Intraspecific variation of anatomical and chemical defensive traits in Maritime pine (*Pinus pinaster*) as factors in susceptibility to the pinewood nematode (*Bursaphelenchus xylophilus*)

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27**Key Message**: Migration ability of the PWN through wood branch tissues of adult
28Maritime pine trees significantly differed among Iberian provenances and this variation
29was related to differences in anatomical and chemical defensive traits.

Abstract

The pinewood nematode or pine wilt nematode (PWN; *Bursaphelenchus xylophilus*) is one of the most dangerous threats to European coniferous forests, especially for the susceptible Maritime pine (*Pinus pinaster*), a valuable forest resource in South Western Europe. The PWN is vectored by beetles of the genus *Monochamus* (Coleoptera, Cerambycidae) and once inoculated in healthy branches it quickly migrates downward to the main trunk through the resin canal system. Therefore, the anatomy of the resin canal system may modulate the migration and proliferation rates. Using material from nine Maritime pine Iberian provenances established in a common garden trial we investigated whether these provenances differed in their (i) resin canal anatomy, (ii) concentration of chemical defences (non-volatile resin and total polyphenolics) in stems and (iii) ability of the PWN to migrate through the pine woody tissues in '*in vitro*' bioassays. Whether variation in anatomical and chemical defensive traits affects the variation in PWN migration across populations was also investigated. Significant intraspecific variation in anatomical and chemical defensive traits and in nematode migration rates through pine tissues was observed. Moreover, the variation in nematode migration rate among pine provenances was related to differences in both anatomical and chemical features. Overall, this study highlights the role of plant genetics in the development of defensive traits against this harmful coniferous pest. The observed intraspecific variation should be taken into account when considering breeding as a strategy to provide areas of high risk of PWN with resistant genetic material.

Keywords: Anatomical defences; Maritime pine (*Pinus pinaster*); Nematode migration rate; Non-volatile resin; Pinewood nematode (PWN; *Bursaphelenchus xylophilus*); Polyphenolics; Population differentiation; Resin canals

Introduction

Range expansions of non-native pests and pathogens to new host plant species is becoming one of the characteristic environmental changes of the Anthropocene. A paradigmatic example is the pinewood nematode (PWN; *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle) which, out of its native range, causes a devastating wilt disease that kills several *Pinus* spp. trees within weeks or a few months (Kuroda 2008b). By blocking the water conductance in the xylem and inducing tracheid cavitation, the PWN has caused extensive damage in the pine forests of Japan, China, Korea, and Taiwan affecting several pine species including *Pinus densiflora, P. thunbergii*, *P. massoniana* and *P. koraiensis* (Webster and Mota 2008). The PWN is nowadays considered one of the main threats to European coniferous forests (Vicente et al. 2012). After its introduction in the late 90's in a *P. pinaster* stand in the West coastal area of the Iberian Peninsula (Mota et al. 1999), it has rapidly spread over the entire Portuguese territory (Vicente et al. 2012), and reached Spain in just a few years (Robertson et al. 2011).

The PWN is vectored by beetles of the genus *Monochamus* (Coleoptera, Cerambycidae), which inoculate the nematodes in branches of healthy trees during the insect´s maturation feeding (Sousa et al. 2001). It is now well accepted that, once a healthy branch is attacked by the insect, the PWN quickly migrates downward to the main stem and colonizes the whole tree through the resin canal system (Ichihara et al. 2000a; Kuroda 2008b; Son et al. 2010), particularly through the thicker resin canals of the phloem and cortex (Kawaguchi 2006). The anatomy of the resin canal system is, therefore, thought to influence the ability of the nematodes to migrate through the tree and colonize healthy tissues (Kuroda 2008b); accordingly, the number and size of resin

canals has been shown to determine the migration rates of the nematodes in *P. thunbergii* (Kawaguchi 2006). Moreover, nematode migration has been related to the virulence of the PWN (Kuroda 2008b; Son et al. 2010). In particular, nematode migration has been found to be slower or even completely blocked in resistant conifer species (Oku et al. 1989; Nunes da Silva et al. 2013), and in resistant genetic variants (Kuroda 2004; Kuroda et al. 1991). Some examples that contradicted this were also reported, however, indicating that migration and colonization ability do not completely determine susceptibility of pines to the PWN (Mori et al. 2008; Eo et al. 2011). The accumulation of chemical defensive compounds that repeal, immobilize or disrupt the life cycle and reproduction of nematodes (Suga et al. 1993; Hanawa et al. 2001; Zhang et al. 2013), and the ability to activate defensive responses to the infection (Ichihara et al. 2000b), may also play a key role. Reduced nematode migration and proliferation rates within the plant tissues seem to be crucial for pine resistance to the PWN (Kuroda 2008b).

The PWN affects several pine species differently, with *P. densiflora* and *P. pinaster* being extremely susceptible and *P. taeda, P. strobus* and *P. pinea* highly resistant (Dwinell 1984; Woo et al. 2008; Dayi and Akbulut 2012). Variation of resistance within pine species to the PWN has been also reported in some previous studies (Kuroda 2004; Franco et al. 2011; Akiba et al. 2012), and prompted the launch of different breeding initiatives aimed to provide resistant genetic material to be used in areas of high risk of PWN damage (Toda and Kurinobu 2002; Nose and Shiraishi 2008; Ribeiro et al. 2012). Maritime pine (*Pinus pinaster* Aiton) is the most affected tree species in Portugal (Vicente et al. 2012), and the only one in Spain in which this nematode was reported (Robertson et al. 2011). Maritime pine occupies large areas in south west Europe and North Africa (more than 4 million ha). Within its natural

distribution range (Fig 1), Maritime pine has a fragmented distribution, with numerous relatively small and isolated populations (Bucci et al. 2007). Reduced gene flow among populations has favoured a strong differentiation between them, which is well documented in terms of genetic (González-Martínez et al. 2002; Burban and Petit 2003) and phenotypic variation of different adaptive traits (Chambel et al. 2007; Corcuera et al. 2012; Santos del Blanco et al. 2012), other relevant traits for timber production (de la Mata and Zas 2010a; Lamy et al. 2012), and herbivore and pathogen resistance (Arrabal et al. 2005). For example, intraspecific variation in resin flux (Tadesse et al. 2001), accumulation of non-volatile resin, total polyphenols and condensed tannins in stems and needles (Sampedro et al. 2011), and resin terpene profiles (Arrabal et al. 2005; Sampedro et al. 2010) have all been reported. Variation in susceptibility to several insect herbivores (Jactel et al. 1996; Burban et al. 1999; Zas et al. 2005) and fungal pathogens (Solla et al. 2011; Vivas et al. 2012) has also been well documented. However, despite this knowledge, and the enormous threat that the PWN poses to *P. pinaster*, the question of whether there is intraspecific variation in resistance/susceptibility to the PWN and in other putatively-related resistant traits remains unexplored.

Taking advantage of a *P. pinaster* common garden test which includes plant material from nine Iberian provenances, in this study we explore whether: i) there is intraspecific variation in the resin canal anatomy and the concentration of two quantitative resistance traits (non-volatile resin and total polyphenolics); ii) the ability of the PWN to migrate through the pine woody tissues varies across provenances, and iii) the variation in anatomical and chemical defensive traits could explain any variation in PWN migration rate across provenances.

Material and Methods

Plant material and experimental site

Our study was carried out using plant material belonging to a provenance trial of Maritime pine (*P. pinaster*) established in 2001 in the interior area of Galicia (NW Spain) by the Forestry Research Centre of Lourizán (Xunta de Galicia, Pontevedra, Spain). The trial was part of a series of provenance trials designed to search for alternative materials to be used in inland Galicia, a transitional region between the typical Atlantic and Mediterranean climates of the Iberian Peninsula, for which adapted forest reproductive materials for reforestation purposes are lacking (de la Mata and Zas 2010a). The trial, sited at Guntín (Lugo, Spain; N 42° 53.853' W 007° 41.049'; 540 m above sea level) followed a randomized complete block design and included nine Iberian provenances: seven Mediterranean provenances of Central and Eastern Spain (Bajo Tietar (BT), Sierra de Gredos (GR), Montaña de Soria-Burgos (SB), Serranía de Cuenca (SC), Sierra de Albarracín (AL), Sierra de Gata (SG) and Sierra de Segura Alcaraz (SS)) and two Atlantic origins represented by genetically improved materials (Coastal Galicia (CG) and Leiria (LE)) (Figure 1; see also Table S1 in Online Supplementary Material). The CG provenance (NW Spain) comprises the F1 open-pollinated offspring of plus trees selected for timber production within the coastal area of Galicia (de la Mata and Zas 2010b). The LE origin was represented by a collection of families derived from crosses between plus trees selected within the Leiria provenance (Portugal) that were obtained within the frame of the Maritime pine breeding program developed in Western Australia (Butcher 2007). More details of this genetic trial can be consulted in de la Mata and Zas (2010a; 2010b).

Sampling

During May and June 2011, when trees were 10 years old, two 2-year-old branches from 10 individual trees per provenance were sampled for chemical and anatomical analyses, and for determining the ability of the PWN to migrate through wood tissues. Trees were randomly selected from the dominant trees of the trial, avoiding trees with any visual disorder (e.g. defoliation, discoloration, wounds, etc). In each branch, the internode corresponding to the 2009 growth (ca. 1.0-2.0 cm of diameter) was sampled and immediately transported to the laboratory inside ice coolers (4ºC maintaining high humidity). The branch internode was then divided into three groups for (i) chemical, (ii) histological, and (iii) nematode migration assessments. Chemical analyses and histology were done at Misión Biológica de Galicia (CSIC, Spain). Nematode migration bioassays were performed at Centro de Biotecnologia e Química Fina (ESB-UCP, Porto, Portugal). In order to allow nematode migration assessment to be done within 12 h after branch cutting, field sampling was performed over four different dates with a 4-7 day interval. Sampling dates were considered in the statistical analyses, but they did not significantly affect the results.

Chemical analyses

Concentration of non-volatile resin and total polyphenolics was determined in freshly sampled branches (ca. 5 cm long pieces). Concentration of non-volatile resin was determined gravimetrically as described in Moreira et al. (2014). Non-volatile resin was extracted with hexane in an ultrasonic bath first for 15 min at 20ºC and then for 24 hours at room temperature. The extract was filtered (Whatman GFF, Whatman Int. Ltd, Kent, UK) and the extraction process was repeated once again. The concentration of non-volatile resin was estimated by weighing the extracted resin to the nearest 0.0001 g after solvent evaporation, and expressed as mg of non-volatile resin g^{-1} dry weight

(d.w.). The residual plant material was then extracted with aqueous methanol (1:1 vol:vol) in an ultrasonic bath for 15 min, followed by centrifugation and subsequent dilution of the methanolic extract. Total polyphenolic content in the extract was determined colorimetrically by the Folin-Ciocalteu method in a Biorad 650 microplate reader (Bio-Rad Laboratories Inc., Philadelphia, PA, USA) at 740 nm, using tannic acid as standard, and expressed in mg g^{-1} d.w. (see more details in Moreira *et al.*, 2014). A total of 90 samples (10 trees × 9 provenances) were analyzed with three analytical replicates.

Additional fresh branch segments (ca. 5 cm long) were used to determine the non-volatile resin separately in both the phloem-cortex and in the xylem tissues. The phloem-cortex was separated with a surgical knife that enabled its peeling away from the inner lignified wood. Non-volatile resin was determined in the two fractions following the procedure previously described. A total of 69 trees were analyzed for resin in phloem-cortex and xylem (8 or 9 trees × 9 provenances).

Histology

Branch segments of ca. 5 cm were fixed in formalin acetic acid (FAA) immediately after sampling, and then transferred to 70 % EtOH for storage until sectioning and staining (Moreira et al. 2008). Cross-sections, 90 μm thick, were made using a sliding microtome. Sections were stained for 12 h with 0.1% aqueous Safranin according to standard procedures (Ruzin 1999). Photographs were taken with a Nikon Digital Sight DS-U1, mounted on a Nikon SMZ-U binocular microscope at x20 magnification. Resin canals on digital images of two quadrants per sample (covering about 75% of the total transectional area) were counted and diameters radially measured

using the Phloemalizer v.2.12 image analysis software developed at the Pacific Forestry Centre (Victoria, BC, Canada) (Moreira et al. 2012).

The following variables in the cortex and in the xylem were obtained for each cross section: (i) resin canal density, through the number of longitudinal resin canals per unit area, (ii) mean interior area of individual canal (μm^2), and (iii) relative conductive area (%), obtained by dividing the total transectional area occupied by the resin canals by the total area of the tissue assessed, then multiplying by 100. Digital image analysis was done at the Misión Biológica de Galicia (Pontevedra, Spain).

Nematode migration rate

The ability of virulent PWN strains to migrate through the branches of each maritime pine origin was determined by migration bioassays tests, as described in Lima *et al.* (2012). Pinewood nematode inocula were prepared by multiplying two virulent isolates of *B. xylophilus* (*Bx*HF and *Bx*8A, both originating from Setúbal Peninsula region, Portugal) on a culture of *Botrytis cinerea* growing on sterilized barley seeds. The nematodes were extracted from the grains using the Baermann funnel method, counted on a dissecting microscope, and adjusted into a solution of 10 nematodes μl^{-1} in deionised water. All the branches were washed with deionised water and subjected to an ultrasonic bath for 5 min to eliminate any air bubble that could prevent nematode migration, after which they were cut into 5 cm segments. Natural basal and distal ends of the branch segments were identified through visual inspection of the bark scales orientation, and segments were placed vertically on 50 ml centrifuge tubes (previously cut by the 15 ml mark). The basal section of the segments (ca. 1 cm) was immersed in 3 ml of deionised water, segments attached to the centrifuge tube with parafilm and then 200 nematodes (20 µl of solution) were inoculated on the distal surface of each

segment. Once the nematode solution was absorbed, the distal surface of the segment was sealed with parafilm to avoid desiccation. The segments containing the nematodes were incubated at 25 ºC in the dark for 24 h. After this time, the nematodes that migrated through the branch segments and reached the water from the basal section were counted on a dissecting microscope. The migration test was performed within the first 36 h after branch sampling in 5 replicated segments per tree (N = 450). Tests were performed at the Centro de Biotecnologia e Química Fina (ESB-UCP, Porto, Portugal).

Statistical analyses

Variation in chemical and anatomical traits among maritime pine provenances was analyzed with a one-way ANOVA. The analysis of the migration rate was performed with a repeated measure ANOVA in which the 5 samples of each tree were considered repeated measures of the same subject, accounting, therefore, for any autocorrelation among them. A compound symmetric covariance structure among repeated measures was assumed. The models also included the random effect of the sampling date (four levels) and the covariation with the stems mean diameter. Models were fitted with the PROC MIXED procedure in SAS 9.2 (SAS Institute, Cary, NC, Littell et al. 2006). When necessary, dependent variables were transformed (*log(x)* or $\sqrt{x}$) to achieve normality. Least square means (± standard errors) were obtained from these models for each provenance. Pearson correlation analyses were performed in order to explore the relationships between nematode migration rates and the chemical and anatomical traits. Correlation analyses were performed with the PROC CORR procedure in SAS 9.2.

Results

Variation in growth among pine provenances

Total height of Maritime pine trees in the common garden test significantly differed depending on the plant origin (Table 1), Atlantic (Leiria (LE) and Coastal Galicia (CG)) growing faster than Mediterranean provenances (Figure S1). Among the Mediterranean provenances, Sierra de Gata (SG) and Montaña de Soria Burgos (SB) were the slowest in growing (Figure S1). No significant differences among pine provenances were observed for breast height diameter (Table 1).

Variation of chemical and anatomical defensive traits

Maritime pine provenances significantly differed in the mean area of cortex resin canals, density of xylem resin canals and the concentration of defensive chemicals (Table 1). No significant differences between provenances were observed for the density and relative conductive area occupied by cortex resin canals and for the mean and relative conductive areas of the xylem resin canals (Table 1). Trees from the Bajo Tietar (BT) provenance showed the greatest concentration of non-volatile resin in the whole stem, whereas those from Sierra de Segura Alcaraz (SS) and Sierra de Gredos (GR) showed the lowest (Figure 2a). Sierra de Segura Alcaraz trees showed the lowest concentration of total polyphenols, and those from Leiria (LE) the highest (Figure 2b). Trees from BT, LE and GR provenances stood out for the large size of their cortex resin canals (Figure 2c). Mean size of cortex resin canals of BT and LE trees was ca. 2-fold greater than the mean size of cortex resin canals in trees from Coastal Galicia (CG). Trees from LE and SG showed the lowest and highest densities of resin canals in the xylem, respectively (Figure 2d).

Concentration of non-volatile resin in the phloem-cortex significantly differed among pine provenances (Table 1) and showed a strong positive correlation with resin in the whole stem at the provenance level ($r = 0.863$, $N = 9$, $p = 0.003$). However, at the

phenotypic level, concentration of non-volatile resin in the phloem-cortex was not related with that in the whole stem ($r = 0.099$, $N = 65$, $p = 0.433$). Concentration of non-volatile resin in the xylem did not significantly differ across provenances (Table 1).

Variation in nematode migration ability

Nematode migration through the branch segments varied significantly across Maritime pine provenances, with some origins presenting 2-fold more recovered nematodes than others (Figure 3). In particular, trees from LE allowed the highest nematode migration in the bioassays (Figure 3), in comparison with trees from genuine Mediterranean origins such as SS and Sierra de Albarracín (AL), in which nematode migration was clearly restricted (Figure 3).

Relationship between migration rate and anatomical and chemical traits

Nematode migration through the branch segments of the different Maritime pine provenances was significantly and positively correlated to the mean area of the resin canals in the cortex and the concentration of polyphenols (Table 2; Figure 4). No other chemical or anatomical traits were significantly related to the nematode migration rates (Table 2). From the correlation analyses it could be inferred that the mean canal area, rather than the density of resin canals, was the main determinant of relative conductive area of resin canals in the cortex. On the contrary, in the xylem the relative conductive area was influenced by the density but not by the mean area of canals (Table 2). Interestingly, a negative relationship between the relative conductive area of resin canals in the cortex and the density of resin canals in the xylem was observed (Table 2). No significant relationships were observed between the concentration of non-volatile resin and any of the measured defensive traits except for total polyphenols (Table 2).

Despite the observed significant relations at the population level, phenotypic correlations at the individual level between nematode migration rates and anatomical and chemical traits were not significant ($p > 0.05$).

Discussion

Our study provides three noteworthy results: first, anatomical and chemical traits putatively related to conifer resistance against biotic threats were differentially expressed depending on the origin of the maritime pine seeds; second, nematode migration throughout pine tissues significantly varied among the different Maritime pine provenances assayed; and third, variation of nematode migration rates among pine provenances was related to the variation of anatomical and chemical traits across different pine origins.

Variation of chemical and anatomical defensive traits

Variation of non-volatile resin and total polyphenol concentrations between pine provenances agree with our previous greenhouse studies showing that these two traits are highly variable within a single Maritime pine population (Sampedro et al. 2011) and between populations (López-Goldar et al., unpublished). In the present study, variation of these two chemical defensive traits between provenances did not show a clear geographical pattern. However, it is worth mentioning that both traits were positively related at the provenance level despite they were not related at the phenotypic level ($r = -0.021$, $p = 0.846$, $N = 88$), suggesting no overlap in the functionalities of chemical defences of different type, as observed in other studies (Koricheva et al. 2004). These chemicals may be present in extremely large concentration in pine tissues (in the order of dozens of mg g^{-1}), and their accumulation is known to be costly for the plants, as it

has been found to be associated with a reduction of plant growth potential (Moreira et al. 2014; Sampedro et al. 2011). In this study, although non-volatile resin was negatively correlated with tree diameter at the phenotypic level ($r = -0.24$, $N = 85$, $p = 0.030$), growth potential and concentration of defensive chemicals were not related across provenances ($r = 0.56$, $N = 9$, $p = 0.180$; $r = 0.37$, $N = 9$, $p = 0.326$ for non-volatile resin and total polyphenolics, respectively). In consequence, physiological constrains at the individual level seems not to have influence the co-differentiation among population in these traits.

Maritime pine provenances differed in the density and size of resin ducts, but different patterns were observed depending on the tissue. Cortex resin canals were variable in size (mean transectional area) rather than in number across provenances, whereas xylem resin canals were more homogeneous in size but were highly variable in number. Previous studies in Maritime pine have shown that cortex resin ducts are influenced by resources availability (e.g. soil nutrients), whereas xylem resin ducts appear to be more sensitive to the biotic environment (e.g. herbivory), with proliferation of traumatic xylem resin ducts in response to herbivore damage (Moreira et al., 2008). Despite the environmental influence on resin canal traits, the variation observed in the present study was attributable to genetic differentiation processes among the studied populations, as the environmental conditions within the common garden test were fairly homogenous.

Intraspecific variation in anatomical defensive structures has been reported for other coniferous species (e.g. Martín et al. 2010; Moreira et al. 2012; Esteban et al. 2012), but no information regarding across provenances genetic variation of resin canal anatomy is available for Maritime pine. Maritime pine populations are known to greatly vary in several life-history traits such as growth (de la Mata and Zas 2010a),

reproduction (Santos del Blanco et al. 2012), fire tolerance (Fernandes and Rigolot 2007), drought tolerance (Gaspar et al. 2013) and cold tolerance (Prada et al., 2014). Based on the relationships between the phenotypic expression of these traits and the environmental conditions in the place of origin it has been inferred that the differentiation among provenances could be related to adaptive processes. The between provenances variation observed in defensive traits in the present study might have also originated from adaptive processes, as geographically distant Maritime pine populations might have been subjected to different selection pressures by biotic threats. However, inferring the adaptive value of the observed differences would be difficult as the biotic environment in which the different origins have evolved is largely unknown. Additionally, many other factors, including demographic processes (Bucci et al. 2007), environmental effects driving population differentiation in defensive traits (Martín et al. 2010; Estaban et al. 2012) or trade-offs among different fitness related traits (Moreira et al. 2014) could also be relevant.

Variation in nematode migration ability

Maritime pine provenances differed in terms of PWN migration ability in stem tissues. Using a simple *in vitro* bioassay (Lima et al. 2012), similar to that used in other related studies (Son et al. 2010), we found that migration rates of the PWN through branches of some provenances were more than 2-fold higher than in other provenances. Nematode migration is a key component of the capability of nematodes to colonize healthy pines, and decreased migration rates can be assumed to be related, at least in part, to pine resistance against nematodes (Kuroda 2008b; Son et al. 2010). The observed variation among maritime pine provenances is a first step to providing some insight about the variation in susceptibility to the PWN along the vast and heterogeneous area occupied

379by this pine species in the Iberian Peninsula. It should be noted that the migration tests
380were done on excised branch segments. Although we were especially careful to avoid
381desiccation and physical deterioration of the samples, we cannot assert that the
382migration ability of the PWN in excised tissues would be identical from that in living
383branches. Further research should test this uncertainty.

384 Interestingly, the Leiria (LE) material showed the highest nematode migration
385rate. This provenance naturally distributes in an area especially damaged by the PWN
386(Rodrigues 2008), close to where the PWN was first detected in Europe (Mota et al.
3871999). The potentially high susceptibility of LE, inferred by the high nematode
388migration observed, is consistent with the rapid expansion and devastating effects of the
389PWN across Portugal. The LE material analyzed here was derived from a breeding
390program developed in Western Australia upon material selected within the Leiria
391Portuguese provenance. The breeding program was designed to improve stem
392straightness and tree vigour (Butcher 2007), as was the case for the breeding program
393developed in Spain with the CG material (e.g. Zas and Merlo 2008). The material
394studied here from LE and CG can be considered, thus, representative of the material
395used for reforestation purposes in Atlantic areas of the Iberian Peninsula.

396 Although the LE material was also the fastest grower in the provenance trial (de
397la Mata and Zas 2010a), growth potential and nematode migration rates were not
398significantly related ($r = 0.56$, $N = 9$, $p = 0.118$). Other fast-growing origins appeared to
399restrict nematode migration to a higher extent. Of particular note in this respect was the
400other Atlantic selected material (CG), which also stands out as being fast growing (de la
401Mata and Zas 2010a). While this origin is assumed to be genetically close to the Leiria
402provenance (Bucci et al. 2007), CG was much more resistant than LE in terms of
403nematode migration rate.

Overall, differences among populations in nematode susceptibility did not follow any clear climatic or geographical patterns. Mediterranean origins, for instance, showed a large variation in nematode susceptibility, and included populations that appeared to be highly susceptible. Simulation studies predict that the PWN could easily spread under a warming climate if both vectoring insects and susceptible trees exist (i.e. Fernández and Solla 2008). Besides, temperature is regarded as the most important factor for the progression of the disease (Evans and Futai 2008), because high summer temperatures and large seasonal variation in water availability increase the risk of wilt expression (Evans et al. 2008). As summer temperatures and summer drought are stronger in these Mediterranean origins, the apparent high susceptibility to the PWN found in the present study can be seen as a warning signal of the risk of expansion to these Mediterranean areas.

Relationship between migration rate and anatomical and chemical traits

Results, indicating a positive relation between the mean area of axial resin canals in the cortex and nematode migration, agree with previous findings (Kawaguchi 2006), and suggest that cortex resin canals may be the most important paths of nematode dispersal in two-year old branches. Nematode migration rate was previously related to the size of the resin canals of the cortex (Kawaguchi 2006), and cortex canals are known to be one of the most important routes for nematode dispersal inside the tree (Ichihara et al. 2000a; Kuroda 2008b; Son et al. 2010). However, cortex tissues are ephemeral in pine species, dying in a few years and leading to periderm formation. Resin canals in the cortex would thus become dysfunctional in older branches, and PWN would likely move to the vertical resin canals of the xylem through the horizontal radial canals. Consequently, in older branches both vertical and radial xylem resin canals could be

also relevant for PWN migration. Nematodes are able to migrate through the xylem canal system and xylem tracheids (Kuroda 2008a; Son et al. 2010). Our results suggest, however, a secondary relevance of those migration paths, at least in two-year-old branches, and a more relevant role of resin canals in the cortex.

We found a positive relationship between nematode migration and polyphenol concentration of branch segments, contradicting previous research reporting that PWN resistant species or varieties accumulate more phenolic compounds than susceptible ones in response to PWN infection (Kuroda et al. 2011; Nunes da Silva et al. 2013). Some particular phenolic compounds found in resistant pine species, i.e. stilbenoids, have been reported to show nematicidal activity to the PWN in *in vitro* assays (Suga et al. 1993), although their role as a factor of resistance *in vivo* has been questioned (Zhang et al. 2013). On the other hand, phenolic compounds are also known to accumulate in response to the PWN infections when compared to control trees (Futai 2003). Most studies reporting positive relations between phenolic concentrations and PWN resistance failed to differentiate whether this link was due to variation in constitutive (as observed here) or induced levels of polyphenols. Further research will be needed to clarify the role of phenolic compounds in the intraspecific variation in PWN resistance in Maritime pine.

Relationships between nematode migration rates and anatomical and chemical traits were only significant at the provenance level, but not at the individual (phenotypic) level. This result calls for caution when interpreting our results and suggest that other unstudied phenotypic traits may be also relevant for nematode migration at the individual level. Despite this lack of phenotypic relationships, genetic differentiation among provenances in cortex resin canals and total polyphenolics parallels that in nematode migration rate. It should be noted, however, that the

significant relationships observed at the population level appear to be highly influenced by the particular population of Leira, which has outstanding values for all these traits. Further research including more Maritime pine origins and other anatomical and chemical traits is needed to clarify the complex equation of nematode susceptibility.

Overall, this study highlights the relevance of tree genetics, anatomy and chemical defensive traits as resistance factors against the PWN. These traits could be extremely valuable for future breeding initiatives aimed at obtaining resistant genetic material.

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667Figure 1. Maritime pine natural distribution range in the Iberian Peninsula (light blue), 668provenances (red dots) included in the study and location of the provenance trial (blue 669square). See Table S1 in Supplementary material for more details on the geographic and 670climatic characteristics of the studied provenances. The entire territory of Portugal is 671now considered to be affected by the PWN (dashed area). The two asterisks in the map 672indicate the locations in which the PWN was detected in Spain. The distribution map of 673*P. pinaster* was obtained from EUFORGEN 2009, www.euforgen.org. (*): The CG and 674LE materials come from breeding programs developed in Galicia (NW Spain) upon 675material selected in the coastal area of this region, and in Western Australia based on 676original material selected within the Leiria Portuguese population, respectively (See 677details in the main text).

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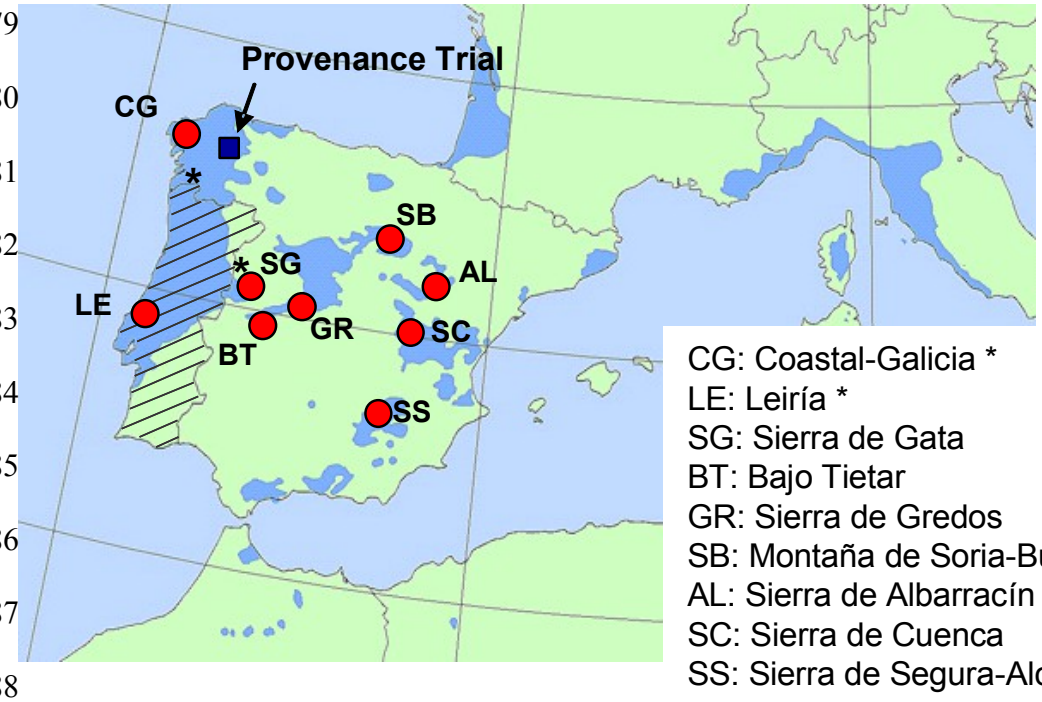

691Table 1. Provenance effect on pine growth, the concentration of defensive compounds 692and the number and size of the resin canals in the cortex and xylem of 10 year old 693Maritime pines growing in a common garden test. Degrees of freedom (factor, error), F 694ratios and associated p-values are shown. N = 9 provenances.

	D.f.	**F value**	**P > F**
Pine growth			
Diameter	8, 77	1.39	0.215
Height	8, 77	4.51	**<0.001**
Non volatile-resin			
Whole stem	8, 80	2.09	**0.046**
Phloem and cortex	8, 57	2.65	**0.015**
Xylem	8, 57	0.39	0.922
Total Polyphenolic Compounds	8, 80	2.44	**0.020**
Cortex resin canals			
Density	8, 80	1.00	0.444
Mean area	8, 80	2.62	**0.013**
Relative conductive area	8, 80	1.62	0.132
Xylem resin canals			
Density	8, 80	2.81	**0.009**
Mean area	8, 80	0.40	0.805
Relative conductive area	8, 80	1.60	0.137

695

696Table 2. Pearson correlation coefficients between traits related to the quantitative 697investment in chemical defences and to the anatomy of the resin canal system in nine 698Iberian Maritime pine provenances growing in a common garden. Traits were assessed 699in two-year-old branches. Significant correlations ($p < 0.05$) are highlighted in bold 700font.

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	Secondary chemicals		Cortex resin canals			Xylem resin canals		
	RES	PH	Rel. Conduc. Area	Density	Mean area	Rel. Conduc. Area	Density	Mean area
Migrating nematodes	0.33	**0.75**	0.40	-0.36	**0.61**	-0.31	-0.57	0.41
Non-volatil resin (RES)		0.46	0.12	-0.21	0.30	0.39	0.18	0.47
Total polyphenols (PH)			0.42	-0.08	0.57	0.10	-0.12	0.46
Cortex resin canals								
Relative conductive area				0.31	**0.76**	-0.48	**-0.63**	0.18
Density					-0.34	-0.25	-0.08	-0.44
Mean area						-0.13	-0.40	0.52
Xylem resin canals								
Relative conductive area							**0.90**	0.45
Density								0.02
Mean area								

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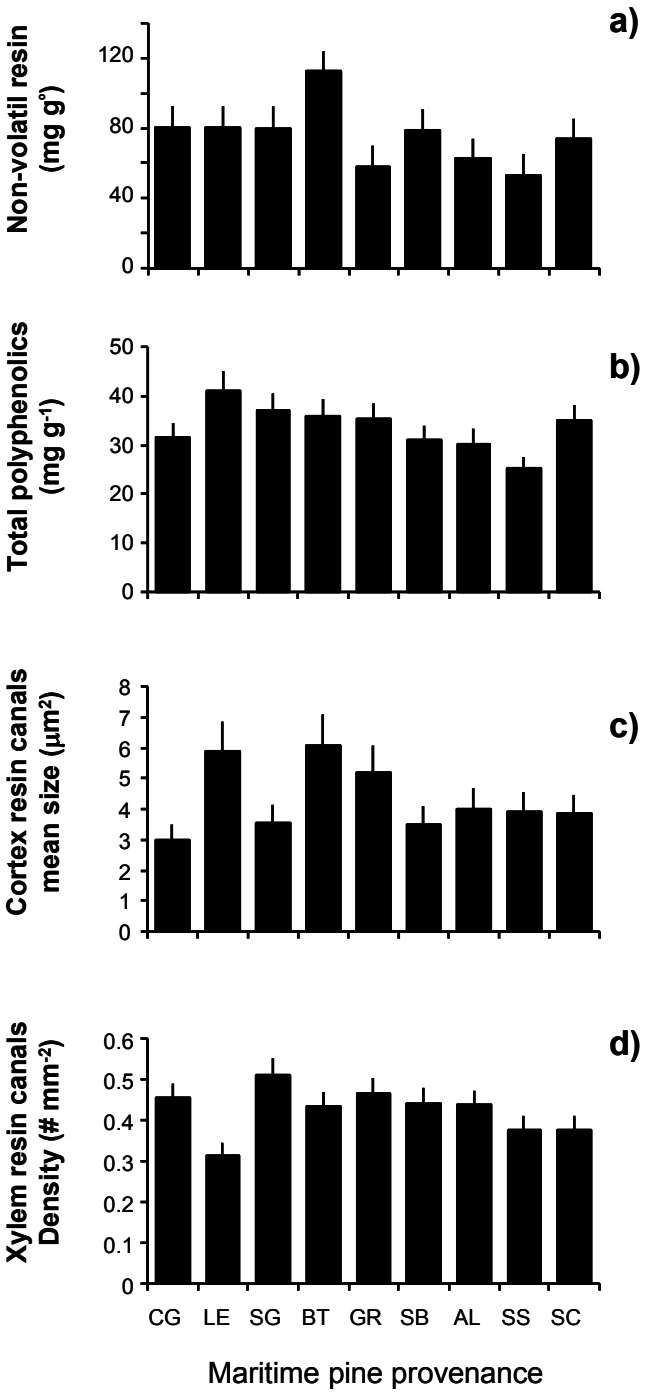
703Figure 2. Concentration of non-volatile resin (a) and total polyphenolics (b), and mean
704size of individual cortex resin canals (c), and the density of resin canals in the xylem (d)
705in two-year old branches of nine Maritime pine provenances. The provenances assayed
706were Coastal Galicia (CG), Leiria (LE), Sierra de Gata (SG), Bajo Tietar (BT), Sierra de
707Gredos (GR), Montaña de Soria-Burgos (SB), Sierra de Albarracín (AL), Serranía de
708Cuenca (SC) and Sierra Segura-Alcaraz (SS). Each provenance was represented by 10
709individuals established in a common garden test located in NW Spain. Mean ± s.e. are
710shown.



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730Figure 3. Migration ability of the pinewood nematode through branch segments of nine
731Maritime pine provenances measured in bioassays. Each provenance was represented by
73210 individuals established in a common garden test located in NW Spain. A total of 200
733nematodes were inoculated in 5-cm long branch segments and migration rate was
734measured after 24 h. Five replicated bioassays were performed for each individual.
735Provenances are ordered southwards. Mean ± s.e.

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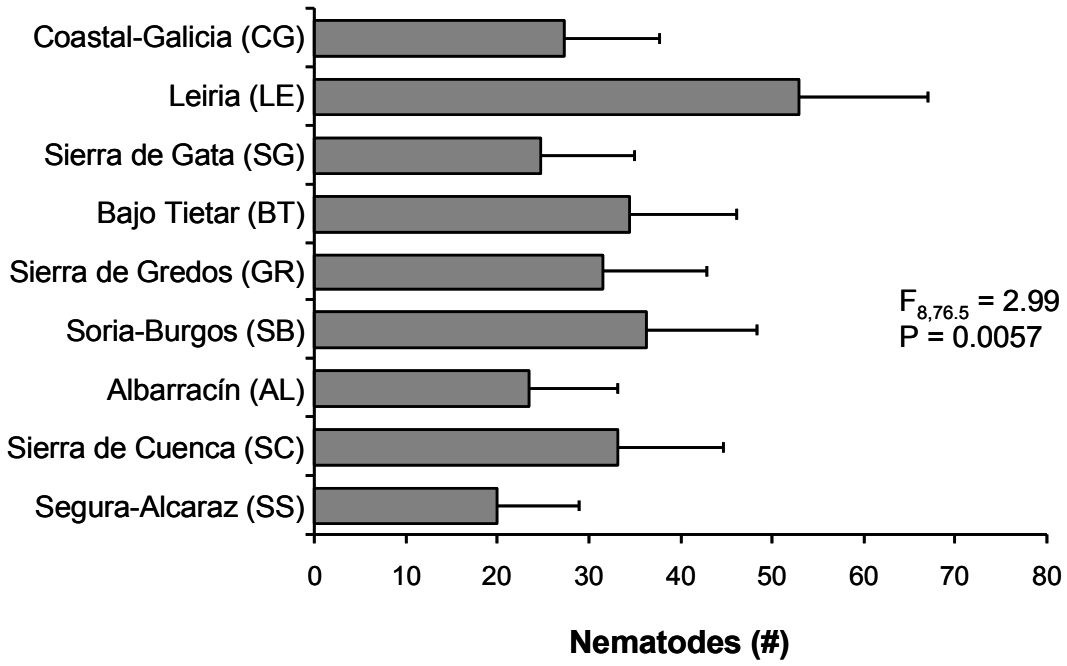

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748Figure 4. Relations between PWN migration rates through two-year-old branch 749segments in bioassays and (a) mean area of cortex resin canals and (b) concentration of 750total polyphenols. Dots are mean values ± s.e. of nine Maritime pine provenances. N = 75110 replicate trees per provenance.

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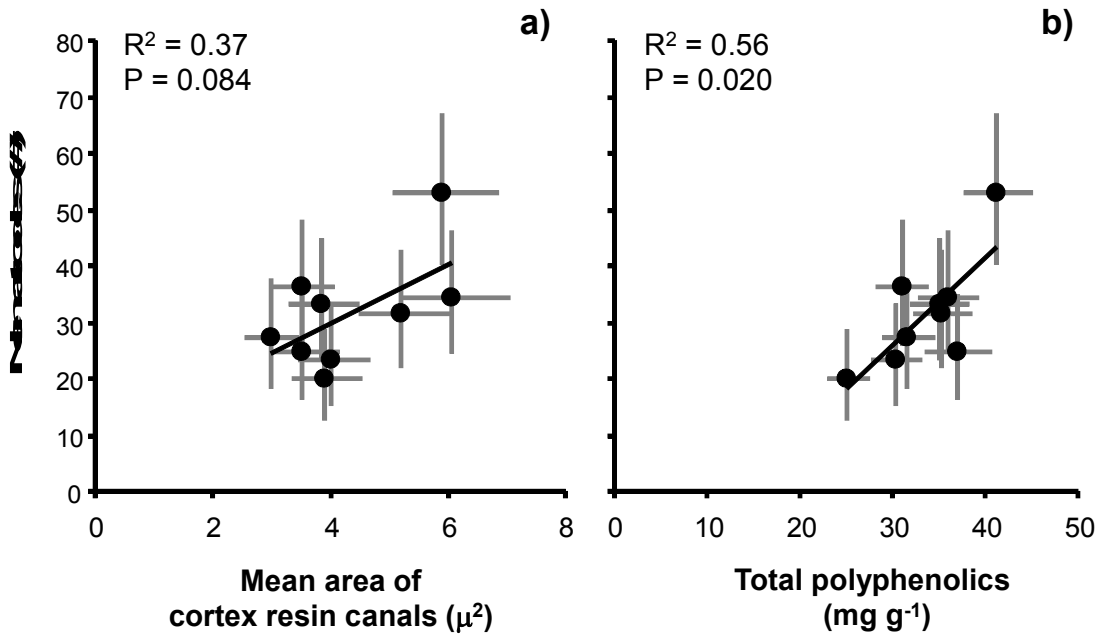

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Electronic Supplementary Material 1

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765Rafael Zas, Xoaquín Moreira, Miguel Ramos, Marta R.M. Lima, Marta Nunes da Silva, Alejandro Solla, Marta W. Vasconcelos, Luis Sampedro.
7662014. Intraspecific variation of anatomical and chemical defensive traits in maritime pine (*Pinus pinaster*) and its relationship to the pinewood
767nematode (*Bursaphelenchus xylophilus*) migration rate

768

769TABLE S1. Main geographic and climatic features characteristic of the studied provenances.

	Code	Longitude (W)	Latitude (N)	Altitude (m)	Annual precipitation (mm)	Summer precipitation (mm)	Annual mean temperature (ºC)	Mean of minimum monthly temperatures (ºC)	Thermal oscillation (ºC)
Galicia-Costa	CG	8°09’ - 9º10’	41º57’ - 43º31’	0-600	1600	200	14.5	7.6	10.1
Leiria	LE	8°96’	39°00’ - 40°30’	80	790	105	15.9	11.9	na
Sierra de Gata	SG	6°07’ - 7°01’	40°09’ - 40°29’	350-900	924	87	15.4	0.8	12.3
Bajo Tiétar	BT	5°23’ - 5°53’	39°50’ - 40°05’	400	1060	70	14.4	1.5	13.2
Sierra de Gredos	GR	4°17’ - 5°10’	40°07’ - 40°27’	600-1400	1398	83	13.4	-0.2	11.6
Montaña de Soria-Burgos	SB	2°27’ - 3°27’	41°43’ - 41°56’	800-1200	686	105	11.3	-2.5	11.2
Sierra de Albarracín	AL	1°12’ - 1°51’	40°03’ - 40°25’	1000-1400	878	155	9.6	-4	12.1
Serranía de Cuenca	SC	0°53’-2°25’	39°25’-40°37’	800-1200	684	101	12.3	-1.1	13.1
Sierra Segura-Alcaraz	SS	1°57’-3°00’	37°46’-38°46’	800-1400	787	65	13.7	2.4	11.7

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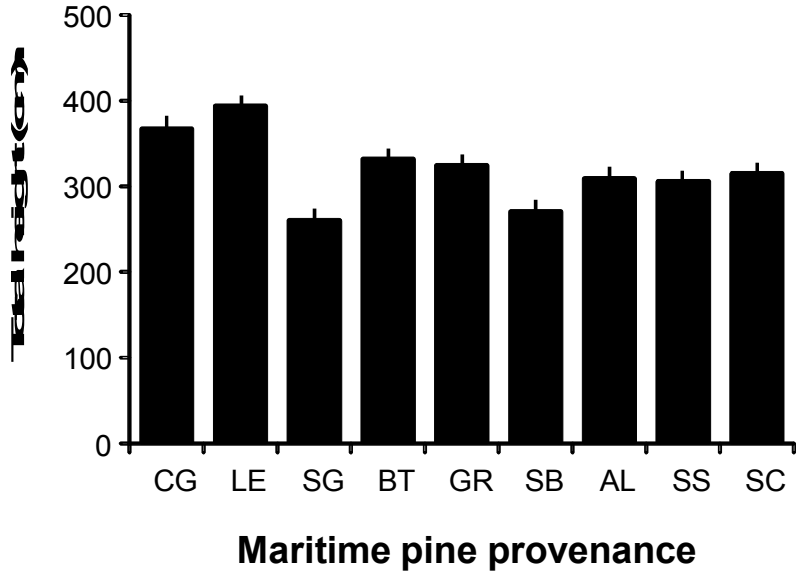
Electronic Supplementary Material 2

Rafael Zas, Xoaquín Moreira, Miguel Ramos, Marta R.M. Lima, Marta Nunes da Silva, Alejandro Solla, Marta W. Vasconcelos, Luis Sampedro. 2014. Intraspecific variation of anatomical and chemical defensive traits in maritime pine (*Pinus pinaster*) and its relationship to the pinewood nematode (*Bursaphelenchus xylophilus*) migration rate

Figure S1. Total height at sampling of nine Maritime pine provenances. The provenances assayed were Coastal Galicia (CG), Leiria (LE), Sierra de Gata (SG), Bajo Tietar (BT), Sierra de Gredos (GR), Montaña de Soria-Burgos (SB), Sierra de Albarracín (AL), Serranía de Cuenca (SC) and Sierra Segura-Alcaraz (SS). Each provenance was represented by 10 individuals established in a common garden test located in NW Spain. Mean ± s.e. are shown.



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