

## Phenotyping *Castanea* hybrids for *Phytophthora cinnamomi* resistance

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*Castanea sativa* is susceptible to *Phytophthora* spp., a serious root pathogen causing ink disease, while *C. crenata* and *C. mollissima* show resistance to infection. Interspecific controlled crosses were established for introgression of resistance genes from the resistant species into the susceptible *C. sativa*, and two mapping populations were created. *Phytophthora cinnamomi* resistance of each progeny was evaluated by root and excised shoot inoculation tests. The number of days of survival after root inoculation was the best discriminator of resistance to *P. cinnamomi* while the percentage of shoots with internal lesions was the symptom most associated with survival. The lesion progression rate in the excised shoot inoculation test was strongly and negatively correlated with survival in the root inoculation test. The excised shoot inoculation test appears to be a reliable approach for screening the resistance of chestnut genotypes to *P. cinnamomi*. Strong genetic correlations were obtained between survival and ink disease symptoms and among symptoms, indicating that common or linked genes might influence resistance to *P. cinnamomi*. The most resistant genotypes selected from this study will be tested for other commercial variables, such as ease of vegetative propagation and stock–scion compatibility.

**Keywords:** *Castanea* hybrids, heritability, phenotypic and genetic correlations, *Phytophthora cinnamomi*

### Introduction

The genus *Castanea* belongs to Fagaceae, a plant family that dominates much of the climax hardwood forests of the northern hemisphere (Manos *et al.*, 2008). The European chestnut (*Castanea sativa*) is considered to be the only native species in Europe. Chestnuts are multipurpose trees being used in the food industry, for their edible nuts, in the wood industry, as timber and also for ecological and landscaping purposes, having a major economic importance in the Mediterranean region.

Chestnut fruit production has declined considerably in southwestern Europe due to social changes and cultural development, and particularly to the emergence of heavily damaging diseases. Ink disease, caused by *Phytophthora* spp., is one of the most destructive diseases affecting *C. sativa*. *Phytophthora cinnamomi* is an aggressive root pathogen, originally from the southeast Asian tropics (Hardham, 2005). However, because *P. cambivora* is also responsible for ink disease, it is difficult to infer the exact time of *P. cinnamomi* introduction in Europe. Nowadays, *P. cinnamomi* is widespread and continues to be destructive in forests of Mediterranean countries, Australia, southeast USA,

southern California and more recently it was recognized as a danger to forests in western North America (Robin *et al.*, 2012).

*Phytophthora cinnamomi* has an exceptionally wide host range, being able to invade more than 3000 plant species around the world (Hardham, 2005; Cahill *et al.*, 2008). Currently, it is the most important *Phytophthora* pathogen of forest trees; besides chestnut, *P. cinnamomi* causes root diseases in eucalyptus, oaks, pines and members of the Ericaceae family, as well as several agricultural crops (Robin *et al.*, 2012).

Disease symptoms in chestnut are similar to other species: ink disease causes root rot, with necrosis of the tap root, which then extends to the lateral roots and the collar. *Phytophthora cinnamomi* infection induces necrosis of the cambial and xylem tissues, causing interference with transpiration from roots to shoots, and consequently causes wilting of leaves and dieback of young shoots (Marçais *et al.*, 1996; Robin *et al.*, 2001; Vannini & Vettraiño, 2001; Hardham, 2005; Gomes-Laranjo *et al.*, 2009).

The pathogen spreads slowly through root-to-root contact and more rapidly in the presence of water. Human activities that move soil and the planting of infested nursery stock intensify pathogen spread (Robin *et al.*, 2012). With changing climates, *P. cinnamomi* is expected to expand its area of destruction, mainly in Europe and North America (Robin *et al.*, 2012).

In Portugal, ink disease has become widespread since *P. cinnamomi* was first recorded in 1838. Despite the

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protection measures taken, it is still a great threat to chestnut orchards, as fruit and timber production is negatively impacted. Chestnut production is an important source of income for rural populations and so new plantings have been carried out while old orchards are being restored. In Europe, chestnut breeding for ink resistance began with the introduction of the Asian chestnut germplasm that is resistant to the main diseases: ink disease and blight (*Cryphonectria parasitica*). Japanese and Chinese species (*Castanea crenata* and *Castanea mollissima*, respectively) were introduced in 1917 in several southern European countries (Elorrieta, 1949). However, the low value of the Asian species as timber and fruit producers was notable. They also presented low compatibility for grafting with local sweet chestnut varieties (Elorrieta, 1949). In Portugal, the first interspecific hybridizations were initiated in 1948 by Bernardino Barros Gomes to introduce resistance to ink disease in *C. sativa* (Guerreiro, 1957). The objectives of these programmes were to breed for resistance to ink disease, as well as to produce rootstock or varieties selected for early nut production or better wood production as compared with Asian species (Fernández-López, 2011).

In 2006 interspecific controlled crosses were performed between *C. sativa* × *C. crenata* (SC) and *C. sativa* × *C. mollissima* (SM) in order to introgress the resistance from Asian species into the European (Costa *et al.*, 2011). The main goal of this on-going programme was to produce a hybrid segregating population to perform DNA marker–phenotype association analysis to identify genomic regions related to ink disease resistance (quantitative trait loci, QTL). For this purpose, it is crucial to determine accurately both genotype and phenotype of each hybrid progeny. However, there are some limitations for the determination of the resistance of *Castanea* spp. to *P. cinnamomi* (phenotyping).

Different methods have been used for screening the resistance of chestnut to *Phytophthora* spp. by different authors: (i) root inoculation using seedlings (Vettraino *et al.*, 2001a,b; Santini *et al.*, 2003; Robin *et al.*, 2006; Jeffers *et al.*, 2009); (ii) root inoculation using cuttings (Miranda-Fontañá *et al.*, 2007) or (iii) plantlets from micropropagation (Cuenca *et al.*, 2009); and (iv) direct inoculation on the top of excised or intact stems/shoots from seedlings or clones selected in the field (Guedes-Lafargue & Salesses, 1999; Fernández-López *et al.*, 2001; Vettraino *et al.*, 2001b; Robin *et al.*, 2006; Miranda-Fontañá *et al.*, 2007; Cuenca *et al.*, 2009). There are advantages and drawbacks to each method; in particular, shoot inoculation is easy to achieve and enables the screening of a high number of individuals at low cost (Fernández-López *et al.*, 2001), but a criticism of the method is that *P. cinnamomi* is a root pathogen.

The objectives of the present study were (i) to select the best resistance discriminators from root and excised shoot inoculation tests and clarify their correlations; and (ii) to assess the resistance to *P. cinnamomi* and evaluate its heritability in progenies of two *Castanea* sp. segregating populations.

## Materials and methods

### Plant material

Two full-sib progenies were obtained from artificial controlled crosses, SC (*C. sativa* × *C. crenata*) and SM (*C. sativa* × *C. mollissima*) in 2006 and 2009. *Castanea sativa* female flowers were isolated before pollination season by placing paper bags on the branches and cutting off the closer catkins. The parental line of *C. sativa* was the same for both crosses. Catkins from *C. crenata* and *C. mollissima* were collected and dried the day before pollination. On the day of pollination, pollen was removed from catkins and was placed on the stigmas using a paintbrush. Pollinated flowers were covered with paper bags, which were kept in place until the end of the pollination season. Subsequently, paper bags were replaced by net bags in order to collect the seeds. The crosses were performed at the germplasm bank of Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal (47°17'10"N, 7°44'43"W), located at 423 m a.s.l. on a slope facing southwest stand.

A total of 66 F<sub>1</sub> genotypes were tested for *P. cinnamomi* infection by either root inoculation or excised shoot inoculation or by both (Table 1). For the root inoculation test, 137 plantlets were produced from 20 genotypes by *in vitro* propagation from buds of mother plants. At the time of inoculation, plantlets were different ages, as determined by the number of days after acclimatization, but were most frequently 80 days old; aerial parts were 16–66 cm on average.

The excised shoot inoculation tests were carried out in the spring and autumn of 2012 for a total of 47 and 60 progenies, respectively (Table 1). A total number of 829 shoots were collected from the mother plants. Both experiments were performed in a controlled chamber with temperatures ranging between 18 and 22°C, photoperiod 16 h light/8 h dark and 65% relative humidity.

### Inoculum of *P. cinnamomi*

In all experiments, the same isolate of *P. cinnamomi* was used (IMI 340340), which was selected as the most virulent following tests using several isolates (Abreu *et al.*, 1999). The high pathogenicity of this isolate in European chestnuts was also confirmed by Dinis *et al.* (2011).

For the root inoculation test, the *P. cinnamomi* inoculum was prepared by growing mycelia on sterilized millet seeds (*Panicum mileaceum*), which were thoroughly moistened with V8 medium broth. Afterwards, this mixture was incubated for 3 weeks in darkness at 25°C.

For the excised shoot inoculation test, *P. cinnamomi* was grown on potato dextrose agar for 6 days in darkness at 25°C.

**Table 1** Number of individuals from *Castanea sativa* × *C. crenata* (SC) and *C. sativa* × *C. mollissima* (SM) crosses tested by root inoculation and excised shoot inoculation

| Test method                        | SC | SM | Total |
|------------------------------------|----|----|-------|
| Root inoculation                   | 16 | 4  | 20    |
| Excised shoot inoculation (total)  | 45 | 18 | 63    |
| Excised shoot inoculation (spring) | 30 | 17 | 47    |
| Excised shoot inoculation (autumn) | 42 | 18 | 60    |
| Total                              | 48 | 18 | 66    |

### Root inoculation test

Between November 2011 and September 2012, four root inoculation experiments were carried out using clonal plantlets placed in sterile substrate. For each experiment, one or two plantlets of each genotype were used as a control, without inoculation. For root inoculation, *P. cinnamomi*-infected millet seed inoculum was carefully placed into the substrate (600 mL) of each pot, at a concentration of 5% (v/v). Mostly, eight replicates per genotype were inoculated, but, due to limitations of *in vitro* propagation, this was not always possible. Therefore, the mean number of plants per genotype was 6.85 and the design of the test was unbalanced.

Inoculated plants and controls were placed separately in different trays and each pot was flooded for 1 h, three times a week, to stimulate zoospore release and promote disease development.

*Phytophthora cinnamomi* was recovered from water collected from the flooding process, using a modified baiting technique adapted from Jung *et al.* (1996).

The experimental design was adapted from Miranda-Fontaiña *et al.* (2007). For each individual, the days of survival after inoculation were recorded until 100 days after inoculation (dai). After death, plantlets were removed from the soil and the roots were gently washed to observe and record ink disease symptoms. The level of root rot was assessed on a scale from 1 to 6, according to Miranda-Fontaiña *et al.* (2007), where 1 indicates the least severe level of root rot and 6 indicates the most severe level. The degree of root collar rot was assessed by measuring the area of the lesion in a transverse section of the collar and expressing this as a percentage of the total area. The percentage was then used to rate the level of root collar rot on a scale of 1–6 (1, no rot; 2, 0.1–9.9% rot; 3, 10–19.9% rot; 4, 20–29.9% rot; 5, 30–49.9% rot; and 6, >50% rot). Shoot internal and external lesions were recorded as the percentage length of internal and external lesion of the longest shoot, respectively. Biomass parameters were also evaluated for each plantlet: leaf and shoot dry weight (g) and root dry weight (g). In order to determine the dry weight, leaves and shoots were separated from roots and both parts were dried at 60°C for 2 days.

At the end of each experiment, plantlets that did not die during the experiment were analysed without destruction and were transplanted to new pots that were placed in a greenhouse with controlled conditions (20–25°C). In spring of 2013 the number of plantlets that showed budburst was recorded.

### Excised shoot inoculation test

The excised shoot inoculation tests took place in spring 2012, using 47 genotypes (eight genotypes were common to the root inoculation test), and in autumn 2012, using 60 genotypes (16 common to the root inoculation test) (Table 1). Excised shoots, the majority with a length of 15 cm, were collected from each mother plant. The mean number of excised shoots inoculated per genotype was 7.94 in spring and 7.60 in autumn. All apart from the two upper leaves were removed to reduce evapotranspiration. The diameter of the top of each excised shoot was recorded before inoculation. Mycelial plugs of *P. cinnamomi* were then placed on the top of the shoots and were covered with an aluminium sheet to avoid desiccation. Replicates were distributed randomly in three trays with perlite and water, in an environmental controlled chamber. Five days after inoculation, the aluminium sheets were removed from each shoot, when colonization by the pathogen had occurred. Resistance to *P. cinnamomi*

was evaluated by measuring the visible external lesion length (LL) at 5, 7, 9, 12 and 14 dai. The lesion progression rate (cm/day) was calculated for each genotype, using the following formula:

$$\begin{aligned} &[(LL_{5 \text{ dai}}/5) + (LL_{7 \text{ dai}} - LL_{5 \text{ dai}})/2 + (LL_{9 \text{ dai}} - LL_{7 \text{ dai}})/2 \\ &+ (LL_{12 \text{ dai}} - LL_{9 \text{ dai}})/3 + (LL_{14 \text{ dai}} - LL_{12 \text{ dai}}/2)]/5. \end{aligned}$$

### Statistical analysis

Analysis of variance (ANOVA) was conducted for root and shoot variables using linear mixed effects models of the general form  $y = X\beta + Z\gamma + \varepsilon$ , where  $y$  is the vector of observations;  $X$  and  $Z$  are design matrices of the parameters associated to fixed and random effects, respectively;  $\beta$  and  $\gamma$  are vectors of fixed effects (including the general mean) and random effects, respectively; and  $\varepsilon$  is the vector of residual errors. In preliminary data analyses, resistance from biparental crosses was evaluated separately for each cross by specifying a two-level 'Family' effect (SC and SM). Because no significant differences were found between the two full-sib families, the family effect was dropped from the model. For the root inoculation test data, Age, Genotype, Inoculation, Inoculation Date and Genotype  $\times$  Inoculation Date were fitted as fixed effects ( $\beta$  vector); for the excised shoot inoculation test data, Length, Genotype, Season and Genotype  $\times$  Season were treated as fixed effects. *F*-tests were used to test the significance of the fixed effects and of genotype mean comparisons, the latter implementing the Tukey adjustment and a matching letter display (adapted from Piepho, 2012). If significant, the covariate age was included in multiple comparison of means and in least squares means (LSM) estimation (at Age = 80 days; not for level of root rot and level of root collar rot); and similarly for the covariate Length (at Length = 15 cm). To gain insight into the variation pattern, a further partition of *F*-tests was performed on the shoots test data, to search for the season and day within season where differences between genotypes were maximized. The restricted error maximum likelihood (REML) estimation was used, with a significance level of  $\alpha = 0.05$  and the Satterthwaite approximation for degrees of freedom (d.f.) when data were missing. Plots of the Studentized residuals showed conformation to the assumptions of the mixed model, except for survival, level of root rot, level of root collar rot and lesion length, which were modelled with a log-normal distribution; shoot internal lesion and shoot external lesion, which were arcsine-transformed and modelled with a Gaussian distribution, for all statistical tests (LSM are reported on the original variable scale). Whenever appropriate, variance heterogeneity between inoculation dates or seasons (root and excised shoot inoculation test, respectively) was fitted by adjusting different residuals by group ( $\varepsilon$  vector).

Phenotypic and genetic correlations were estimated for different variables measured in the same plants of the root inoculation test (based on all observations); and between pairs of variables from the two tests (based on genotype LSM). Phenotypic correlations were analysed with the nonparametric (distribution-free) Spearman's correlation coefficient. Genetic correlations were obtained from the additive genotypic covariance matrix in multivariate repeated measures analysis using REML estimation. The transformation  $\log(2y + 1)$  was applied to data, in order to meet the model assumptions.

Genotype-level narrow-sense heritabilities were estimated with the REML approach for root and shoot variables as  $h^2 = \sigma_a^2/(\sigma_a^2 + \sigma_e^2)$  where additive genetic variance  $\sigma_a^2$  was the

genotype variance component, implemented with an additive relationship matrix obtained with pedigree information;  $\sigma_e^2$  was the residual variance component; and  $\sigma_a^2 + \sigma_e^2$  was the phenotypic variance. Age (covariate) and Inoculation Date (root inoculation test) and the covariate Length (excised shoot inoculation test) were included as fixed effects in the heritability calculation. Data transformations and distributions used to model the response variables were applied as explained above. Approximate standard errors (SE) of heritabilities were obtained by Taylor series expansion. All analyses were conducted using SAS v. 9.3 software (GLIMMIX, CORR and INBREED procedures).

## Results

### Root inoculation test

All control plantlets survived until the end of the experiment. No symptoms associated with *P. cinnamomi* were observed in the roots, collar or shoots of the control plantlets, indicating that cross contamination did not occur. A reduction in the growth of inoculated plantlets was observed, when compared with noninoculated plantlets. Over the course of the experiment, the average growth of *P. cinnamomi*-inoculated and control plantlets was 1.23 cm and 1.71 cm, respectively.

At the end of experiment, only 18 inoculated plantlets (13.14%) survived, corresponding to seven different genotypes. After death, plantlets showed the typical visual symptoms of *P. cinnamomi*: chlorosis and wilting of leaves and die-back of shoots. Susceptible plantlets showed high levels of root rot, characterized by long necrotic lesions in roots, which in some cases extended to collar and shoots.

Analysis of variance revealed a highly significant effect of age at inoculation on survival and shoot internal lesion (Table 2). Genotype mean values obtained for survival and adjusted for the age covariate effect, showed that there were big differences in the resistance pattern obtained between different genotypes; the most susceptible genotype (SC918) survived only 10 days, while the resistant ones survived up to 70 dai (Table 3) and the average time of survival was  $33.44 \pm 3.36$  days (Table 2). Fifty-five percent of the overall genotypes had

a survival below average and 20% did not survive more than 15 dai; however, 45% of them (nine genotypes) survived beyond 33 days. At the end of the experiment the majority of individuals belonging to seven genotypes (SC24, SM904, SC51, SC55, SC09, SC01, SC57) survived after root inoculation and sprouted in the following spring.

The ANOVA (Table 2) for the effects of Age, Genotype, Inoculation (i.e. inoculation or non-inoculation), Inoculation Date and the interaction between Genotype and Inoculation ( $G \times I$ ) revealed that these effects were significant for most of the variables analysed. For all plantlets tested (with root inoculation and control), there was a highly significant effect of Age, Genotype, Inoculation, Inoculation Date and  $G \times I$  on survival and shoot internal lesions. Inoculation had the strongest effect on all variables, as expected. Genotype had a highly significant influence on survival, level of root rot and shoot internal lesion, while level of root collar rot was less influenced by Genotype. On the other hand, the Genotype effect had no significant influence on shoot external lesions (Table 2).

The severity of symptoms decreased progressively from the point of inoculation (roots) to shoots. Depending on the genotype, the root rot was either limited and localized on some roots or invasive to the entire root system, causing wilting of leaves (Fig. 1b). Almost all of inoculated plantlets (99.3%) and all genotypes showed symptoms of root rot. The overall mean level of root rot was  $4.80 \pm 0.12$  (Table 2). Figure 1c shows a healthy root and root collar while Figure 1d shows an example of level 5 on the scale for root rot: large roots with few lesions of less than 2.5 cm, as described by Miranda-Fontaiña *et al.* (2007). Observation in detail (Fig. 1g) shows that, at level 5, the lesions on the roots were not continuous. Genotype mean levels ranged from 2.69 (SC51) to 5.67 (SC32) (Table 3). At the time of death, SC51, SC24 and SC57 genotypes displayed confined, small lesions in a few roots, showing resistance to the spread of the pathogen through the roots. These genotypes are significantly different from SC32, SC916, SC919 and SM919 genotypes, which exhibited a high

**Table 2** Effect of the Age, Genotype, Inoculation, Inoculation Date, and Genotype  $\times$  Inoculation interaction on days of survival and ink disease symptoms for all root-inoculated plantlets and controls

| Effect                        | Days of survival | Level of root rot | Level of root collar rot | Shoot internal lesion | Shoot external lesion |
|-------------------------------|------------------|-------------------|--------------------------|-----------------------|-----------------------|
| Age <sup>a</sup>              | 22.06***         | 0.02              | 3.63                     | 13.04***              | 5.92*                 |
| Genotype                      | 11.01***         | 3.63***           | 2.09*                    | 5.40***               | 1.54                  |
| Inoculation                   | 488.00***        | 2229.32***        | 210.98***                | 126.28***             | 63.74***              |
| Inoculation date              | 7.69***          | 2.87*             | 3.90*                    | 7.49***               | 9.11***               |
| Genotype $\times$ Inoculation | 11.26***         | 5.21***           | 2.67**                   | 6.41***               | 1.24                  |
| Mean value $\pm$ SE           | $33.44 \pm 3.36$ | $4.80 \pm 0.12$   | $4.70 \pm 0.24$          | $69.45 \pm 6.51$      | $16.73 \pm 2.01$      |

<sup>a</sup>Age: days after acclimatization.

F-test significance is indicated by asterisks: \* $P < 0.05$ ; \*\* $P < 0.01$ ; and \*\*\* $P < 0.001$ . Least squares mean values  $\pm$  standard error (SE) adjusted for the covariate Age for survival, shoot internal lesion and shoot external lesion are represented on the original variable scale (only for inoculated plantlets). Level of root rot and root collar rot was registered using a scale from 1 to 6 and shoot internal and external lesion were calculated by the percentage length of internal and external lesion of the longest shoot, respectively.

**Table 3** Ranking of genotypes tested by root inoculation, according to the least squares means (LSM) of days of survival, levels of root and collar rot, and shoot internal and external lesion, on the original variable scale (adjusted for the covariate Age at 80 days)

| Genotype | Days of survival |      | Level of root rot |      | Level of root collar rot |      | Shoot internal lesion |      | Shoot external lesion |      |
|----------|------------------|------|-------------------|------|--------------------------|------|-----------------------|------|-----------------------|------|
|          | LSM              | Test | LSM               | Test | LSM                      | Test | LSM                   | Test | LSM                   | Test |
| SC01     | <u>62.36</u>     | ab   | <u>4.44</u>       | ac   | <u>4.64</u>              | ac   | <u>31.32</u>          | cf   | <u>23.63</u>          | ab   |
| SC09     | <u>65.29</u>     | ab   | <u>4.87</u>       | ac   | <u>4.74</u>              | ac   | <u>32.76</u>          | ef   | <u>15.00</u>          | bc   |
| SC19     | 18.69            | def  | 5.48              | a    | 5.25                     | a    | 69.72                 | cde  | 15.60                 | bc   |
| SC24     | <u>77.45</u>     | abcd | <u>2.74</u>       | e    | <u>2.08</u>              | bc   | <u>53.50</u>          | bdf  | <u>15.50</u>          | bc   |
| SC32     | 18.53            | def  | <u>5.67</u>       | a    | 6.00                     | a    | 100                   | bd   | 14.00                 | bc   |
| SC36     | 44.51            | abce | 5.48              | acd  | 4.78                     | ab   | 29.95                 | bdf  | 23.50                 | b    |
| SC51     | <u>72.29</u>     | af   | <u>2.69</u>       | de   | <u>1.93</u>              | bc   | <u>58.48</u>          | bdf  | <u>7.71</u>           | bc   |
| SC55     | <u>70.30</u>     | ab   | <u>4.07</u>       | bc   | <u>2.06</u>              | b    | <u>14.58</u>          | f    | <u>17.00</u>          | bc   |
| SC57     | <u>59.22</u>     | abc  | <u>3.19</u>       | cd   | <u>3.93</u>              | ab   | <u>23.85</u>          | ef   | <u>8.00</u>           | c    |
| SC903    | 12.49            | ef   | 4.86              | ac   | 5.60                     | a    | 72.64                 | abc  | 12.30                 | bc   |
| SC904    | 12.64            | f    | 4.41              | ac   | 3.93                     | ab   | 92.09                 | abe  | 16.10                 | bc   |
| SC912    | 16.68            | def  | 5.44              | a    | 4.68                     | a    | 100                   | bd   | 12.60                 | bc   |
| SC914    | 28.48            | cf   | 5.01              | ac   | 4.34                     | ab   | 52.07                 | adf  | 19.00                 | ab   |
| SC916    | 34.20            | bf   | 5.67              | ab   | 3.02                     | ab   | 41.49                 | bdf  | 14.40                 | bc   |
| SC918    | 10.25            | f    | 4.60              | ac   | 5.12                     | a    | 100                   | bd   | 9.77                  | bc   |
| SC919    | 16.25            | def  | 5.57              | a    | 5.81                     | a    | 100                   | ab   | 16.70                 | ab   |
| SM901    | 25.13            | cf   | 5.23              | acd  | 4.53                     | ab   | 45.79                 | bdf  | 4.16                  | bc   |
| SM904    | <u>75.70</u>     | a    | <u>5.01</u>       | acd  | <u>3.68</u>              | ab   | <u>41.89</u>          | ef   | <u>8.25</u>           | ac   |
| SM906    | 14.98            | bf   | <u>5.15</u>       | acd  | 6.00                     | ab   | 100                   | abc  | <u>23.20</u>          | bc   |
| SM919    | 15.04            | ef   | 5.54              | a    | 5.73                     | a    | 100                   | b    | 20.50                 | ab   |

LSM: least squares means.

Estimated least squares greater than 100%, due to the model adjustment for the covariate Age at 80 days, were replaced by 100%. Means followed by different letters in the same column are significantly different at  $P \leq 0.05$ , using the Tukey method; the letter display of the mean comparisons was obtained by adapting the macro described in Piepho (2012) (for the multiple-comparison tests, the normality assumption was approached as described in the statistical analysis section). The LSM of variable for the seven most resistant genotypes are underlined. Level of root rot and root collar rot was registered using a scale from 1 to 6, and shoot internal and external lesion were calculated by the percentage length of internal and external lesions of the longest shoot, respectively. The SC918 genotype showed the highest susceptibility to *P. cinnamomi* whereas SC24 was the most resistant.

level of root rot, indicating that the pathogen invaded the entire root system.

Most of the inoculated plantlets exhibited root collar rot (84.7%), with an overall average score of  $4.70 \pm 0.24$  on the root collar rot scale (Table 2) and genotype mean levels ranging from 1.93 (SC51) to 6.00 (SC32) (Table 3). Although the genotypes SC32, SC919 and SM919 again showed the highest levels of rot, no significant differences were observed among the genotypes, except for SC55.

The shoot internal (Fig. 1e) and external (Fig. 1f) lesions developed in 81.8% and 73.7% of inoculated plantlets, respectively, with mean lesion lengths (as % of shoot length) of  $69.45 \pm 6.51$  and  $16.73 \pm 2.01$ , respectively (Table 2). Similar to results obtained for root rot, shoot internal and external lesions were found at their highest levels in genotypes SC32, SC919 and SM919 (Table 3). However, depending on genotype, a large variation was observed in the mean shoot internal lesion, ranging from 14.58% (SC55) to 100% (SC918). The average variation of the percentage of shoot external lesion was lower than shoot internal lesion, ranging from 4.16% (SM901) to 26.63% (SC918) (Table 3).

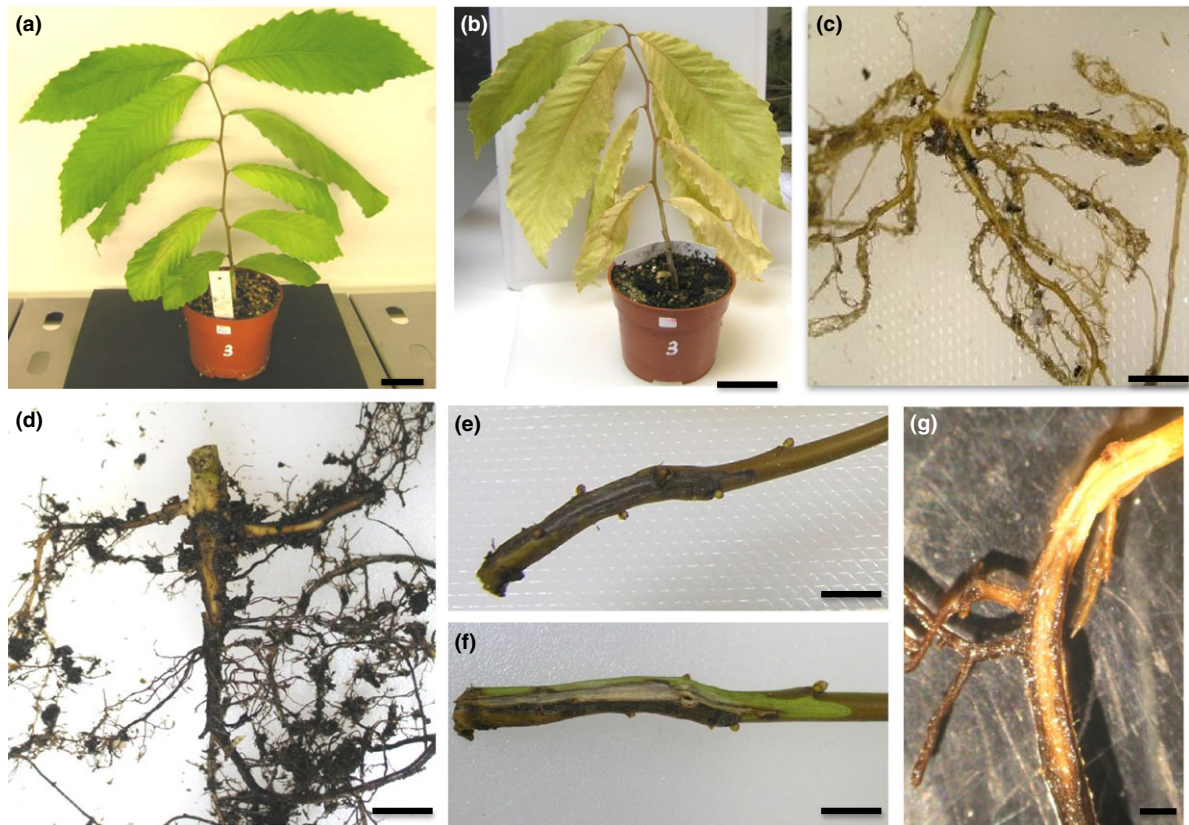
The phenotypic and genetic correlation coefficients were estimated for all the variables recorded on

inoculated plantlets. Both correlation coefficients showed a great similarity among pairs of variables, in regard to direction and to magnitude (Table 4).

The phenotypic correlations showed that survival had highly significant negative correlations with three of the four ink disease symptoms analysed: level of root collar rot, shoot internal lesion and shoot external lesion. Both phenotypic and genetic correlations showed that shoot internal lesion was the main symptom negatively associated to survival. The level of root rot was the least important symptom associated to survival, with nonsignificant phenotypic correlation (Table 4).

The phenotypic correlations evaluated between survival and the biomass parameters were positive and highly significant (Table 4). Correlations were lower for leaves and shoots than for roots, while the symptoms and biomass parameters were not strongly correlated. The phenotypic correlation coefficients observed between symptoms were positive and highly significant, especially level of root collar rot with shoot external lesion, followed by shoot external lesion with shoot internal lesion. The weakest correlated symptoms were level of root rot with shoot external lesion (Table 4).

The highest genetic correlations were found among symptoms and among biomass parameters. The genetic



**Figure 1** Root-inoculated plantlet (SC914, replica 3) at the time of inoculation (a) and after death (b), showing typical symptoms of *Phytophthora cinnamomi*. (c) Healthy root and root collar from a control plantlet, without inoculation. (d) Root with high level of root rot (level 5) and low level of collar rot (level 2). (e) External lesion of the longest shoot and (f) internal lesion of the longest shoot. (g) Detail of a fine root with partial root rot. Scale bar sizes: a, b = 5 cm; c, d = 2.5 cm; e, f = 1 cm; g = 4 mm.

**Table 4** Phenotypic (above the diagonal) and genetic (below the diagonal) correlation coefficients determined between pairs of the variables measured in root inoculation test ( $n = 108\text{--}137$ )

|                           | Days of survival | Leaf and shoot dry weight | Root dry weight | Level of root collar rot | Level of root rot | Shoot external lesion | Shoot internal lesion |
|---------------------------|------------------|---------------------------|-----------------|--------------------------|-------------------|-----------------------|-----------------------|
| Age                       | 0.31***          | 0.38***                   | 0.60***         | -0.01                    | -0.02             | -0.05                 | -0.22*                |
| Days of survival          |                  | 0.43***                   | 0.50***         | -0.42***                 | -0.16             | -0.44***              | -0.69***              |
| Leaf and shoot dry weight | 0.51             |                           | 0.75***         | 0.02                     | 0.18              | -0.13                 | -0.26*                |
| Root dry weight           | 0.64             | 0.99                      |                 | 0.02                     | 0.18              | -0.10                 | -0.24*                |
| Level of root collar rot  | -0.88            | -0.08                     | -0.23           |                          | 0.49***           | 0.76***               | 0.61***               |
| Level of root rot         | -0.86            | -0.05                     | -0.21           | 1.00                     |                   | 0.40***               | 0.44***               |
| Shoot external lesion     | -0.90            | -0.13                     | -0.29           | 1.00                     | 1.00              |                       | 0.66***               |
| Shoot internal lesion     | -0.97            | -0.36                     | -0.50           | 0.96                     | 0.95              | 0.97                  |                       |

Phenotypic correlations were analysed with Spearman's correlation coefficient. Genetic correlations were adjusted for the covariate Age effect (Age = 80 days after acclimatization). Significance is indicated by asterisks: \* $P < 0.05$ ; and \*\*\* $P < 0.001$ .

correlation coefficients among symptoms ranged from 0.95 to 1.00 (level of root collar rot with shoot external lesion), in agreement with phenotypic correlations.

The heritability values for chestnut resistance to *P. cinnamomi* varied between 0.34 and 0.90, with low standard errors. Survival showed the highest heritability ( $0.90 \pm 0.04$ ) with low residual variance and thus the highest potential to be inherited. Among symptoms, the highest heritability value was obtained for shoot internal

lesion whereas shoot external lesion showed the lowest value, with the lowest variance explained by both components, genetic and residual (Table 5).

#### Excised shoot inoculation

At 5 dai in both seasons, *P. cinnamomi* had induced visible necrotic lesions of varying length, depending on the genotype. At 14 dai a very low percentage (0.36%)

**Table 5** Narrow-sense heritabilities ( $h^2$ ) and their standard errors (in parentheses) estimated for the variables (root inoculation test)

| Variable                 | Variance component                |                           | $h^2$       |
|--------------------------|-----------------------------------|---------------------------|-------------|
|                          | Additive genetic ( $\sigma_a^2$ ) | Residual ( $\sigma_e^2$ ) |             |
| Days of survival         | 0.92 (0.34)                       | 0.10 (0.01)               | 0.90 (0.04) |
| Level of root rot        | 0.03 (0.02)                       | 0.04 (0.01)               | 0.46 (0.16) |
| Level of root collar rot | 0.25 (0.11)                       | 0.22 (0.03)               | 0.54 (0.11) |
| Shoot internal lesion    | 0.45 (0.19)                       | 0.15 (0.02)               | 0.75 (0.09) |
| Shoot external lesion    | 0.01 (0.01)                       | 0.03 (0.00)               | 0.34 (0.14) |

$n = 108-137$ .

of shoots did not show any lesion and 8.80% of shoots showed total necrosis (100% of shoot length).

ANOVA revealed that lesion length was significantly affected by Genotype, which had the strongest effect, and also by Season and the interaction of both (data not shown). In contrast to diameter, the Initial Shoot Length had a significant effect on the lesion length (data not shown), and therefore was used as a covariate effect in the analysis.

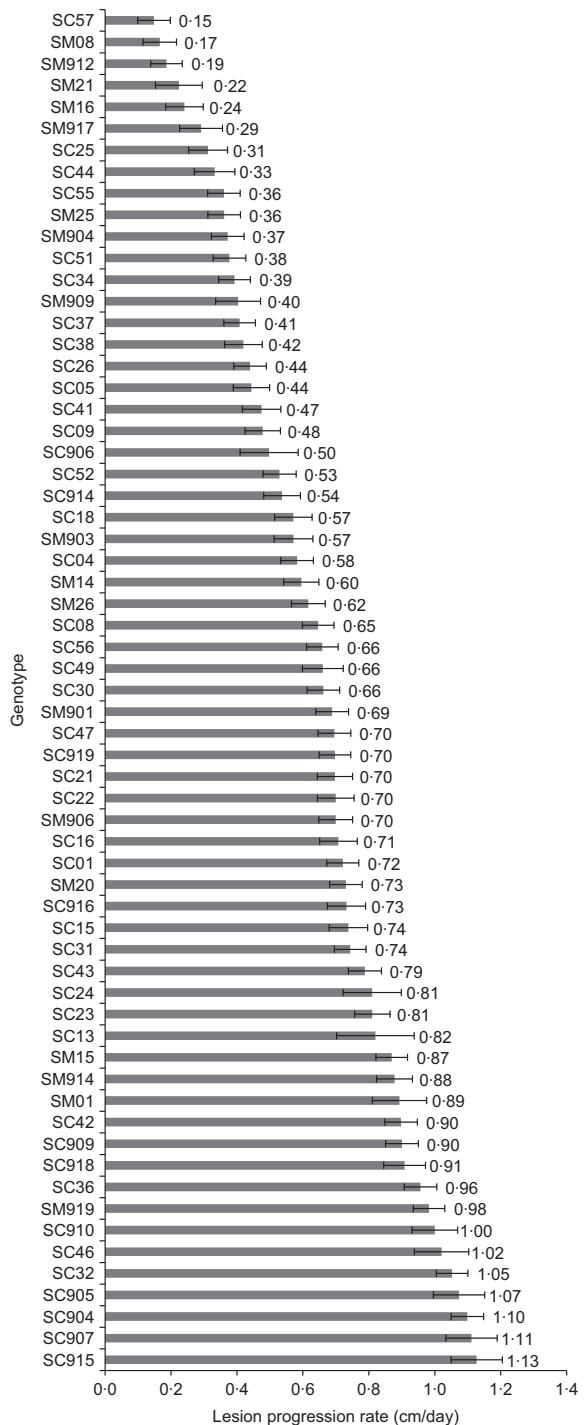
Further analysis showed that differences observed between genotypes for lesion length were very high for all time points of measurements and in both seasons (data not shown). Moreover, differences observed between genotypes for lesion length were maximal at 5 dai ( $F = 19.80$ ,  $P < 0.001$ ) in spring ( $F = 33.11$ ,  $P < 0.001$ ) after bud burst.

Different responses to *P. cinnamomi* were observed in the progenies: a continuous range of resistance-susceptibility levels among genotypes was observed. For the majority of genotypes, the lesion length in the shoots increased over time. In addition, for the most resistant genotypes the lesion length stopped at a given time point, until the end of the experiment. Therefore, the lesion progression rate (cm/day) was calculated for each genotype. Genotype mean values, estimated across the two seasons and adjusted for the covariate Initial Length, are shown in Figure 2. The lesion progression rate varied from 0.15 to 1.13 cm per day across genotypes and seasons; SC57 was the most resistant genotype while SC915 was the genotype with the greatest lesion progression rate (i.e. most susceptible).

### Correlations between inoculation tests

The estimation of phenotypic and genetic correlations between the root inoculation test and excised shoot inoculation test was possible using the mean response of common genotypes in both tests ( $n = 17$ ). In this way, some differences were obtained in the phenotypic and genetic correlations between survival and symptoms (Tables 4 & 6).

Phenotypic correlations obtained among all variables from both inoculation tests showed that lesion progression rate was the parameter with the strongest



**Figure 2** Mean values in lesion progression rate, adjusted for the covariate Initial Length, for 63 genotypes tested with excised shoot inoculation. The genotypes were ranked by lesion progression rate, from the most resistant to the most susceptible. SC915 genotype showed the highest susceptibility to *Phytophthora cinnamomi* whereas SC57 was the most resistant. Bars represent standard errors.

correlation with survival ( $r = -0.85$ ,  $P < 0.001$ ). Shoot internal lesion was correlated moderately with lesion progression rate, as well as level of root collar rot

**Table 6** Phenotypic (above the diagonal) and genetic (below the diagonal) correlation coefficients determined between pairs of the variables measured in root inoculation test: Days of survival, Level of root collar rot, Level of root rot, Shoot external lesion and Shoot internal lesion; and between the variables measured in root inoculation test and Lesion progression rate from excised shoot inoculation test ( $n = 17$ )

|                          | Days of survival | Level of root collar rot | Level of root rot | Shoot external lesion | Shoot internal lesion | Lesion progression rate |
|--------------------------|------------------|--------------------------|-------------------|-----------------------|-----------------------|-------------------------|
| Days of survival         |                  | -0.63**                  | -0.36             | -0.47                 | -0.74**               | -0.85***                |
| Level of root collar rot | -0.67            |                          | 0.59*             | 0.58*                 | 0.56*                 | 0.62*                   |
| Level of root rot        | -0.56            | 0.82                     |                   | 0.09                  | 0.36                  | 0.44                    |
| Shoot external lesion    | -0.45            | 0.62                     | -0.05             |                       | 0.75***               | 0.45                    |
| Shoot internal lesion    | -0.87            | 0.44                     | 0.39              | 0.66                  |                       | 0.67**                  |
| Lesion progression rate  | -0.83            | 0.62                     | 0.73              | 0.34                  | 0.75                  |                         |

Significance is indicated by asterisks: \* $P < 0.05$ ; \*\* $P < 0.01$ ; and \*\*\* $P < 0.001$ .

(Table 6). Genetic correlation coefficients were in agreement with phenotypic correlations and the heritability for lesion progression rate was  $0.67 \pm 0.04$ .

## Discussion

This study addresses two types of inoculation test used to determine the response of individual chestnut plantlets from two mapping populations to inoculation with *P. cinnamomi*. Lesion progression rate was the variable selected to perform DNA marker–variable association, for future QTL identification.

Although more than one isolate of *P. cinnamomi* is commonly used in this type of study, only one isolate was used in the present investigation in order to maximize the number of replicates of each chestnut genotype screened, thus making the analysis more robust and accurate. Several previous studies of *P. cinnamomi* on chestnut (Abreu *et al.*, 1999; Dinis *et al.*, 2011) enabled the most virulent isolate to be selected for the present investigation. Frampton *et al.* (2013) also used a single isolate of *P. cinnamomi* in soil inoculation of *Abies* spp. seedlings. Moreover, Fernández-López *et al.* (2001) and Miranda-Fontaiña *et al.* (2007) observed that there was no significant interaction between isolates and genotypes in the rot symptoms evaluated, indicating no specificity of those isolates in chestnut.

In previous studies, the origin and physiological conditions of plant material, replicate number, time point of lesion measurements and test conditions varied for both inoculation tests (Vettraino *et al.*, 2001a,b; Robin *et al.*, 2006; Miranda-Fontaiña *et al.*, 2007; Cuenca *et al.*, 2009). Therefore, in the present study, the experiments were designed in order to obtain the most reliable results possible: a high number of clonal plantlets per genotype and the use of the same controlled environmental conditions for both inoculation tests. Clonal testing of progeny from mapping populations is the most efficient way to minimize the effect of environmental variation and obtain better estimates of the phenotypic value (Bradshaw & Foster, 1992). Minimizing environmental variation and therefore increasing heritability, increases the robustness and the ability for QTL detection.

In the root inoculation test, control plantlets grew more than inoculated plantlets, as expected. The lesions in roots and shoots caused by *P. cinnamomi* may hinder water and nutrient absorption, and as a consequence, cause a reduction in the photosynthesis rate and growth. Robin *et al.* (2006) and Miranda-Fontaiña *et al.* (2007) also reported a reduction in growth of chestnut plants inoculated with *P. cinnamomi*. Cahill *et al.* (1989) observed that *P. cinnamomi* inoculation stopped root growth in a group of plant species within 24–48 h.

Previous studies have indicated that days of survival should be the main discriminator of *Phytophthora* spp. resistance in chestnut (Vettraino *et al.*, 2001a), in *Abies* spp. (Frampton *et al.*, 2013) and in *Eucalyptus* sp. (Stukely & Crane, 1994). However, other authors have considered the level of root or collar rot as the main indicator of resistance to *Phytophthora* spp. in chestnut (Robin *et al.*, 2006; Miranda-Fontaiña *et al.*, 2007; Cuenca *et al.*, 2009). The present study showed that variable ‘Days of survival’ was the most important indicator of resistance to *P. cinnamomi* because differences in response between genotypes were maximized; the presence of high levels of root and collar rot in almost all inoculated plantlets indicated that these symptoms were not good discriminators for resistance. Miranda-Fontaiña *et al.* (2007) also reported high levels of root and collar rot in a high percentage of chestnut plants. Likewise, Cuenca *et al.* (2009) observed root rot in 60% of the resistant *C. crenata* plants. Survival has not been considered as the main descriptor of resistance to *P. cinnamomi* in chestnut, mainly because of the high mortality of control chestnut plants before and during the experiment due to biotic or abiotic factors or cross contamination (Miranda-Fontaiña *et al.*, 2007; Cuenca *et al.*, 2009). In this study, all control plantlets survived until the end of the experiment, indicating that *P. cinnamomi* cross contamination and other biotic and abiotic stresses, such as flooding, did not occur. In future, similar studies should include preventive measures, such as the use of sterile substrates and avoiding excess flooding time during the experiments. Twenty to thirty minutes flooding is sufficient for *P. cinnamomi* cysts to germinate and release zoospores (Hardham, 2005). In the present investigation, long survival was considered evidence of high resistance



and thus seven genotypes (35%) were selected as the most resistant.

In this study, shoot internal lesion was evaluated for the first time as a parameter to assess chestnut resistance to *Phytophthora* spp. It was chosen because it indicates the spread of the pathogen from the roots and collar to the aerial vascular system. This is important for determining the degree of plant resistance, as the rapid invasion of the pathogen into the phloem and xylem may affect water and nutrient movement through the shoots, causing death.

With regard to biomass parameters, the phenotypic correlation was stronger between survival and root dry weight than with leaf and shoot dry weight. Cuenca *et al.* (2009) also observed a good correlation between fresh root weight and survival. A healthy and developed root system is an important factor for resistance to *P. cinnamomi*.

The lesion caused by the inoculation of excised shoots is considered to be an indirect measure of *Phytophthora* spp. resistance. The length of the lesion is negatively proportional to resistance to the pathogen (Fernández-López *et al.*, 2001). The results showed that, similar to root-inoculated plants, the resistance to *P. cinnamomi* in the shoots is related to the confinement of the lesion to point of inoculation. For the most resistant genotypes, the surrounding tissues dried, limiting the progression of the lesion.

In the present study, differences observed between genotypes for lesion length were very high for all time points of measurement and in both seasons. Nevertheless, the results revealed that spring was the better season to perform excised shoot inoculation tests. After budburst, plants have good physiological conditions that may allow a better resistance response. It was found that the best time to take measurements in future investigations would be 5 dai, when differences in lesion lengths between genotypes were maximized.

Thus, the present study has shown that the strongest and most significant phenotypic and genetic correlations were obtained for lesion progression rate and survival; therefore, these would be the best variables to measure in future investigations.

The estimation of heritabilities and genetic correlations (genetic parameters) is an important strategy for plant breeding. Phenotypic variables with higher heritabilities (in this study: survival, shoot internal lesion and lesion progression rate) have the potential to be inherited to varying degrees in populations exposed to differential natural selection pressures in distinct environments (White *et al.*, 2007).

Resistance to *P. cinnamomi* is a polygenic and quantitative trait (Irwin *et al.*, 1995) that was evaluated in the present study by measuring several variables. The strong genetic correlations observed between survival and symptoms suggest common genetic determinants. Similarly, survival had a strong genetic correlation with lesion progression rate in the excised shoot inoculation test. The assessment of survival by root inoculation

testing is expensive and laborious and cannot always be determined in a population. The strong favourable genetic correlation observed between the two variables suggests that indirect selection could be made by lesion progression rate, which is easily measured. In this study, the resistance phenotype of each progeny was accurately determined by measuring lesion progression rate. Genetic studies are in progress to determine genotypes associated with these resistant phenotypes, and two QTL related to *P. cinnamomi* resistance have already been identified.

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