were selected, the genetic selection corresponds to a selection proportion of 15.6% and the predicted genetic gain for survival would be of 40.82%. This last result means that seedlings obtained from the cross of the 15 selected mother trees would allow for 4.2% increase over the predicted survival mean (11.04%), reaching 15.55% (Table 3).

3.3 Evaluation of family performance for survival

Figure 2 shows the evolution concerning the EBLUPs for survival and predicted survival means of the 15 top-ranking families at 157 DAI on the four dates of observation. For all observation dates, complete EBLUPs for family survival effects and predicted survival family mean rankings are presented in supplementary data 2. For each DAI, the family rankings varied according to the EBLUPs for survival or the predicted survival means. There are some families with negative EBLUPs at 27 DAI that reached the top at 157 DAI (families 144, 260, 440, 447), whereas others remained at the top for all dates (families 152 and 77).

Table 3 compares family survival results at 157 DAI (obtained from model 3) and the family EBLUP rankings position for height and diameter at inoculation time (obtained from models 1 and 2 and ordered from the largest to smallest) for 27 families. These families cover the whole range of susceptibility, including the 22 most resistant ones (i.e., highest EBLUPs survival). The 22 top-ranked families for EBLUP survival were mostly ranked within the first third for predicted survival but rarely in the first third for EBLUPs for height and diameter (i.e., they were generally not among the most vigorous ones). However, two of the three top families for EBLUP survival, predicted and observed survival, families 152 and 282, are also ranked among highest EBLUP for height and diameter. Thus, a high survival rate can be associated not only to genetically superior families for both PWN resistance and vigor traits but also to genetically superior families for only resistance, height, or diameter.

Figure 3 gathers some of the studied families that exemplify different behaviors concerning the predicted survival means on the four observation dates. Families 152 and 421 started with similar predicted survival rates; however, after 56 DAI, the performance of latter family decreased. Families 278 and 131 were among those with lower predicted survival rates at the end of the trial. Additionally, family 260 showed a slight decrease in survival after 36 DAI.

4 Discussion

This study was performed using half-sibs from a previous phenotypic selection of healthy adult trees identified in a forest area in Portugal that was highly infested by PWD. Even after the natural preselection of this disease, genetic variability in the susceptibility to PWD was found after artificial

Table 2   Results of the fitting of the probit model (model 3) for PWD survival at 27, 38, 56, and 157 DAI: block effects test and respective p values; family variance component estimate ($\hat{\sigma}_{fam}^2$); average prediction error variance estimate ($\widehat{APEV}$) for the family effects; the heritability of family means (h_f^2); the estimated coefficients associated with height ($\hat{\beta}_1$) and diameter at the base ($\hat{\beta}_2$); and p values of the hypothesis tests for parameters, σ_{fam}^2, β_1 and β_2; predicted ($\bar{\bar{\pi}}$, %) and observed ($\bar{o}$, %) family means survival

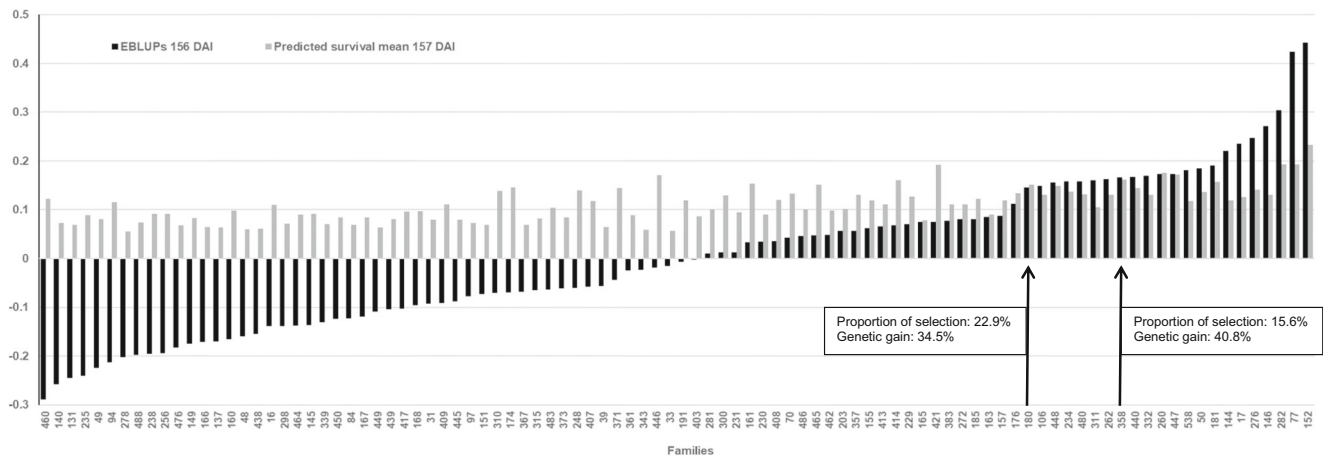| DAI	F value block effects (p value)	$\hat{\beta}_1$ (p value)	$\hat{\beta}_2$ (p value)	$\hat{\sigma}_{fam}^2$ (p value)	$\widehat{APEV}$	h_f^2	$\bar{\bar{\pi}}$ (%)	$\bar{o}$ (%)
27	3.92 (0.0091)	0.027 (0.0137)	0.088 (0.4300)	0.0912 (< 0.0001)	0.0473	0.486	84.98	84.76
38	0.80 (0.4934)	0.011 (0.2642)	0.200 (0.0428)	0.0633 (0.0002)	0.0347	0.456	47.44	47.46
56	0.22 (0.8854)	0.003 (0.7336)	0.289 (0.0046)	0.0772 (< 0.0001)	0.0390	0.500	28.90	29.07
157	0.94 (0.4226)	0.002 (0.8506)	0.357 (0.0023)	0.0627 (0.0017)	0.0399	0.368	11.04	11.23

Fig. 1 EBLUPs ranking for survival (in probit scale) for the 96 studied families and their corresponding predicted survival means at 157 DAI (results obtained from the fitting of model 3)

inoculation with PWN. Thus, this significant genetic variation indicates that genetic improvement can help control this disease in forests. In fact, several authors have shown the importance of breeding in improving plant and forest resistance to different pathogens (Carson and Carson 1989; Burdon 2001; Sniezko 2006; Telford et al. 2015), including nematodes (Boerma and Hussey 1992; Toda and Kurinobu 2002; Nose and Shiraishi 2008).

In this research, a moderate family heritability for survival after inoculation was obtained for all observation dates when compared to the family heritability observed for vigor traits at the time of inoculation. Although an appropriate experimental design was applied in the greenhouse, noise factors may explain these moderate values such as the exact number of PWNs in suspension and the total number of nematodes that effectively enter the plant. On the other hand, at 157 DAI, high average prediction error variance estimate combined with lower survival justifies the lowest value obtained for heritability. The differences found between the observed and predicted family survival means also reflect the low values obtained for heritability. In other studies, using different pines for PWN inoculation, higher values for heritability were reported. For example, Toda and Kurinobu (2002) and Menéndez-Gutiérrez et al. (2018) obtained a mean family heritability of 0.89 for nursery survival and 0.59 for greenhouse mortality, respectively. However, it is important to refer that, in both studies, different methodologies for data analysis and heritability computation were applied.

Concerning the preliminary approach using phenotypic correlations between vigor traits at inoculation date and survival rate, generally low positive Spearman correlations were found. On the contrary, in a study using maritime pine provenances, Menéndez-Gutiérrez et al. (2017) found high phenotypic correlations between vigor traits and mortality. These Spearman correlations between mortality and height and mortality and diameter were negative and positive, respectively.

However, in a study using maritime pine half-sibs, Menéndez-Gutiérrez et al. (2018) found low positive correlations between total height and mortality. Hakamata et al. (2013) reported positive effects of height and stem diameter at ground level on survival in 1-year-old Japanese black pine seedlings 2 months after inoculation but these authors indicated that no clear conclusions were drawn from the family effects.

Concerning the methodology used in the current study for data analysis (model 3), survival appeared to be influenced by plant height and diameter at the plant base, albeit in two distinct phases. In the early stage, approximately 1 month after inoculation, the results showed that taller plants are less vulnerable than smaller ones. After this date, a significantly positive effect on survival was observed in plants with larger diameters. These findings are in accordance with other author's reports about PWN movements inside the plant. Kuroda (2008) found that, in the early phase of disease development in *Pinus* species, PWNs move exclusively in the resin canals and do not feed on or kill the cambial cells. In the final stages of pine wilt disease, as the tree is approaching death, the PWN population increases and is present in the cambial zone. Son et al. (2010) reported that, after tangential artificial inoculation of 3-year-old *P. thunbergii* seedlings, PWNs moved faster in the vertical than in the horizontal direction through the resin canals in both the cortex and the xylem. These authors showed that the migration was slower in cortical tissues. For Kuroda (2008), reduced nematode migration and proliferation rates within the plant tissues seem to be crucial for pine resistance to PWN. However, Mori et al. (2008) found that inhibiting the proliferation of PWN was essential for the expression of resistance but inhibiting the migration of PWN was not closely related to resistance. Even in the resistant group, PWNs did not remain around the inoculation point. These authors studied migration and proliferation of *B. xylophilus* using 2-year-old clonally propagated *P. thunbergii* with pre-evaluated individual resistance levels. In a study of

Table 3 Results for 27 out of 96 studied families at 157 DAI concerning survival and vigor traits: EBLUPs of the family effects for survival (in probit scale) and predicted family means survival ($\bar{\bar{\pi}}_{.j}$, %) and their respective rankings (both obtained from model 3); observed family means survival (o_j, %); EBLUPs ranking positions for family height (h) and diameter (d) at the inoculation time (the EBLUPs were obtained from models 1 and 2, respectively, and ordered from the largest to smallest); and predicted genetic gains (ΔG, %)

Family	157 DAI					At inoculation time	
	Survival					EBLUPs ranking position	
	EBLUP	Ranking	$\bar{\bar{\pi}}_{.j}$ (%)	Ranking	o_j (%)	h	d
152	0.443	1	23.34	1	38.33	12	24
77	0.424	2	19.31	3	33.33	33	58
282	0.304	3	19.31	2	30.00	16	26
146	0.271	4	13.03	30	21.67	57	86
276	0.247	5	14.04	18	21.67	81	65
17	0.236	6	12.64	33	20.00	69	80
144	0.221	7	11.89	38	18.33	91	85
181	0.191	8	15.67	10	21.67	21	37
50	0.185	9	13.67	22	20.00	41	53
538	0.181	10	11.80	42	16.67	59	84
447	0.173	11	17.27	6	23.33	7	18
260	0.173	12	17.59	5	23.33	54	14
332	0.170	13	13.08	28	18.33	75	52
440	0.167	14	14.47	16	20.00	49	43
358	0.166	15	16.16	8	21.67	14	22
262	0.163	16	13.09	26	18.33	27	60
311	0.161	17	10.46	49	15.00	90	87
480	0.158	18	13.18	25	18.33	56	51
234	0.158	19	13.76	21	18.33	42	44
448	0.156	20	14.92	14	20.00	36	32
106	0.149	21	13.03	29	18.33	84	54
180	0.145	22	15.08	13	20.00	34	27
…	…	…	…	…	…	…	…
421	0.075	29	19.15	4	21.67	1	3
…	…	…	…	…	…	…	…
446	− 0.019	50	17.13	7	16.67	2	2
…	…	…	…	…	…	…	…
131	− 0.245	94	6.94	85	0.00	83	42
140	− 0.257	95	7.32	79	0.00	62	29
460	− 0.289	96	12.29	34	3.33	3	1
Mean			*11.04*		*11.23*		
$\bar{\bar{\pi}}_{15}(\%)$			*15.55*				
ΔG_{15} (%)			*40.82*				
$\bar{\bar{\pi}}_{22}(\%)$			*14.85*				
$\Delta G_{22}(\%)$			*34.52*				

four pine species widely distributed in Europe, *P. pinaster*, *P radiata*, *P. sylvestris*, and *P. pinea*, Silva et al. (2015) also indicated that biometry of the xylem resin canals should not be considered the only factor influencing the migration capacity of PWNs through stems. All these findings support the decision in the present study to select the top-ranking families

based on the EBLUPs of survival and not the predicted family survival means, which also depend on the height and diameter. In fact, families 421 and 446 clarified this situation, as both had high predicted family survival means, probably due to the strong genetic height and diameter effects but intermediate EBLUP ranking positions at 157 DAI (Table 3). Among the
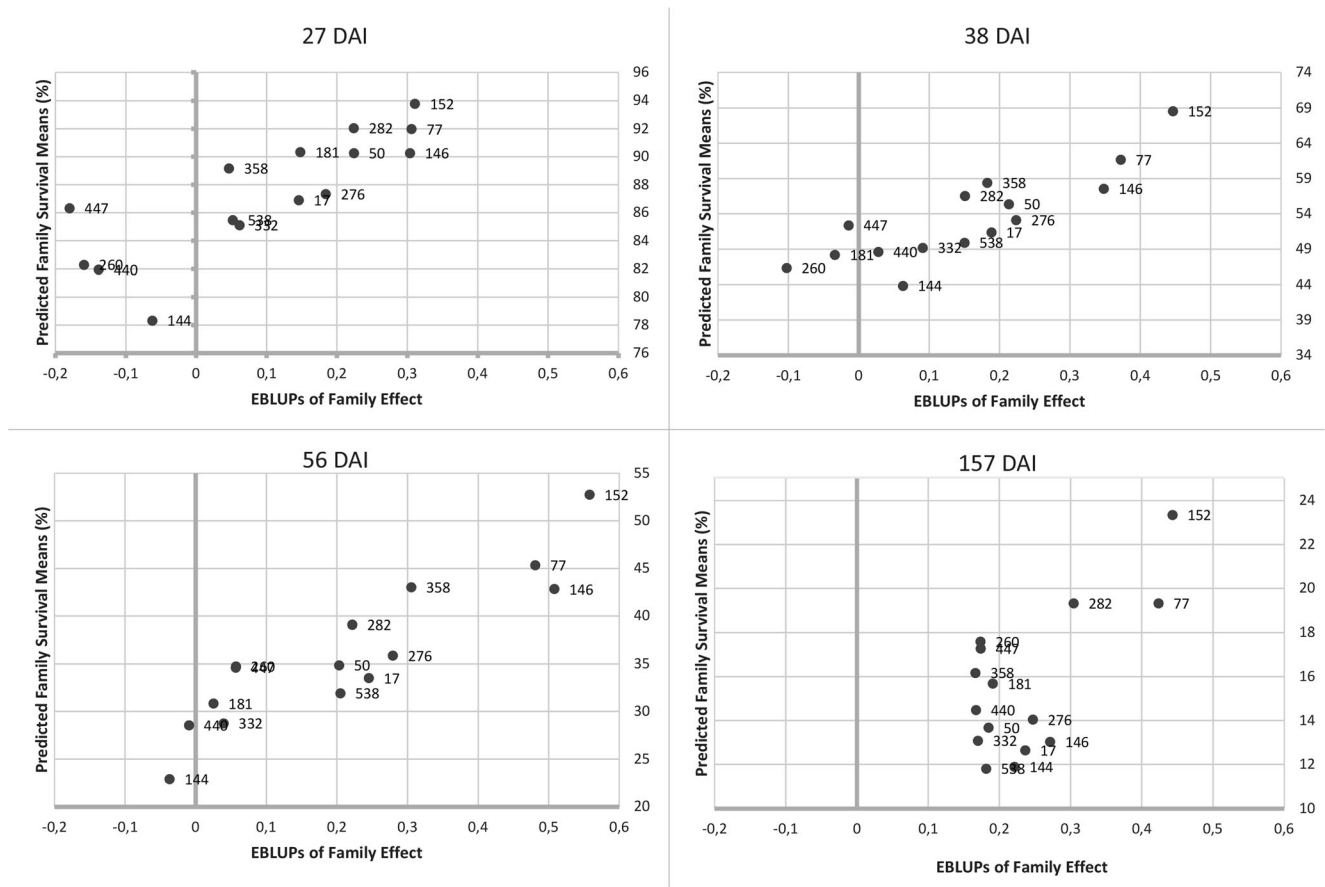
Fig. 2 Evolution of the 15 top resistant families to PWD (identified at 157 DAI) on the four dates of observation (27, 38, 56, and 157 DAI) concerning the relation between the EBLUP for survival and the predicted survival mean obtained from the fitting of model 3

15 top-ranking families at 157 DAI, only six were the same regarding EBLUPs and predicted survival means. Although

Fig. 3 Predicted survival means (obtained from the fitting of model 3) for eight families exemplifying different behaviors along the four dates of observation

several studies on PWD genetic resistance established a direct relationship between resistance and survival (Toda and Kurinobu 2002; Mori et al. 2008), the current results show that selection for PWN resistance should be performed on the genetic ability to resist PWN (survival family effects) and not only based on plant height and diameter. These traits might be related to the dispersion of the nematode inside the plant. Besides, from a practical point of view, selecting the surviving families with higher vigor does not act to prevent the PWD spread. In fact, these trees should be out of genetic selection ranks because they might be a source of inoculum to be spread by the insect vector.
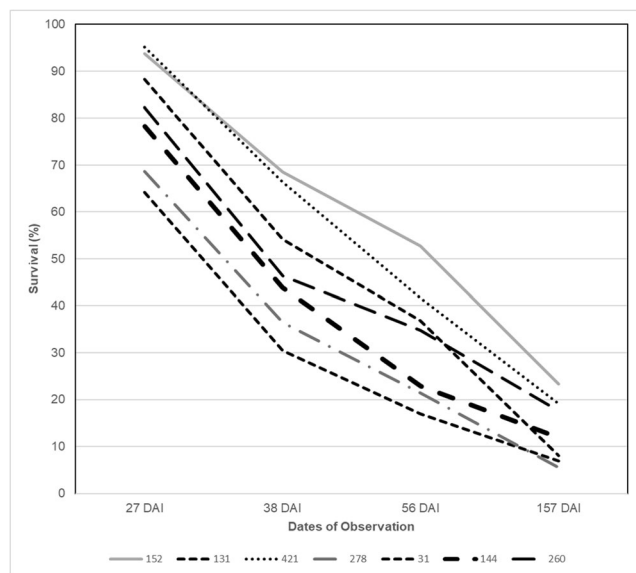
The selection of a nematode isolate to be used as inoculum is a critical part of any disease screening genetic program. Thus, a virulent isolate is important for detecting genotypes with higher resistance levels (Burdon 2001; Sniezko 2006). It should be reinforced that although a single isolate was used in this study, its virulence was proven not only by the low survival results observed in this inoculation trial, but also by the fact that this isolate was obtained from a symptomatic adult maritime pine located in the central region of mainland Portugal that was severely affected by PWD. Nematodes were subcultured on fungal plates and allowed to grow in wood

before inoculations to prevent any reduction in virulence. In addition, Mallez et al. (2015) used microsatellite markers and reported very low or complete absence of genetic diversity of PWN populations obtained from 34 different native and invaded areas in the USA, Japan, and Portugal. Nevertheless, these authors also considered that measurements of neutral genetic diversity have limited value for the prediction of quantitative PWN genetic variability.

The relevance of artificial studies with young plant material versus natural situations is another issue to be considered. According to Telford et al. (2015), to improve the resilience of forests to biotic threats, the degrees to which variations in resistance mechanisms are heritable and durable must be established through appropriately structured growth chamber glasshouse and field trials. In this sense, research on the susceptibility of maritime pine to PWN should continue in the field to assess the durability of resistance. However, these field trials should be carefully established because PWN is a quarantined organism that is vectored by an insect with large dispersion potential.

The low survival observed in families at the end of this trial is consistent with the low levels of resistance reported in other host species in response to non-native pathogens and diseases with previously established genetic improvement programs (Sniezko 2006). For *P. radiata* D. Don, Matheson et al. (2011) reported early estimates of resistance levels as low as 3% to pitch canker (*Fusarium circinatum*). In other studies, also regarding pine pitch canker, Matheson et al. (2006) reported that 85% of *Pinus radiata* were susceptible to initial infection, and this finding was associated with high mortality levels. After 10 years of genetic improvement through open-pollinated progeny tests using artificial inoculations to select half-siblings of Japanese red and black pines in nursery conditions, Toda and Kurinobu (2002) estimated, 3 months after artificial inoculation, average survival rates of 0.62 to 0.47, respectively. In similar tests using non-selected families, average survival rates varied from 0.55 to 0.22 for red and black pines, respectively. These authors considered that the annual climatic conditions in the nursery might justify the fluctuations in survival rate. However, concerning the survival rate, the comparison among different studies should be established carefully. Different experimental conditions during the trial (such as the length of the experiment, the PWN isolate virulence and concentration applied, the inoculation methods) and criteria for evaluating survival are some examples that may interfere with survival assessment for a tree species.

Finally, it is important to emphasize that the results obtained in this study represent an important contribution to maintaining maritime pine forests as before the wilt disease dispersal in Portugal. In fact, for the first time, these preliminary results noted the possibility to proceed to the second step in the program to improve the resistance of maritime pine to PWD in Portugal, which involves the establishment of a first clonal seed orchard using the best 15 out of the 96 studied trees. The economic and environment importance of maritime pine in Portugal justifies this investment. Besides, these procedures are in accordance with those proposed by Sniezko et al. (2014). In addition, other greenhouse tests should be performed to continue the genetic evaluation and include other trees from the maritime pine reference population in the breeding population.

Compliance with ethical standards

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

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