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Screening of Maritime pine (*Pinus pinaster*) for resistance to *Fusarium circinatum*, the causal agent of Pitch Canker disease

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Summary

Pitch canker, caused by the fungus *Fusarium circinatum*, is an introduced non-native disease on pines in natural and planted stands of Europe. Research has not been conducted to test whether a European native pine species shows genetic variation in susceptibility to this disease. Half-sib families from 39 *Pinus pinaster* clones and seedlings from one unimproved seed source (control) were evaluated for resistance. Pitch canker resistance was not genetically related to tree growth, but seed weight and germination rates were predictive of time-to-death. Heritabilities and associated genetic gains calculated from the greenhouse experiment were consistent, h_i^2 = 0.18 and 0.45 for time-to-death and for tree mortality, respectively. These heritabilities are high enough to allow pitch canker to be reduced through appropriate genetic strategies. Results indicated that selection for growth of *P. pinaster* trees in breeding programs would not necessarily imply an increase of susceptibility to *F. circinatum*. This research may allow the use of native pine individuals as breeding stock or as sources to produce seeds with moderate levels of tolerance to *F. circinatum*.

Introduction

Fusarium circinatum Nirenberg and O'Donnell (teleomorph *Gibberella circinata*), known to cause pitch canker, is a fungus with great virulence on most of the *Pinus* species. This pathogen was first recorded in the US in 1946 and since then sporadic outbreaks and epidemics have been reported in numerous countries (Wingfield *et al.*, 2008). In 2004, the fungus was first isolated in the European continent affecting nurseries and forest plantations of *Pinus radiata* and *Pinus pinaster* in northern Spain (Landeras *et al.*, 2005). In Italy, the pathogen has been reported on *Pinus halepensis* and *Pinus pinea* (Carlucci *et al.*, 2007). Recently, the fungus was isolated in Portugal from *P. radiata* and *P. pinaster* seedlings (Bragança *et al.*, 2009). The suitability of Western Europe for *F. circinatum* to spread indicates that further disease outbreaks could be expected (Watt *et al.*, 2011). Within the Iberian Peninsula, the impact of *F. circinatum* on conifer productivity has not been quantified, but the strict quarantine and sanitary measures undertaken to eradicate the pathogen and/or to avoid its spread are causing substantial economic losses to the forest industry and public forest services.

Maritime pine (*P. pinaster*) is a native conifer of the Western Mediterranean basin that is of great importance to the economy. In the Iberian Peninsula, *P. pinaster* covers 1.6 million ha and is the most common tree used for reforestation. The presence of *F. circinatum* in the northern Iberian Peninsula has become the main threat for *P. pinaster* forests and nurseries in this area (Iturritxa *et al.*, 2011). Commercial plantations of *P. pinaster* in France and Italy are also at risk. At present, no means of disease control exist, although proper nursery and silvicultural management, adequate quarantine measures and genetic selection for genotypes that are less susceptible to the pathogen

would reduce the economic impact of the disease (Wingfield *et al.*, 2008).

Different families of *Pinus* inoculated with *F. circinatum* have consistently shown significant differences in susceptibility (Dwinell and Barrows-Broaddus, 1979; Barrows-Broaddus and Dwinell, 1984; Gordon *et al.*, 1998a, 1998b; Storer *et al.*, 1999; Schmale and Gordon, 2003; Aegerter and Gordon, 2006; Roux *et al.*, 2007). This suggests that there may be some resistance to *F. circinatum* in *P. pinaster*. However, research has yet to be conducted to test native pine species and their susceptibility to this fungal disease. In some studies based on artificial inoculations of seedlings, maritime pine appears to be more tolerant to *F. circinatum* than *P. radiata* (Bragança *et al.*, 2009) and with similar tolerance as *Pinus nigra* and *Pinus sylvestris* (Pérez-Sierra *et al.*, 2007). The intra-specific genetic variation in resistance to *F. circinatum* has not been quantified in *P. pinaster*. The hypothesis tested is that *P. pinaster*, characterized by high across- and within-population variation in adaptive traits (González-Martínez *et al.*, 2004), has a high genetic variation in tolerance to *F. circinatum*. Information gathered from this work will determine the tolerance of *P. pinaster* to this fungus and if the species can be improved through selection and breeding.

Materials and methods

Plant material

Plant material consisted of 39 *P. pinaster* half-sib families obtained from genotypes selected for superior growth and form in mature plantations of *P. pinaster* in Galicia (North-West Spain). These families were selected because information about their susceptibility to other pests and pathogens was available (Zas *et al.*, 2005, 2007; Solla *et al.*, 2011). Seeds from open-pollinated cones from the 39 genotypes were collected in autumn 2007 from Sergude seed orchard (Xunta de Galicia, Consellería de Medio Rural, 42° 49′ N, 8° 27′ W). One unimproved seed lot was also included in the experiment.

Greenhouse experiment design

To assess seed infestation by *F. circinatum*, seed lots from each family (~100 seeds per lot) were placed and cultured in FSM selective medium. This medium is composed of 15.0 g peptone, 20.0 g agar, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g Pentachloronitrobenzene and 10 ml of a streptomycin sulphate stock solution (30 mg ml^{-1}) in 1 l of de-ionized water (Aegerter and Gordon, 2006). Seven days after incubation at 20°C, examination resulted in no *F. circinatum* recovery from any of the seed lots. In December 2008, additional seeds were used for the greenhouse experiment. Seeds were individually weighed in order to check the influence of seed weight on susceptibility. Pre-weighed seeds were individually sown in nursery trays (40 wells of 250 cm^3 per tray) containing sandy soil and peat (4 : 1 v/v, pH 5.5) and covered with a thin layer of white sand. Plants were grown in a greenhouse at temperatures fluctuating in the range of 23 ± 5°C and under 70 per cent full sunlight. Seedlings were watered as needed every day (~4 mm day^{-1}). The experimental layout consisted of a randomized complete block design with 48 trays (replicates) each including one plant of each of the 40 families. A total of 1920 seeds comprising 48 trays × 40 families were used. Seeds that did not germinate were not replaced. Trays were set on three different greenhouse beds that were considered main blocks for the analysis. Each main block included 16 trays or replicates.

Susceptibility test and tree assessment

The germination status of each seed was examined every 7 days for 3 months. Plant height was measured the day before inoculation. On June 24 2009, when the plant material was ~6 months old and ~12 cm tall, 1200 seedlings (30 of the 48 trays) were inoculated. Inoculations included 30 seedlings per family, distributed in 10 trays per main block. The *F. circinatum* strain used (MAT-1, code Fc7-1) was provided by Carmen Muñoz (Escuela Universitaria de Ingeniería Técnica Forestal, Universidad Politécnica de Madrid) and isolated in 2005 from a stem canker on a *P. pinaster* tree in Asturias, northern Spain. The virulence of the strain used was confirmed in several previous pathogenicity tests (Vivas *et al.*, 2009). Since the virulence of *F. circinatum* in Spain is homogeneous (Martínez-Álvarez *et al.*, 2009; Iturritxa *et al.*, 2011) and because different *F. circinatum* strains do not reveal significantly different rankings of susceptibility among the same host genotypes (Gordon *et al.*, 1998b; Matheson *et al.*, 2006), only one isolate was used.

Cultures of the strain used were grown on potato dextrose agar at 25 ± 1°C under dark for 7–10 days. Mycelium and conidia were scraped off the agar surface with a sterile scalpel that was used to make a 1-mm-long slit wound into the succulent seedling stem tissue to introduce the inoculum (Correll *et al.*, 1991). This technique placed about 700–1000 conidia into the wound. The wound was made parallel to the axis of the main stem and ~5 cm above the soil level (Correll *et al.*, 1991; Muñoz and Ampudia, 2005). Identical wounds were made with a sterile scalpel to 720 non-inoculated control seedlings representatives of each half-sib family tested.

Once a week, inoculated seedlings were scored for disease symptoms until 8 weeks. At each assessment, seedlings were assigned to one of the following categories (Correll *et al.*, 1991): 0 = healthy foliage with no stem necrosis; 1 = healthy foliage with necrosis only at the point of inoculation; 2 = healthy foliage with at least 2 cm necrosis beyond the point of inoculation; 3 = wilting of needles and necrosis girdling the stem and 4 = stem girdled and foliage dead distal to the point of inoculation (dead plant) (Figure 1). Necrosis length was not quantified due to the small stem size.

Seedlings scored with 4 were removed, and a 5-cm-long stem segment near the point of inoculation was cut-off. Needles were removed and the stem was surface disinfested by immersion in Tween 20, then 30 sec in 70 per cent ethanol and 1 min in 1 per cent sodium hypochlorite.

Figure 1. Classification of *Fusarium circinatum* disease symptoms in *Pinus pinaster* seedlings (Correll *et al.*, 1991): 0 = healthy foliage with no stem necrosis; 1 = healthy foliage with necrosis only at the point of inoculation; 2 = healthy foliage with at least 2 cm necrosis beyond the point of inoculation; 3 = wilting of needles and necrosis girdling the stem and 4 = stem girdled and foliage dead distal to the point of inoculation (dead plant).

Stems were cultured at 22°C for 7 days on FSM selective medium (Aegerter and Gordon, 2006). Samples were transferred to KCl medium (0.6 g KCl and 15.0 g agar in 1 l of de-ionized water) to facilitate identification. Eight weeks after inoculation, all remaining seedlings were harvested and cultured as described above.

Data processing and statistical analysis

Seedling height and time-to-death data were analysed with a mixed model including the tray and family as random factors and the seed weight and germination date as fixed covariables. Incidence and percentage of dead trees (mortality) were calculated for each family and main block, including only those seedlings from which *F. circinatum* was re-isolated. Angular transformed percentage values $[y = \arcsin(x/100)^{1/2}]$ were analysed with a mixed model including family and main block as random factors. Variance components were estimated by restricted maximum likelihood. The statistical significance of the variance components for each random factor was assessed using likelihood ratio tests, where the differences in two times the log-likelihood of the models including and excluding that random factor are distributed as one-tailed χ^2, with one degree of freedom (Fry, 2004). When genetic variance was significant, the corresponding narrow sense heritability was calculated, assuming the pine families as true half-sibs and thus estimating the additive variance as four times the family variance. In the case of mortality, heritability was estimated as follows:

$$h_i^2 = 4V_f/(V_f + V_p + V_e),$$

in which V_f represents the family variance component, V_p represents the variance among groups of five plants of the same block and family (Aegerter and Gordon, 2006) and V_e represents the residual variance. All mixed models were fitted with the MIXED procedure of SAS (Littell *et al.*, 2006).

Because some seedlings remained alive at the end of the experiment, their time-to-death data were unknown. For the genetic analyses described above, the time-to-death of these trees was assumed as the final date of the experiment; probably biasing the results. Thus, survival analysis techniques were used to further describe and model time-to-death data (survival time), where delayed mortality of plants is interpreted as higher tolerance to the pathogen (Esker *et al.*, 2006; Solla *et al.*, 2011). Seedlings that were alive at the end of the experiment, but had *F. circinatum* recovered, were considered censored since their time-to-death was unknown. Survival analysis, which is commonly used in ecological and medical experiments to analyse the time-to-death or time-to-event data (Kleinbaum and Klein, 2005), is unique in that it allows for censoring of observations and analysis of failure times (i.e. deaths) that are not normally distributed. To compare tolerance to *F. circinatum* among families, the Kaplan–Meier estimate was used to obtain survival probabilities (Kleinbaum and Klein, 2005), which is a nonparametric procedure. 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 (Esker *et al.*, 2006). Goodness-of-fit of modelling and median life expectancies were obtained with the 'Life Tables & Distributions' procedure of Statistica v7.0 (Stat Software Inc., Tulsa, OK), and survival time data were analysed through the 'Comparing Multiple Samples' procedure of this statistical package, with 'Family' as the grouping variable.

The relationships between parameters of seed weight, time to germination, tree height, percentage of infected seedlings (incidence), time-to-death and percentage of seedling mortality were examined by means of Pearson correlation coefficients both at the family (N = 40) and the individual level (N = 1200).

Results

Seed weight, time to germination and tree height varied significantly among families ($P < 0.001$; Table 1). Germination started 17 days after sowing for most families but lasted up to 129 days for some other families (Table 1). The first seedlings killed by *F. circinatum* were observed 14 days after inoculation, and mortality peaked 28 days after inoculation (Figure 2).

Table 1: Family means of early performance (seed weight, time to germination and tree height) and susceptibility to *Fusarium circinatum* (incidence, time-to-death and mortality) variables in 40 *Pinus pinaster* progenies, ranked by mortality

Family code	Seed weight (mg)	Time to germination (days)	Tree height (cm)	Incidence (%)	Time-to-death (days)	Mortality (%)
2002	65.5 (30–84)	29.0 (17–73)	11.9 (6–18)	71	48	33
1049	65.7 (30–81)	27.1 (17–66)	11.8 (7–16)	58	47	38
1020	77.1 (36–93)	20.3 (17–38)	13.2 (8–24)	73	46	46
2070	75.7 (63–88)	25.6 (17–87)	12.2 (8–19)	62	46	46
1007	82.7 (44–107)	32.8 (17–94)	13.8 (8–18)	75	43	47
1033	53.1 (34–76)	37.8 (17–129)	10.7 (5–17)	76	46	48
2053	54.7 (27–74)	23.7 (17–38)	13.0 (9–19)	62	44	50
1046	71.9 (32–88)	26.1 (17–73)	12.3 (6–20)	65	43	50
2017	82.8 (62–105)	23.5 (17–31)	12.4 (7–18)	81	45	52
1030	79.9 (55–109)	26.1 (17–52)	14.3 (9–22)	70	44	52
1043	68.1 (45–91)	30.3 (17–94)	11.9 (7–17)	76	44	56
2004	65.8 (32–82)	24.3 (17–66)	13.1 (8–18)	74	44	56
2021	59.5 (30–90)	37.5 (17–87)	13.2 (9–25)	74	43	56
2043	70.0 (35–94)	22.6 (17–80)	12.2 (10–15)	72	42	56
1036	71.4 (43–89)	29.1 (17–87)	11.7 (8–17)	88	44	60
2082	54.9 (20–85)	30.1 (17–129)	11.0 (6–18)	83	44	61
1050	61.9 (35–81)	23.0 (17–94)	12.6 (7–18)	73	41	62
1003	96.1 (60–119)	26.8 (17–80)	13.1 (9–17)	81	42	63
1059	66.6 (42–85)	19.4 (17–38)	11.5 (7–17)	76	38	64
2026	53.6 (40–71)	20.3 (17–38)	12.5 (7–24)	69	40	65
2050	83.1 (52–97)	20.5 (17–31)	11.9 (7–16)	77	41	67
2064	75.8 (51–92)	30.1 (17–73)	12.4 (9–19)	79	41	67
2013	72.2 (33–100)	25.3 (17–87)	12.0 (7–17)	88	39	67
Control	47.9 (24–65)	48.9 (17–129)	11.1 (8–15)	75	38	69
1011	71.3 (32–95)	28.0 (17–80)	12.4 (9–21)	78	39	70
2076	56.8 (29–66)	37.0 (17–94)	10.5 (7–14)	79	39	71
2072	46.5 (21–88)	34.8 (17–94)	10.4 (7–15)	83	36	71
2042	61.7 (31–97)	31.6 (17–80)	11.2 (7–15)	84	41	74
2001	78.0 (44–102)	21.7 (17–45)	12.5 (8–18)	84	39	74
2031	88.6 (10–114)	21.1 (17–59)	13.1 (7–18)	85	39	74
2054	60.2 (34–79)	39.8 (17–122)	11.2 (8–14)	74	38	74
2041	78.8 (36–131)	23.7 (17–52)	12.6 (8–20)	89	36	74
1035	83.9 (59–104)	18.4 (17–24)	12.7 (8–16)	93	40	76
2062	53.7 (20–87)	37.3 (17–122)	10.6 (7–15)	86	37	76
1004	71.3 (39–90)	29.4 (17–80)	12.8 (8–17)	88	38	77
1056	71.7 (60–85)	27.4 (17–73)	12.6 (7–20)	85	39	78
2045	61.0 (31–81)	40.4 (17–87)	12.0 (9–19)	87	34	78
2051	62.3 (32–84)	20.4 (17–45)	12.1 (7–16)	81	36	81
2077	58.4 (35–88)	52.0 (17–94)	11.0 (7–13)	92	36	81
2040	61.2 (33–85)	25.6 (17–80)	13.6 (7–17)	92	35	81
Average	68.0***	28.7***	12.2***	79*	41***	63*

Numbers in brackets indicate range values. Incidence refers to percentage of infected seedlings. Asterisks indicate levels of significance between families at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Re-isolation of the pathogen from the inoculated trees was 98 per cent, and *F. circinatum* was not recovered from the controls. About 80 per cent of all seedlings were symptomatic, and overall mortality was 63 per cent (Table 1). Disease incidence, time-to-death and seedling mortality varied significantly among families (Table 1). By the end of the experiment, 15 of 40 families showed a high disease incidence, with more than 70 per cent mortality. Seedling mortality varied among families between 33 and 81 per cent (Table 1). Heritability estimates were low for height, incidence and time-to-death but high for mortality (Table 2).

Examination of the log-likelihood of the data under the null model indicated that the exponential function described the survival data better than other models examined (results not shown). Cumulative proportions of survival, compared through the Kaplan–Meier estimate, were significantly different among families ($P = 0.0069$) (Figure 3). The unimproved control family had 31 per cent seedling survival at the end of the study, not different to the mean survival of the 39 improved families (37 per cent) (Figure 3). The four families showing the highest life expectancies were 1020, 1049, 2002 and 2070, coinciding with those showing the higher survival rates.

The germination rate varied among families ranging from 54 to 100 per cent. Germination rate was predictive of time-to-death (Pearson's $r = 0.37$, $P = 0.018$) and of seedling mortality ($r = -0.32$, $P = 0.043$). Families and individuals from heavy seeds germinated significantly sooner and were significantly taller than families and individuals from light seeds (Table 3). At the family level, there were no significant relations between early performance (seed weight, time to germination and tree height) and susceptibility variables. At the individual level, however, genotypes with heavy seeds died later than genotypes with light seeds (Tables 1 and 3).

Discussion

Screening of selected *P. pinaster* families used for reforestation purposes in NW Spain showed that genetic variation in response to *F. circinatum* does exist. Although heritability of time-to-death was moderate (~0.2), heritability of mortality was high enough (~0.5) to allow screening for resistance. The heritability of mortality obtained in this study for *P. pinaster* was in the same range as the heritabilities reported for *P. radiata* (Aegerter and Gordon, 2006; Matheson *et al.*, 2006). This indicates a strong genetic control of the observed variation and confirms our hypothesis that selection of resistance is possible. The mechanism for resistance in *P. pinaster* is unknown, however, specific genes governing the formation of lignified cell wall, periderm restoration and the timing and density of traumatic resin canal formation (Dwinell and Barrows-Broaddus, 1979; Barrows-Broaddus and Dwinell, 1984; Kim *et al.*, 2010) may be responsible for the different susceptibility of the trees to *F. circinatum*.

Although 15 per cent of the inoculated trees were asymptomatic, the fungus was re-isolated from the latent lesions. This indicates that infection occurs without necessarily resulting in lesion development (Barrows-Broaddus and Dwinell, 1983; Kim *et al.*, 2008). Others have observed that asymptomatic infected seedlings become symptomatic at some point when the fungus switches from a latent to an active form of infection (Wingfield *et al.*, 2008). This change of behaviour supports previous concerns that infected seedlings can remain symptomless and thereby serve as vectors for long distance transport of the pathogen

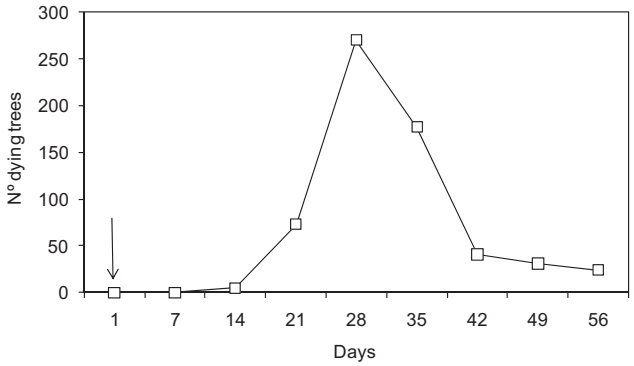

Figure 2. Mortality of *Pinus pinaster* seedlings after inoculation with *Fusarium circinatum* ($n = 984$). Arrow indicates inoculation on 24 June 2009.

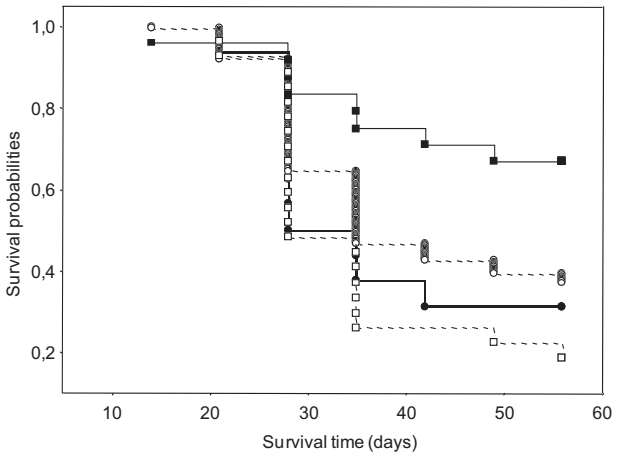

Figure 3. Plot of survival probabilities using the Kaplan–Meier estimate ($P = 0.0069$) of the survival function for *Pinus pinaster* inoculated with *Fusarium circinatum* at time 0. Lines correspond to all genetic entries (open circles), control family (filled squares), highly susceptible 2051 family (open squares) and moderately tolerant 2002 family (filled squares).

Table 2: Mixed model summary used to calculate heritability in 40 *Pinus pinaster* families inoculated with *Fusarium circinatum*

	Family			Tray/block			Residual	
Variable	σ^2	F/χ^2	*P*-value	σ^2	F/χ^2	*P*-value	σ^2	h_i^2
Tree height	0.29 ± 0.12	17.5	0.0000	0.97 ± 0.3	95	0.0000	5.53 ± 0.26	0.20
Incidence	0.001 ± 0.005	0	0.5000	0.022 ± 0.024	21.7	0.0000	0.057 ± 0.009	0.07
Time-to-death	6.37 ± 2.78	13.1	0.0001	33.47 ± 9.99	131.1	0.0000	138.49 ± 6.48	0.18
Mortality	0.011 ± 0.006	5.3	0.017	0.05 ± 0.051	60.7	0.0000	0.037 ± 0.006	0.45

Height and time-to-death data were analysed assuming a randomized complete block design with 30 blocks (trays), whereas the incidence and mortality were analysed assuming a randomized complete block with three main blocks (greenhouse beds). The family and the tray/block effects were considered random effects, and variance components (σ^2) and corresponding likelihood ratio significance tests (χ^2) are shown.

Table 3: Pearson values from familiar (above the diagonal, $n = 40$) and individual (below the diagonal, $n = 984$) correlations among early performance and susceptibility variables of *Pinus pinaster* trees

	Seed weight (mg)	Time to germination (days)	Tree height (cm)	Incidence (%)	Time-to-death (days)	Mortality (%)
Seed weight (mg)	X	−0.53***	0.60***	ns	ns	ns
Time to germination (days)	−0.19***	X	−0.52***	ns	ns	ns
Tree height (cm)	0.26***	−0.16***	X	ns	ns	ns
Incidence (%)	–	–	–	X	−0.58***	0.76***
Time-to-death (days)	0.10**	ns	ns	–	X	−0.87***
Mortality (%)	–	–	–	–	–	X

Asterisks indicate levels of significance at $^{**}P < 0.01$ and $^{***}P < 0.001$; ns = non significant; – = invalid correlation.

(Storer *et al.*, 1998). This avenue of dissemination could contribute to the establishment of pitch canker in Spain as has occurred in California (Gordon *et al.*, 1996). Further studies are necessary to determine in which conditions non-active lesions of *P. pinaster* caused by *F. circinatum* become active.

The likelihood of *F. circinatum* spreading from Spain to Southern France on *P. pinaster* is high. However, this has not yet occurred. Within the native populations of *P. pinaster* in Spain, a certain level of resistance to the pathogen exists. Inoculation tests conducted for *P. radiata* resulted in 100 per cent of plant mortality 8 weeks after inoculation (Hodge and Dvorak, 2000; Muñoz and Ampudia, 2005; Bragança *et al.*, 2009), in contrast to the lower mortality obtained here. In areas in which *P. radiata* is heavily affected by pitch canker, most of the *P. pinaster* trees remain asymptomatic (Berra and Urkola, 2010). Although the fungus can be frequently isolated from seeds and flowers (Pintos *et al.*, 2008), reported mortality of mature trees is rare. Possibly, the introduction is too recent. For example, in California, it has been reported that the disease intensity increases only after many trees have become infected (Storer *et al.*, 2002).

The relationship between the susceptibility to *F. circinatum* of half-sib families in the greenhouse and the susceptibility of the orchard clones in other pine species (Barrows-Broaddus and Dwinell, 1984; Gordon *et al.*, 1998a; Kim *et al.*, 2008) indicates that inoculations of *P. pinaster* seedlings could be used to test for resistance. Experiments in which mortality could be compared among field trees and nursery plants would give more support to this possibility. However, field research on *F. circinatum* in south-western Europe is subject to strict quarantine measures presenting limitations for long-term experiments. By law, removal of infected trees in the field is compulsory (Anonymous, 2010).

Given the positive correlations between seed weight and tree height, and between seed weight and time-to-death, one would expect that survival rates might simply vary according to differences in vigour between seedlings. The lack of correlation between tree height and time-to-death, at both the family and individual levels, argues against this hypothesis. In the same way, it should be noted that the unimproved control seed lot (not selected for tree growth) had an intermediate level of susceptibility to the pathogen. The lack of correlation between tree growth and susceptibility would provide a practical advantage for tree breeders, i.e. selection for growth of *P. pinaster* trees in breeding programs would not necessarily imply an increase of susceptibility to *F. circinatum*. Increased susceptibility of *P. pinaster* with increased tree vigour has been previously reported in relation to *Dioryctria sylvestrella* and *Hylobious abietis* attacks (Kleinhentz *et al.*, 1998; Zas *et al.*, 2005) and to infection by *Melampsora pinitorqua* (Desprez-Loustau and Wagner, 1997).

The importance of seed weight (size) in governing the fitness of a tree has been supported by extensive empirical evidence, i.e. larger seeds promote germination and favour growth and survival (Castro *et al.*, 2006). Although weak, the significant correspondence between seed weight and time-to-death supports this concern. The germination rate could also be considered a proxy for seedling vigour. Most of the seedlings were 6 months old when inoculated, and it is possible that differences of 1 or 2 weeks between times to germination would have an influence on the seedlings' susceptibility. Early performance parameters of seedlings should be used with caution when analyzing susceptibility tests (Solla *et al.*, 2011).

Conclusion

At the present, the best long-term solution of controlling pitch canker in nurseries and forest plantations lies in the selection and breeding for tolerant trees. Our results provide evidence that rapid screening in greenhouse conditions is possible. The native 1020, 1049, 2002 and 2070 pine individuals could be used as breeding stock or as sources to produce seeds with moderate levels of tolerance to *F. circinatum*. The substantial levels of resistance among these *P. pinaster* families may provide an alternative species to landowners currently using the more susceptible *P. radiata*.

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Conflict of interest statement

None declared.

References

Anonymous 2010 Real Decreto 65/2010, de 29 de enero, por el que se modifica el Real Decreto 637/2006, de 26 de mayo, por el que se establece el programa nacional de erradicación y control del hongo de la coníferas "*Fusarium circinatum*" Nirenberg et O´Donnell. *Bol. Off. Estado*. **44**, 16157–16159.

Aegerter, B.J. and Gordon, T.R. 2006 Rates of pitch canker induced seedling mortality among *Pinus radiata* families varying in levels of genetic resistance to *Gibberella circinata* (anamorph *Fusarium circinatum*). *For. Ecol. Manage.* **235**, 14–17.

Barrows-Broaddus, J. and Dwinell, L.D. 1983 Histopathology of *Fusarium moniliforme* var. *subglutinans* in four species of southern pines. *Phytopathology*. **73**, 882–889.

Barrows-Broaddus, J. and Dwinell, L.D. 1984 Variation in susceptibility to the pitch canker fungus among half-sib and full-sib families of Virginia pine. *Phytopathology*. **74**, 438–444.

Berra, D. and Urkola, A. 2010 Epidemiología de *Fusarium circinatum* en plantaciones forestales de Gipuzkoa. In *Proceedings of the XV Congreso de la Sociedad Española de Fitopatología, 2010*. Sociedad Española de Fitopatología (ed.). Vitoria-Gasteiz, Spain, 282 pp.

Bragança, H., Diogo, E., Moniz, F. and Amaro, P. 2009 First report of pitch canker on pines caused by *Fusarium circinatum* in Portugal. *Plant Dis.* **93**, 1079.

Carlucci, A., Colatruglio, L. and Frisullo, S. 2007 First report of pitch canker caused by *Fusarium circinatum* on *Pinus halepensis* and *P. pinea* in Apulia (Southern Italy). *Dis. Notes*. **91**, 1683.

Castro, J., Hódar, J.A. and Gómez, J.M. 2006 Seed size. In *Handbook of Seed Science and Technology (Seed Biology, Production, and Technology)*. A.S. Basra (ed.). Harworth Press, New York, NY, pp. 397–428.

Correll, J.C., Gordon, T.R., Mccain, A.H., Fox, J.W., Koehler, C.S., Wood, D.L. *et al.* 1991 Pitch canker disease in California: pathogenicity, distribution, and canker develpoment on monterey pine (*Pinus radiata*). *Plant Dis.* **75**, 676–682.

Desprez-Loustau, M.L. and Wagner, K. 1997 Influence of silvicultural practices on twisting rust infection and damage in maritime pine, as related to growth. *For. Ecol. Manage.* **98**, 135–147.

Dwinell, L.D. and Barrows-Broaddus, J.B. 1979 Susceptibility of half-sib families of slash and loblolly pine to the pitch canker fungus, *Fusarium moniliforme* var *subglutinans* (Abstr.). *Phytopathology*. **69**, 527.

Esker, P.D., Gibb, K.S., Padovan, A., Dixon, P.M. and Nutter, F.W.J. 2006 Use of survival analysis to determine the postincubation time-to-death of papaya due to yellow crinkle disease in Australia. *Plant Dis.* **90**, 102–107.

Fry, J.D. 2004 Estimation of genetic variances and covariances by restricted maximum likelihood using PROC MIXED. In *Genetic Analysis of Complex Traits using SAS*. A.M. Saxton (ed.). SAS Institute, Cary, NC, pp. 11–34.

González-Martínez, S.C., Mariette, S., Ribeiro, M.M., Burban, C., Raffin, A., Chambel, M.R. *et al.* 2004 Genetic resources in maritime pine (*Pinus pinaster* Aiton): molecular and quantitative measures of genetic variation and differentiation among maternal lineages. *For. Ecol. Manage.* **197**, 103–115.

Gordon, T.R., Storer, A.J. and Okamoto, D. 1996 Population structure of the pitch canker pathogen, *Fusarium subglutinans* f. sp. *pini*, in California. *Mycol. Res.* **100**, 850–854.

Gordon, T.R., Okamoto, D., Storer, A.J. and Wood, D.L. 1998a Susceptibility of five landscape pines to pitch canker disease, caused by *Fusarium subglutinans* f. sp. *pini*. *Hortscience*. **33**, 868–871.

Gordon, T.R., Wikler, K.R., Clark, S.L., Okamoto, D., Storer, A.J. and Bonello, P. 1998b. Resistance to pitch canker disease, caused by *Fusarium subglutinans* f. sp. *pini*, in Monterey pine *(Pinus radiata)*. *Plant Pathol.* **47**, 706–711.

Hodge, G.R. and Dvorak, W.S. 2000 Differential responses of Central America and Mexican pine species and *Pinus radiata* to infection by the pitch canker fungus. *New For.* **19**, 241–258.

Iturritxa, E., Ganley, R.J., Wright, J., Heppe, E., Steenkamp, E.T., Gordon, T.R. *et al.* 2011 A genetically homogenous population of *Fusarium circinatum* causes pitch canker of *Pinus radiata* in the Basque Country, Spain. *Fungal Biol.* **115**, 288–295.

Kim, Y.S., Woo, K.S., Koo, Y.B. and Yeo, J.K. 2008 Variation in susceptibility of six pine species and hybrids to pitch canker caused by *Fusarium circinatum*. *For. Pathol.* **38**, 419–428.

Kim, K.W., Lee, I.J., Kim, C.S., Eom, I.Y., Choi, J.W. and Lee, D.K. 2010 Resin flow, symptom development, and lignin biosynthesis of two pine species in response to wounding and inoculation with *Fusarium circinatum*. *J. Plant. Pathol.* **26**, 394–401.

Kleinbaum, D.G. and Klein, M. 2005 *Survival Analysis. A Self-Learning Text*. Springer-Verlag, New York, NY.

Kleinhentz, M., Raffin, A. and Jactel, H. 1998 Genetic parameters and gain expected from direct selection for resistance to *Dioryctria sylvestrella* Ratz. (Lepidoptera: *Pyralidae*) in *Pinus pinaster* Ait., using a full diallel mating. *For. Genet.* **5**, 147–154.

Landeras, E., García, P., Fernández, Y., Braña, M., Fernández-Alonso, O., Méndez-Lodos, S. *et al.* 2005 Outbreak of pitch canker caused by *Fusarium circinatum* on *Pinus spp*. in northern Spain. *Plant Dis.* **89**, 1015.

Littell, R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D. and Schabenberger, O. 2006 *SAS System for Mixed Models*. 2nd edn. SAS Institute, Cary, NC.

Martínez-Álvarez, P., Blanco, J., Vallejo, M., Alves-Santos, F. and Diez, J.J. 2009 Pathogenicity of *Fusarium circinatum* Nirenberg & O'Donnell on seeds and seedlings of *radiata pine*. In *Proceedings of the Conference of IUFRO Working Party 7. 02.02 2009*. N. Gürlevik (ed.). SDU Faculty of Forestry Journal, EĞİRDİR, Turkey, pp. 200–205.

Matheson, A.C., Devery, M.E., Gordon, T.R., Werner, W., Vogler, D.R., Balocchi, C. *et al.* 2006 Heritability of response to inoculation by pine pitch canker of seedlings of radiata pine. *Australas. For.* **69**, 101–106.

Muñoz, C. and Ampudia, M. 2005 Ensayos de patogeneidad de *Fusarium circinatum* Nirenberg y O'Donnell en plantas de *Pinus radiata* D. Don. In *Proceedings of the 4° Congreso Forestal Español, 2005*. Sociedad Española de Ciencias Forestales, Gobierno de Zaragoza (ed.). S.E.C.F., Zaragoza, Spain, pp. 1–7.

Pérez-Sierra, A., Landeras, E., León, M., Berbegal, M., García-Jiménez, J. and Armengol, J. 2007 Characterization of *Fusarium circinatum* from *Pinus* spp. in northern Spain. *Mycol. Res.* **111**, 832–839.

Pintos, C., Pérez-Otero, R., Aguín, O., Mansilla, J.P., González-Penalta, B. and Rial, C. 2008 Presencia de *Fusarium circinatum* en estructuras reproductivas de *Pinus pinaster* en Galicia. In *Proceedings of the XIV Congreso de la Sociedad Española de Fitopatología, 2008*. Sociedad Española de Fitopatología (ed.). Sociedad Española de Fitopatología Lugo, Spain, 149 pp.

Roux, J., Eisenberg, B., Kanzler, A., Nel, A., Coetzee, V., Kietzka, E. *et al.* 2007 Testing of selected south african *Pinus* hybrids and families for tolerance to the pitch canker pathogen, *Fusarium circinatum*. *New For.* **33**, 109–123.

Schmale, D.G. and Gordon, T.R. 2003 Variation in susceptibility to pitch canker disease, caused by *Fusarium circinatum*, in native stands of *Pinus muricata*. *Plant Pathol.* **52**, 720–725.

Solla, A., Aguín, O., Cubera, E., Sampedro, L., Mansilla, J. and Zas, R. 2011 Survival time analysis of *Pinus pinaster* inoculated with *Armillaria ostoyae*: genetic variation and relevance of seed and root traits. *Eur. J. Plant Pathol.* **130**, 477–488.

Storer, A.J., Gordon, T.R. and Clark, S.L. 1998 Association of the pitch canker fungus, *Fusarium subglutinans* f.sp. *pini*, with Monterey pine seeds and seedlings in California. *Plant Pathol.* **47**, 649–656.

Storer, A.J., Bonello, P., Gordon, T.R. and Wood, D.L. 1999 Evidence of resistance to the pitch canker pathogen (*Fusarium circinatum*) in native stands of Monterey pine (*Pinus radiata*). *For. Sci.* **45**, 500–505.

Storer, A.J., Wood, D.L. and Gordon, T.R. 2002 The epidemiology of pitch canker of Monterey pine in California. *For. Sci.* **48**, 694–700.

Vivas, M., Vrhovnik, M. and Solla, A. 2009 Fertilización de plántulas de *Pinus pinaster* y su efecto en la susceptibilidad a *Fusarium circinatum*. In *Proceedings of the 5° Congreso Forestal Español, 2009*. Sociedad Española de Ciencias Forestales, Junta de Castilla y León (ed.). S.E.C.F., Ávila, Spain, pp. 1–10.

Watt, M.S., Ganley, R.J., Kriticos, D.J. and Manning, L.K. 2011 Dothistroma needle blight and pitch canker: the current and future potential distribution of two important diseases of *Pinus* species. *Can. J. For. Res.* **41**, 412–424.

Wingfield, M.J., Hammerbacher, A., Ganley, R.J., Steenkamp, E.T., Gordon, T.R., Wingfield, B.D. *et al.* 2008 Pitch canker caused by *Fusarium circinatum* - a growing threat to pine plantations and forest worldwide. *Australas. Plant Pathol.* **37**, 319–334.

Zas, R., Sampedro, L., Prada, E. and Fernández-López, J. 2005 Genetic variation of *Pinus pinaster* Ait. seedlings in susceptibility to the pine weevil *Hylobius abietis* L. *Ann. For. Sci.* **62**, 681–688.

Zas, R., Solla, A. and Sampedro, L. 2007 Variography and kriging allow screening *Pinus pinaster* resistant to *Armillaria ostoyae* in field conditions. *Forestry*. **80**, 201–209.

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