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Survival time analysis of *Pinus pinaster* inoculated with *Armillaria ostoyae*: genetic variation and relevance of seed and root traits

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Abstract Results of a greenhouse *Armillaria ostoyae* inoculation experiment, designed for screening resistant *Pinus pinaster* genotypes and for exploring the role of different phenotypic traits in seedling susceptibility, are reported. The experiment included 39 open-pollinated pine families that comprised a random subset of the breeding population of *P. pinaster* in Galicia (NW Spain). We employed a non-parametric survival-time analysis to analyze patterns of survival times during 14 months after inoculation with a local *A. ostoyae* strain. Results indicate (i) a significant correlation between seed weight and tree susceptibility, with seedlings originating from large seeds being more susceptible, (ii) a positive family mean correlation between secondary root weight and size and median life expectancy, and (iii) genetic variation of tree tolerance to *A. ostoyae*, with some families surviving significantly longer than others. Less susceptible families could be used in breeding programmes or directly in forest plantations to reduce the losses caused by *A. ostoyae*. Large within-family variation in tolerance to the disease was also observed, suggesting that non additive genetic variance was also important. Although being infected, 32 out of the 1200 inoculated trees survived the fungus infection. These tolerant genotypes comprise an attractive collection to further investigate genetic, phenotypic and environmental factors affecting pine susceptibility to *Armillaria* root rot.

Keywords Tree resistance · Pine · Screening · White root rot · Root density

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Introduction

Maritime pine (*Pinus pinaster* Ait.) is of vital importance to the forest economy of Spain, France and Portugal. In Galicia (NW Spain), this species occupies 400 000 ha, nearly 40% of the productive forest area. *Armillaria ostoyae* (Romagn.) Herink is the most common fungal species causing root disease in natural and planted *P. pinaster* forests, leading to

high mortality and important yield losses (Lung-Escarmant and Guyon 2004; Zas et al. 2007). Fungicides are not the best mean of controlling *Armillaria* root disease because, given the size of most forest stands, this approach is both economically and ecologically impractical. In contrast to soil fumigation or fungicide injections, a more sustainable approach to the control of *Armillaria* root disease may be the use of resistant tree species or cultivars (Guillaumin et al. 1991; Baumgartner and Rizzo 2006; Zas et al. 2007; Cruickshank et al. 2010).

A number of inoculation trials involving isolates of *Armillaria* spp. have been conducted on several conifer species (Mugala et al. 1989; Omdal et al. 1995; Morrison and Pellow 2002; Prospero et al. 2004; Hood et al. 2009; Cruickshank et al. 2010). The incidence and mortality reported in those studies were calculated only for trees that were symptomatic or asymptomatic during the study period, whereas a number of infected trees had not show any symptoms at the time when the final disease incidence assessment was performed. Such data are referred to as "censored data" because they are observations that do not fail (trees that did not die) within the time frame of the study. Survival analysis is a method that enables the analysis of all the survival data, i.e. the population of trees that were infected and died, plus the population of trees that were infected but did not die by the end of the study (Esker et al. 2006). Survival analysis is commonly used in ecological and medical experiments to analyze the time-to-death or time-to-event data (Collett 2003). This method offers several advantages over ANOVA approach methods since it handles repeated measurements over time on the same sampling units, including censored observations, and accounts for failure times (i.e., death) that are not normally distributed. Survival analysis has been recently used to assess tree mortality in forest inventories (Woodall et al. 2005), but as far as we know, it has never been applied in screening forest trees for disease resistance.

Seed weight and rapid development of fine roots are amongst the most important traits influencing the early stages of the life cycle of plants (Castro et al. 2006; Cubera et al. 2009). Several studies have found that larger seeds help seedlings to promote germination, growth and survival (Castro et al. 2006 and references therein). On the other hand, fine roots, responsible for the majority of absorption of water and nutrients supplied to the crown, are key factors of plant health and are interpreted as a direct measure of tree vitality (Grossnickle 2005; Cubera et al. 2009; Konôpka and Lukac 2010). Seed weight and fine roots have never been characterized in *P. pinaster* in relation to *A. ostoyae*, and adequate experimental data need to be generated to approach the hypothesis that resistance of *P. pinaster* to *A. ostoyae* would be linked to these phenotypic traits. Our second hypothesis is that *P. pinaster* has enough variability to allow screening for tolerance under controlled conditions. The present investigation reports a greenhouse experiment in which the differential response of seedlings from 39 plus trees to *A. ostoyae* was measured. The experiment aimed at determining the relationships between seed weight, root density and plant susceptibility and at searching for potential genotypes of *P. pinaster* to be used as a source of resistant forest reproductive material for reforestation and for further breeding purposes.

Materials and methods

Plant material

Plant material consisted of open-pollinated families of 39 maternal plus trees selected for superior growth and form in mature plantations of *P. pinaster* in Galicia (NW Spain) by Xunta de Galicia. The plus trees were clonally propagated and planted in a clonal seed orchard (Sergude, 42°49′N, 8°27′W) to provide high genetic quality seeds for reforestation on the Atlantic coast of Galicia. The 39 families comprised a random subset of the F_1 breeding population of *P. pinaster* in Galicia. One additional unimproved seed source, commonly used for reforestation in the coastal area of Galicia, was included as a control.

In February 2006, seeds were individually weighed and sown in 5 l-pots containing soil substrate (pH 5.5–6) composed of a volume of 20% peat and 80% composted pine bark (Dermont II®, Vimianzo, A Coruña, Spain). Seven seeds per pot were placed forming a regular heptagon of about 5 cm each side, and covered with a layer of 0.5 cm of sand. Before sowing and to allow a better inoculation, a plastic pipe (15 cm × 3 cm diameter) was carefully buried into the substrate in the centre of the pot. Plants were grown under a shading mesh providing 25% of full

sunlight at the Centro de Investigación Forestal de Lourizán, Pontevedra (42°24′N, 8°40′W; 90 m a.s.l.; 14.7°C mean annual temperature).

Germination assessment and experimental design

The germination of each seed was determined upon successive assessments every 3–4 days from 3 April 2006 to 2 June 2006. In August 2006 and in accordance with Prospero et al. (2004), only 5 seedlings per pot were left, by carefully pulling from the root collar of the other two seedlings (if any). Seedlings were removed in order to even up the number of plants of all the pots, and the selection of the 5 seedlings per pot was done at random.

The experimental design consisted of a randomized complete block design with 8 blocks, each block including 40 pots arranged at random. Within each pot, all seedlings belonged to the same progeny. To facilitate inoculation and to assure even colonization by the pathogen, more than one seedling per pot was used. Six out of the eight blocks were inoculated with *A. ostoyae* (details later) and the other two remained uninfected. For the susceptibility test, a total of 1200 plants comprising 6 blocks × 40 progenies × 5 seedlings per progeny were used. For the root assessment, a total of 400 plants comprising 2 blocks × 40 progenies × 5 seedlings per progeny were used.

Root assessment

In September 2006, when the plant material was six-months-old and about 30–45 cm tall, the 400 untreated seedlings were uprooted. Because not all plants were uprooted the same day, the dates of harvest were recorded and considered for data analysis. After carefully removing the soil from the root ball of each plant (Fig. 1a), the main root (pivotal root) was separated from the secondary roots (SR) (Cubera et al. 2009). About half of the biomass of SR of each plant were scanned using an Epson Expression 10 000 XL (Epson America, Inc., USA). SR length, SR surface area, and SR mean diameter were obtained from these aliquot root samples using the image analysis software WinRhizo Pro v.2007d (Régent Instruments Inc., Quebec, Canada). The main root, the non-scanned SR and the aliquot of scanned SR were placed inside paper bags, oven-dried at 65°C for 48 h and weighed. Data were expressed as *root density* (mg l^{-1} of soil), *SR length density* (cm l^{-1} of soil), *SR surface area density* (cm^2 l^{-1} of soil), *SR weight* in relation to the total root weight (%), and *SR diameter* (mm) (Cubera et al. 2009).

Inoculum preparation, susceptibility test and tree mortality assessment

In February 2008, when the trees were two-years-old and about 75–95 cm in height, all seedlings from the susceptibility test were inoculated. Inoculations were performed later than the root assessments because at the age of 0.5–1 years, *P. pinaster* is highly susceptible to *A. ostoyae*, and genetic differences among progenies are not quantifiable (personal observation). In addition, root assessments at the age of 2 years would substantially increase the difficulties of fine root separation among plants of the same pot.

An *A. ostoyae* local strain (EFA 1048/07) collected in November 2007 from a naturally infected *P. pinaster* stand at Cotobade (Pontevedra, Spain; 42° 27′N, 8°28′N) was used in the infection trials. For fungal isolation, pieces of fresh wood showing rhizomorphs and white mycelium between the bark and the inner cortex were excised with a razor blade, placed on PDA (Potato-dextrose-agar), and maintained at 24°C in darkness. Species identification of the isolate was carried out by the PCR-RFLP technique (Aguín-Casal et al. 2004). The strain was selected because of its outstanding capacity of producing vigorous rhizomorphs (Fig. 1b), and the fact that the most virulent isolates of *A. ostoyae* are usually the best rhizomorph producers (Omdal et al. 1995; Morrison and Pellow 2002; Prospero et al. 2004). A preliminary test performed in 1-year-old *P. pinaster* seedlings with additional strains (C1 and C18 among others (Prospero et al. 2004, kindly provided by Daniel Rigling, Swiss Federal Research Institute) confirmed the superior virulence of EFA 1048/07 (results not included).

The inoculum was prepared as described by Mansilla et al. (2001), with slight modifications. Fresh stem segments (5–6 cm×1.5–2 cm diameter, bark included) of *Corylus avellana* L. and *P. pinaster* were placed overnight in water in glass boxes (10 rods/box). The following day water was removed and the boxes were autoclaved (45 min, 120°C, and 1.1 bar). After cooling, 250 ml of PDA medium (previously sterilized at 120°C for 30 min and

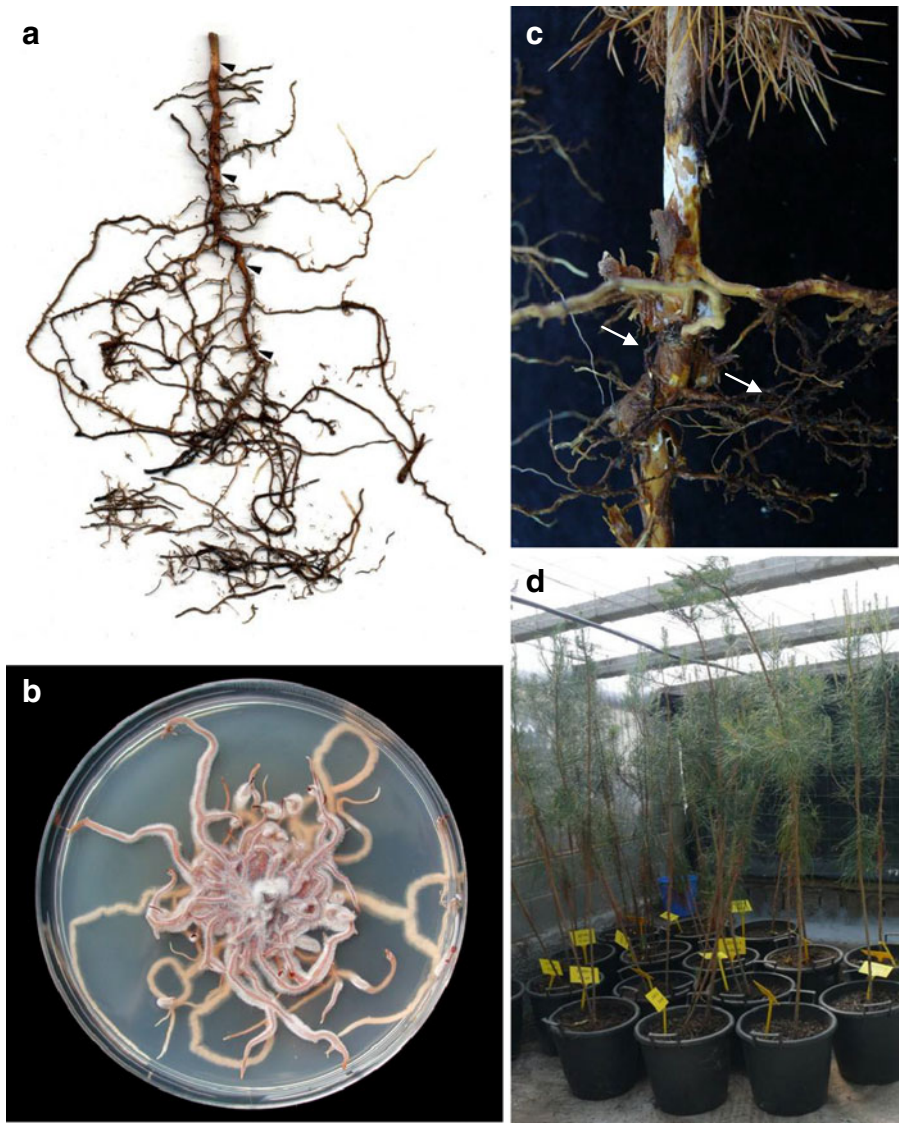

Fig. 1 **a** *Pinus pinaster* root ball, in which the main root is indicated by arrowheads. **b** Isolate of *Armillaria ostoyae* used, 10 days after subculturing in PDA at 24°C. **c** 2.5-year-old *P. pinaster* tree, 5 months after inoculation, with white mycelium of *A. ostoyae* underneath the stem and rhizomorphs indicated by *arrows*. **d** Tolerant *P. pinaster* trees

1.1 bar) were added to each box, the rods being completely submerged in agar. Each box was inoculated by placing five small pieces (3–4 mm side) of an actively growing *Armillaria* culture onto the stem segments. Boxes were incubated in the dark at 25°C for 2 weeks.

For inoculation, one inoculum rod (bark included) of *C. avellana* and one from *P. pinaster* (in all cases completely covered with mycelium or rhizomorphs of *A. ostoyae*) were buried in the centre of the pot in place of the plastic pipe. One inoculum rod per tree species was used in order to increase the chance of a successful inoculation. The top of both sticks was covered with soil. All 1200 seedlings from the susceptibility test were inoculated and kept under greenhouse conditions. To avoid the influence of water availability on tree mortality and on inoculum potential (Wargo and Harrington 1991), plants were watered every 2–3 days by overhead sprinklers until field capacity.

Seedlings were assessed every 2 weeks for symptoms of *Armillaria* root rot (chlorotic foliage and mortality) during 15 months, from February 2008 to May 2009. Seedlings that died were recorded and immediately checked for the presence of *Armillaria* mycelium in the cambial region of the root collar. Dead seedlings were not removed during the experiment. At each assessment, each seedling was

assigned to one of the following categories: dead, dying (i.e. chlorotic foliage), and healthy (no visible above-ground symptoms of an *Armillaria* infection). By the time of mortality, plant height and stem diameter at the soil surface were assessed, and the presence of rhizomorphs was observed on roots and in the soil (Fig. 1c). To confirm the infection, *Armillaria* was isolated from colonized stem and superficial roots, and samples taken from roots and soil analysed through nested-PCR/RFLP (Escofet et al. 2006).

Data processing and statistical analysis

Statistical analyses were performed with Statistica v7.0 (Stat Software Inc., Tulsa, OK, USA). An angular transformation of the percentage values (x) was performed to normalize data before analysis [$y = \arcsin(x/100)^{1/2}$]. To compare values of *seed weight*, *germination date*, *seedling growth*, *root density*, *root length density*, *root surface area density*, *SR weight*, and *SR density* (dependent variables) among *families* and *blocks* (factors), a two-way analysis of variance (ANOVA) was used. When analyzing the root parameters, the *uprooting date* was considered as a covariate. To compare means, LSD tests were performed.

To test the hypothesis that *P. pinaster* seedlings had enough genetic variability to allow screening for tolerance to *A. ostoyae*, a survival time analysis, where tolerance is interpreted as delayed mortality was used (Esker et al. 2006). Time-to-death (survival time) was defined as the day on which a plant died minus the day on which plants were inoculated. Two types of events occurred that constituted the censoring events. The first ones occurred when seedlings died by unknown reasons, i.e. when no mycelium in the cambial region of the root collar was observed. Plants that died by unknown reasons had censored survival times determined to the days-to-death. The second censoring event occurred in May 2009 (day=432), when the study was concluded. Plants that were symptomatic and had tested positive for *A. ostoyae*, but were not dead, were considered censored since their time-to-death was unknown. To compare tolerance to *A. ostoyae* among families, we used the Kaplan-Meier estimate of survival probabilities using the log-rank test (Collett 2003), which is a nonparametric procedure. The survival function is the number of individuals with survival time$\geq t$ (time) divided by the number of individuals in the study (Collett 2003). The Kaplan-Meier estimate of the survival function is a product-limit estimate:

$$\widetilde{S}(t) = \prod_{j=l}^{k} \left(\frac{n_j - d_j}{n_j} \right)$$

where n_j = number of individuals alive just before time $t_{(j)}$, and d_j = number of deaths at $t_{(j)}$ for $t_{(k)} \leq t \leq t_{(k+1)}$. Because survival data tend to be right and positively skewed, the median life expectancies were estimated instead of the mean life expectancies (Collett 2003). The median life expectancy is defined as the time in which 50% of individuals in the study are expected to survive (Collett 2003). To model time-to-death of trees the Weibull, exponential, and Gompertz distributions were examined. Model selection was based on the log-likelihood values, and the mathematical function that had the smallest value was selected. Goodness-of-fit of modelling and median life expectancies were obtained with the *Life Tables & Distributions* procedure, and survival time data were analyzed through the *Comparing Multiple Samples* procedure, with *Family* as the grouping variable.

Proximity of plants within a pot was rated according to: 2=two neighbouring plants surrounding the seedling were alive; 1=only one neighbouring plant surrounding the seedling was alive; and 0=both neighbouring plants surrounding the seedling died. To estimate the relevance of proximity among plants within a pot, we used the Kaplan-Meier estimate of survival probabilities through the *Comparing Multiple Samples* procedure, with *Proximity* as the grouping variable.

The relationships between the average family parameters of seed weight, time to germination, secondary root weight, secondary root diameter, percentage of infected trees, percentage of survival of trees, and median life expectancy were examined by means of Pearson's correlation coefficients.

Results

Seed weight, germination of seeds, and root assessments of seedlings

Seed weight significantly varied among families (P= 0.0022), with family mean values ranging from 53 to 93 mg seed^{-1} (Table 1). Within family variation was

relatively high, e.g. family 1043 had seeds weighing from 43 to 101 mg, and the heaviest seed of family 1033 weighed 2.8-fold more than the lightest seed of the same family (Table 1). The time to germination also varied significantly among families ($P<0.0001$) and within families (Table 1). Germination started just only 15 days after seeding in seedlings from some families, and lasted up to 43 days in seedlings from other families (Table 1).

SR density, SR weight and SR diameter were significantly different among families ($P<0.05$; Table 1). However, no significant differences among families were observed for the density of the main root, the SR length density and the SR surface area density ($P>0.2$; data not shown).

Inoculation success and tree survival

From an initial number of 1184 trees (16 seedlings were lost before the first symptom assessment), 942 trees showed white mycelium at the root collar, thus 80% of the total plants were successfully inoculated (Table 1). Re-isolation of the pathogen from the infected trees was always possible. The extent of rhizomorph production was not assessed, although in all pots inocula of *A. ostoyae* had produced rhizomorphs, frequently attached perpendicularly to the root surface. The number of proximal neighbour plants surrounding the seedlings (normally 2 and less frequently 1 or 0) did not influence the probability of infection or the time of survival after infection by *A. ostoyae* ($P>0.2$).

The first pine seedlings killed by *A. ostoyae* were observed in May 2008, 3 months after inoculation. The rate of mortality was not uniform with marked peaks in June 2008, October 2008 and January 2009 (Fig. 2). By the end of the experiment (i.e. May 2009, 15 months after inoculation) overall mortality of infected trees was about 96% and 43 out of the 1200 trees were alive, 32 of them showing *A. ostoyae* mycelial fans underneath the stem and root bark and rhizomorphs attached to the roots. The PCR-RFLP and nested-PCR/RFLP methods applied to samples taken from roots and soil of all these 32 trees (Fig. 1d) confirmed the presence of *A. ostoyae*.

Genetic variation underlying susceptibility to *A. ostoyae*

Twenty four out of 40 families showed a very high incidence with 100% mortality, in contrast to a quarter of the families showing at least 5% of seedling survival (Table 1). Only five families (1049, 2001, 2045, 2053, and 2070) were moderately tolerant to *A. ostoyae* (12–17% of seedling survival), and comprised 17 out of the 32 infected trees that had survived the inoculations. The unimproved seedlot TC showed intermediate resistance, similar to the average of the 39 improved families (Fig. 3), with 4% of final survival (Table 1).

Mean survival time (± SD) for *A. ostoyae*-infected *P. pinaster* was 186±27 days, and the median survival time was 177±38 days. When comparing median survival times of families, 2001 and 2045 had values of 284 and 230 days, about 60% higher than those of 2031 and 1020 (129 and 130 days, respectively). The five families showing the most delayed mortality were 2001, 2077, 2045, 2053 and 2064, not coinciding with the five families with less infected trees, nor with the families with higher survival rates (Table 1).

Based on examination of the log-likelihood of the data under the null model, the Weibull function described the survival data better than the other models examined (results not shown). Cumulative proportions of survival, compared through the Kaplan-Meier estimate, were significantly different among families ($P=0.0024$) (Fig. 3). When comparing mortality dates of trees within families, a high variation was also observed, e.g. within the most tolerant 2001 family eight trees died during the first 150 days of assessments, whereas four trees were still alive at the end of the experiment.

Relation among variables

Families with heavy seeds germinated significantly earlier than families with light seeds ($P<0.05$) (Table 2). Family mean seed weight was also significantly related to the percentage of infected trees ($P<0.01$) and marginally related to the median life expectancy ($P<0.1$) (Table 2). The median life expectancy of families was significantly ($P<0.05$) and positively related to the average values of secondary root weight, secondary root diameters, and survival of trees (Table 2). Pearson coefficients relating the main root weight with survival and median life expectancy of families were negative and marginally significant ($P=0.08$; results not shown). Secondary root density was only directly related with tree survival ($P=0.04$), and SR length density and SR surface area density

Table 1 Family means (and range of variation) of early performance and susceptibility variables of 39 *Pinus pinaster* open pollinated families and one unimproved seed source (TC)

Family code	Seed weight (mg)	Time to germination (days)	Secondary root weight (%)	Secondary root diam (mm)	Infected trees (%)	Survival of trees (%)	Median life expectancy (days)
1003	85.6 (76–110)	22.6 (20–26)	71	0.94	83	0	180
1004	63.8 (51–93)	23.4 (20–35)	66	0.95	63	0	167
1007	80.0 (76–93)	23.3 (21–27)	72	1.08	87	0	200
1011	66.1 (56–97)	22.3 (19–26)	65	1.01	86	0	148
1020	75.1 (68–98)	22.6 (20–25)	65	1.00	90	0	130
1030	83.6 (72–102)	22.0 (21–28)	65	0.96	73	9	203
1033	52.6 (23–64)	25.0 (15–27)	68	1.01	67	0	154
1035	80.0 (64–110)	20.6 (18–24)	65	0.96	73	9	144
1036	71.8 (57–86)	22.5 (20–27)	69	0.89	83	0	141
1043	73.1 (43–101)	23.9 (18–40)	65	0.89	80	4	165
1046	76.6 (63–83)	21.4 (18–27)	65	0.98	77	0	138
1049	73.3 (58–88)	21.2 (19–35)	67	0.95	87	12	145
1050	62.0 (55–90)	21.8 (19–26)	70	0.99	60	0	237
1056	84.6 (78–95)	21.0 (19–25)	62	0.90	77	0	141
1059	75.1 (70–82)	22.2 (19–25)	58	0.96	90	4	147
2001	73.9 (76–96)	21.8 (19–25)	70	1.13	80	17	284
2002	72.1 (63–86)	23.5 (21–27)	74	0.89	77	0	208
2004	72.9 (63–88)	22.4 (21–24)	65	0.93	77	9	146
2013	80.9 (60–101)	22.4 (18–26)	66	0.93	73	0	194
2017	83.6 (67–94)	20.0 (21–25)	61	1.03	87	0	134
2021	66.8 (55–77)	24.2 (20–40)	66	1.00	87	8	152
2026	63.4 (33–70)	22.5 (19–35)	63	0.97	80	0	136
2031	90.3 (87–115)	22.2 (19–35)	68	0.93	88	0	129
2040	68.9 (58–81)	21.8 (18–35)	64	0.94	83	0	174
2041	93.3 (83–115)	25.2 (18–40)	64	0.93	86	0	146
2042	83.7 (55–96)	23.5 (20–27)	64	0.91	90	0	205
2043	73.9 (46–106)	21.1 (18–40)	57	0.99	73	0	180
2045	65.6 (62–93)	22.4 (19–30)	64	1.12	77	13	230
2050	68.7 (63–95)	21.8 (18–27)	67	0.97	83	0	180
2051	65.7 (55–82)	21.6 (19–43)	66	0.94	80	0	133
2053	59.6 (52–77)	23.4 (20–40)	66	1.02	80	13	233
2054	72.1 (54–76)	23.7 (22–26)	70	0.96	63	0	170
2062	59.2 (42–64)	24.9 (19–40)	70	1.01	82	9	193
2064	78.3 (69–89)	27.1 (19–43)	65	1.05	81	0	203
2070	85.9 (74–91)	21.0 (20–25)	67	0.93	87	15	194
2072	62.6 (50–97)	24.4 (20–23)	68	0.98	73	0	161
2076	67.8 (41–71)	24.5 (22–28)	67	1.04	93	0	189
2077	61.3 (61–74)	32.0 (21–40)	66	1.05	71	5	277
2082	58.3 (53–71)	24.4 (15–40)	68	0.90	77	4	215
TC	74.0 (36–79)	26.9 (21–40)	66	0.98	83	4	160
Average	72.7	23.1	66.1	0.98	80.0	3.4	176.7

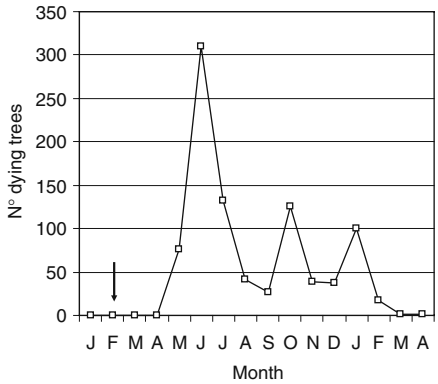

Fig. 2 Seasonal pattern of mortality of *Pinus pinaster* trees after inoculation with *Armillaria ostoyae* (n=910). The *arrow* indicates the inoculation time (February 2008)

were not related with any of the parameters of resistance (P>0.3; results not shown).

Discussion

Seasonal patterns of mortality, similar to the one reported here, were observed by Omdal et al. (1995) on eight forest tree species inoculated with *A. ostoyae*. Minimal mortality occurred during winter months, which coincide with the dormant period of both host and pathogen. The susceptibility of pines to *Armillaria* root rot infection would thus depend on the season in which plants are assessed. The capability of survival time analysis to statistically address this inconvenience justifies the use of this powerful method. In contrast to other research fields such as medicine or ecology, survival analysis has rarely been applied in plant pathology (Esker et al. 2006).

Our study makes three important findings. The first is that there is a significant correspondence between seed weight and tree susceptibility to *A. ostoyae*. The importance of seed weight (size) in governing the fitness of the progeny has been supported by extensive empirical evidence. Several studies have found that larger seeds help seedlings to promote germination, and favour plant growth and survival against water stress (Castro et al. 2006; Ramírez-Valiente et al. 2009). This positive effect of seed weight is logical because larger seeds have higher amounts of resources for the developing seedling, which in turn results in greater access to soil nutrients, water and sunlight (Castro et al. 2006). In terms of disease resistance, however, it has been reported that large seeds of trees are more damaged by seed diseases than are smaller seeds (Pringle et al. 2007). In our case, the positive relation between seed weight and susceptibility could be mediated by other factors such as the germination date and the root growth (explained below), which would probably influence the initial size of seedlings and the amount of roots exposed to the pathogen. A quickly germinating seed that rapidly produces roots is also more likely to encounter a pathogen sooner than a slow-germinating seedling. Another explanation of why lighter seeds and delayed germination will favour tree resistance is

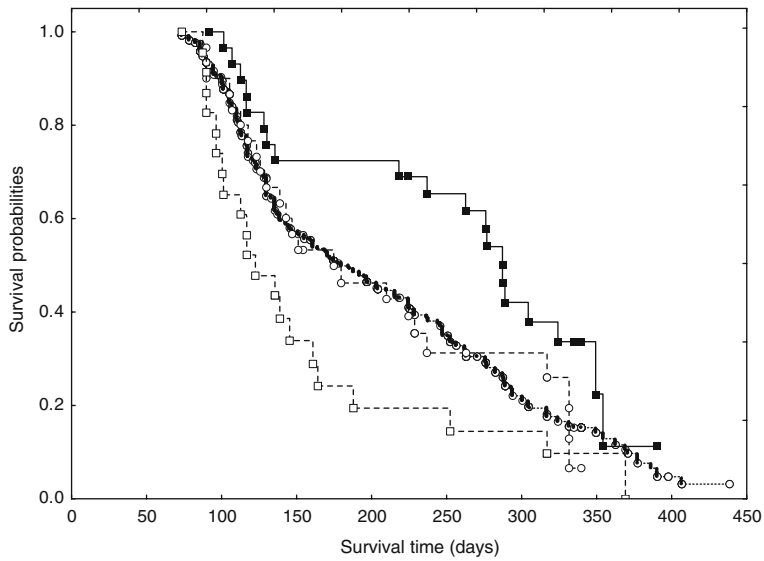

Fig. 3 Plot of survival probabilities using the Kaplan-Meier estimate of the survival function for *Pinus pinaster* inoculated with *Armillaria ostoyae* at time 0. Lines correspond to all genetic entries (●), unimproved seed source (TC) (○), highly susceptible 2031 family (□) and moderately tolerant 2001 family (■)

Table 2 Pearson correlation matrix among early performance and susceptibility variables in 40 *Pinus pinaster* open pollinated families

	Time to germination (days)	Secondary root weight (%)	Secondary root diameter (mm)	Infected trees (%)	Survival of trees (%)	Median life expectancy (days)
Seed weight (mg)	−0.32**	ns	ns	0.39***	ns	−0.26*
Time to germination (days)		ns	ns	ns	ns	0.38**
Secondary root weight (%)			ns	ns	ns	0.32**
Secondary root diameter (mm)				ns	0.33**	0.47***
Infected trees (%)					ns	−0.26*
Survival of trees (%)						0.41***

Asterisks indicate levels of significance at $P<0.10$ (*), $P<0.05$ (**), and $P<0.01$ (***); *ns* = non significant. $N = 40$

the fact that conservative genotypes in terms of growth rates invest their energy more effectively in defence; several ecological models predict a negative association among growth and resistance, both at the phenotypic or/and genetic levels (Stamp 2003). The positive relationship between seed weight and *A. ostoyae* incidence, and the higher survival expectancy of trees obtained from light seeds in comparison to trees obtained from heavy seeds, could suggest that natural selection at sites in which *A. ostoyae* is highly virulent favours trees originating from smaller seeds.

Our second finding is that secondary roots are somehow linked to tree resistance. The ability of *A. ostoyae* to kill a tree will mainly depend on its inoculum potential. Thick roots such as the tap root may provide *A. ostoyae* with enough inoculum to allow quick colonization of the distal portion of the root, allowing the fungus to overcome any resistance offered by even the most resistant of the hosts. Conversely, secondary roots infected and girdled by *A. ostoyae* may not offer enough inoculum potential to overcome even weak host response (Robinson and Morrison 2001). Accordingly, fine roots are not as susceptible to *Armillaria* root disease as woody roots (Baumgartner and Rizzo 2006), and although fine root fragments may serve as sources of inoculum of *A. ostoyae* (Kromroy et al. 2005) their potential to originate infections in coarse roots has not been assessed yet.

Our results could lead to the assumption that contact between the pathogen and the main root and contact between secondary roots and the soil, are key elements for *A. ostoyae* incidence and tree survival. The relation between median life expectancy and secondary root diameter, itself a complex character, means that true interpretation is far from simple. A large secondary root diameter maximizes root-soil contact, favouring an efficient uptake of resources. The amount of fine roots in the soil, expressed as secondary root weight, also determines the ability of the root system to exploit the soil resources, and a high proportion of fine roots have been found to be an advantage for forest trees against decline (Cubera et al. 2009; Konôpka and Lukac 2010). The fungus probably needs a longer time to kill a tree with more secondary roots than a tree with fewer secondary roots. The balance between the spread rate of the fungus in the root system and the rate of initiation and growth of new roots by the host would determine the delay of death of an infected tree (Guillaumin et al. 1991).

Rapid root replacement after infection is important to exploit water and nutrients from surrounding soil, and could represent an efficient mechanism by which plants may tolerate fungal attack. Trees with large initial root volume have been shown to have higher root growth potential (Grossnickle 2005). Thus, a possible mechanism to alleviate root rot damage would be a greater growth allocation to roots relative to shoots, mechanism which will otherwise limit aboveground growth. Traditionally, resistance to *Armillaria* root rot has been related with tree vigour as indicated by its relative growth (Wargo and Harrington 1991). Above-ground growth limited by increased below-ground growth may hinder any

relation between tree vigour and tree resistance, in accordance with our results and previous field observations (Rosso and Hansen 1998). Moreover, tree defence responses to infection will imply immediate, heavy demands of plant resources which can also result in decreased growth (Robinson and Morrison 2001; Solla et al. 2002; Cruickshank et al. 2006).

The third major finding of this study was the significant variation of the median life expectancy observed among families, indicating a genetic component of *P. pinaster* tolerance to *A. ostoyae*. In consequence, less susceptible families could be used in breeding programmes or directly in forest plantations to reduce the losses caused by *A. ostoyae*. Only few experimental data are available on variations in susceptibility and resistance to *Armillaria* within forest tree species (e.g. Proffer et al. 1988; Prospero et al. 2004). Differences in the susceptibility of forest tree species to *Armillaria* spp. are frequently attributed to tree vigour and site conditions but rarely to host genetics (Wargo and Harrington 1991). In the field, variation in susceptibility to *A. ostoyae* has been observed among different provenances of *P. pinaster* (Lung-Escarmant and Taris 1989) and among families within provenances (Zas et al. 2007). Specific genes governing the chemistry of root bark (Myszewski *et al.* 2002), the formation of ligno-suberized boundary zones (Robinson and Morrison 2001; Solla et al. 2002), and the timing and density of traumatic resin canals formation (Cruickshank et al. 2006; Moreira et al. 2008) may be responsible for the different susceptibility of the families in the current study to *A. ostoyae*.

Families varied significantly in the time needed for *Armillaria* root rot to kill the trees, but a large within family variation was also observed. This suggests that the heritability of tolerance to *A. ostoyae* would be low, as already observed in field conditions (Zas et al. 2007). Further research is needed to determine whether specific combining ability is also an important source of variation or whether genetic resistance is mainly limited to specific genotypes and transmitted mostly through vegetative reproduction. It should be pointed out that with the inoculation method used (similar to the natural infections of trees in the field), some trees could survive later than others because rhizomorphs reached the seedlings roots at different times. This difference in timing could also explain why the five families showing the most delayed mortality did not coincide with the five families with higher survival rates or with less infected trees. In order to exploit genetic resistance within breeding programs, results presented here suggest that a combined selection method (resistant genotypes within the most resistant families) should be preferred, but for *P. pinaster* the utility of other breeding alternatives such as cross pollinations or vegetative propagation remain to be explored.

The unimproved seedlot, although showing a lower growth potential than the genetically improved families (data not shown), had an intermediate susceptibility to the pathogen. Our results contrast with previous findings in which the selected material of the F_1 breeding population was clearly more susceptible to insect herbivores than unimproved seed sources (Zas et al. 2005). These findings, together with the commonly observed negative genetic correlation between growth and resistance, have alerted forest tree breeders. Results presented here suggest that simultaneously breeding for improved growth and resistance to *Armillaria* root rot could be possible.

A number of inoculation trials involving isolates of *A. ostoyae* have been conducted on several pine species, and sometimes the pathogen was found to exhibit considerable variation in virulence among the isolates and among the host genetics (Mugala et al. 1989; Omdal et al. 1995; Morrison and Pellow 2002; Prospero et al. 2004). Although no previous work has considered as many tree families as in the current study, further strains could have been tested. We feel confident about the unique strain used as observed mortality due to *A. ostoyae*, starting 3 months after inoculation and reaching up to 80%, was much higher than mortalities reported in other *A. ostoyae* greenhouse pot experiments (<52%), involving 2- or 3-year-old conifer seedlings (Mugala et al. 1989; Omdal et al. 1995; Morrison and Pellow 2002; Prospero et al. 2004). Our strain produced vigorous rhizomorphs within weeks, in contrast to other studies reporting rhizomorph production of *A. ostoyae* 18 months after inoculation (Prospero et al. 2006). Nevertheless, whether the results from this study can be extended to the broader population of *A. ostoyae* in the forest remains to be explored.

In conclusion, this experiment is a first step to show the importance of two new phenotypic traits

modulating tree resistance to *A. ostoyae*. Small seeds and increased fine root production promoted survival during early growth of the pine progenies. We ignore whether these influences will diminish with age or whether their imprint will remain for a prolonged period of time. A larger experiment would provide knowledge to help foresters when selecting suitable seeds or seedlings to be used for reforestation in sites in which *A. ostoyae* is present. Additionally, the present study is the first to show genetic variation, at the family level, within a population of a *Pinus* species in terms of resistance to *A. ostoyae* in greenhouse conditions. The 32 surviving trees will be transplanted, inoculated again and, if they tolerate a second inoculation, vegetatively propagated and thoroughly studied in order to better understand the mechanisms involved in resistance.

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