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TESIS DOCTORAL:

SUSCEPTIBILIDAD DE *Pinus pinaster* Ait. ANTE *Fusarium circinatum* Nirenberg y O'Donnell: VARIABILIDAD Y EFECTOS MATERNOS

Presentada por María Vivas Conejero para
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SUSCEPTIBILITY OF *Pinus pinaster* Ait. TO *Fusarium circinatum* Nirenberg and O'Donnell: VARIABILITY AND MATERNAL EFFECTS

PhD program on "Conservation and Sustainable Management of Forest Systems"

The present thesis fulfils the necessary requisites to obtain the International Doctorate Mention through the University of Valladolid.

María Vivas Conejero

PhD Supervisor Dr. Alejandro Solla Hach

Dedicada a todos los que me habéis acompañado en este camino

Susceptibility of *Pinus pinaster* Ait. to *Fusarium circinatum* Nirenberg and O'Donnell: variability and maternal effects

María Vivas, 2012

Fusarium circinatum is a fungal pathogen, known to cause pitch canker disease on pines. The disease cause important damage and relevant economic looses in nurseries and natural or planted stands of pines. In Europe, pitch canker is an introduced non-native disease. First isolated in northern Spain, affects nurseries and forest plantations of *Pinus radiata* and *P. pinaster*. Maritime pine (*P. pinaster*) is a native conifer of the Western Mediterranean basin used for important reforestation areas in many countries and continents. Nowadays, there is no means to control the pitch canker disease. The aim of the present study is to evaluate the variability of *P. pinaster* susceptibility to the pathogen fungus and the influence of environmental maternal effects. The variability of *P. pinaster* susceptibility to the fungus is first evaluated using plant defense elicitors. Particularly, the ability of different chemical plant elicitors (methyl jasmonate, DL-β-aminobutiric acid and benzothiadiazole) to induce resistance in *P. pinaster* against *F. circinatum* was evaluated. The results suggested that the use of these elicitors to prevent pitch canker disease in *P. pinaster* seedlings should be discarded. Moreover, the variability of half-sib families from 39 *P. pinaster* clones were tested for resistance to pitch canker disease and results indicated that the use of native pine individuals as breeding stock or as sources to produce seeds with moderate levels of tolerance to *F. circinatum* is possible. In other hand, the effects of two contrasting *P. pinaster* seed orchards (Sergude and Monfero, favourable and unfavourable seed orchards,

respectively), in terms of growth and reproduction, were studied, in order to account for variability of susceptibility of *P. pinaster* seedlings to *F. circinatum*. The results showed that maternal environment influenced seedlings susceptibility, so that the necrosis length caused by the fungus was different between seedlings from contrasting maternal environments. Carbohydrate compounds and antioxidant activity of seedlings were different depending on the maternal environments. To sum up, it has been demonstrated that an appropriated genetic selection for less susceptible clones and maternal environments exposed to the appropriated environmental cues can reduce the impact of the disease. These results open possibilities of research to further investigate new means of controlling pitch canker disease.

Keywords: Maritime pine, pitch canker, induce resistance, genetic resistance, maternal effects.

Susceptibilidad de *Pinus pinaster* Ait. ante *Fusarium circinatum* Nirenberg y O'Donnell: variabilidad y efectos maternos

María Vivas, 2012

Fusarium circinatum es un hongo patógeno, conocido por ser el causante del chancro resinoso del pino. Esta enfermedad causa importantes daños y pérdidas económicas en viveros y en zonas naturales o plantaciones de pinos. En Europa, el chancro resinoso es una enfermedad introducida que fue aislada por primera vez en el norte de España y que afectó a viveros y plantaciones de *Pinus radiata* y *P. pinaster*. El pino marítimo (*P. pinaster*) es una conífera natural de la cuenca Oeste Mediterránea utilizado para repoblar importantes zonas en numerosos países y continentes. Actualmente no existe ningún medio para controlar el chancro resinoso. El objetivo del estudio consiste en evaluar la variabilidad de la susceptibilidad de *P. pinaster* al patógeno y la influencia de los efectos del ambiente materno. La variabilidad de la susceptibilidad de *P. pinaster* al hongo se evaluó primero utilizando elicitores de defensa de las plantas. Particularmente, se evaluó la habilidad de metil jasmonato, ácido DL-β-aminobutirico y benzotiadiazol para inducir resistencia en *Pinus pinaster* ante *F. circinatum*. Los resultados sugieren que no se deben utilizar estos inductores para prevenir la enfermedad del chancro resinoso en plántulas de *P. pinaster*. También, se analizó la variabilidad de la resistencia de clones de *P. pinaster* de medios hermanos ante el chancro resinoso y los resultados indican que el uso de determinados clones con niveles moderados de tolerancia a *F. circinatum* como individuos reproductores o como productores de semillas es posible. Por otra parte, se estudian los efectos de dos

huertos de *P. pinaster*, diferentes en términos de crecimiento y reproducción (Sergude y Monfero, favorable y desfavorable respectivamente), con el fin de explicar la variabilidad de la susceptibilidad de la descendencia ante *F. circinatum*. Los resultados muestran que el ambiente materno influye en la susceptibilidad de las plántulas ante *F. circinatum*, de modo que la longitud de la necrosis causada por el hongo es menor en las plántulas provenientes del ambiente materno más favorable. La composición de carbohidratos y la actividad antioxidante de las plántulas mostró diferentes respuestas según los ambientes maternos. En resumen, una selección genética apropiada de clones menos susceptibles y ambientes maternos expuestos a estímulos ambientales apropiados pueden reducir el impacto de la enfermedad. Los resultados amplían las posibilidades de investigación de nuevos métodos de control del chancro resinoso del pino.

Palabras clave: pino marítimo, chancro resinoso, resistencia inducida, resistencia genética, efectos maternos.

LIST OF ORIGINAL ARTICLES

This thesis is based on the following papers and manuscripts, which in the text will be referred to by their Roman numerals I-VI.

I. Vivas, M., Martín, J. A., Gil, L., Solla, A., 2012. Evaluating methyl jasmonate for induction of resistance to *Fusarium oxysporum, F. circinatum* and *Ophiostoma novo-ulmi*. Forest Systems 21(2), 289-299.

II. Vivas, M., Solla, A., 2012. Aplicaciones de BABA y BTH en brinzales de *Pinus pinaster* para la inducción de resistencia ante *Fusarium circinatum*. Cuadernos de la Sociedad Española de Ciencias Forestales 36, 11-16.

III. Vivas, M., Zas, R., Solla, A., 2012. Screening of Maritime pine (*Pinus pinaster*) for resistance to *Fusarium circinatum*, the causal agent of Pitch Canker disease. Forestry 85(2), 185-192.

IV. Vivas, M., Zas, R., Sampedro, L., Solla, A., 2012. Environmental maternal effects in a pine tree: early performance and susceptibility against *Fusarium circinatum*. Preliminary manuscript.

V. Vivas, M., Nunes, C., Coimbra, M.A., Solla, A., 2012. Carbohydrates of *Pinus pinaster* seedlings originating from contrasting maternal environments: do they influence susceptibility to *Fusarium circinatum*? Preliminary manuscript.

VI. Vivas, M., Nunes, C., Coimbra, M.A., Solla, A., 2012. Maternal environments influence the antioxidant activity of *Pinus pinaster* when infected by *Fusarium circinatum*. Preliminary short manuscript.

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INTRODUCTION

Importance and presence of *Fusarium circinatum*

The pathogen fungus *Fusarium circinatum* (teleomorph = *Gibberella circinata*) (Nirenberg and O'Donnell, 1998) is the causal agent of pitch canker disease in many parts of the world (Wingfield *et al.*, 2008). Actually, pitch canker is one of the most important diseases in pine species affecting plants plantations and nurseries. The disease reduces yield and causes high levels of mortality, resulting in important economic losses.

F. circinatum is an endemic pathogen in the south-eastern of United States where it was first recorded in 1946 (Hepting and Roth, 1946). After that date, it has been reported in Haiti (Hepting and Roth, 1953), California (McCain *et al.*, 1987), Japan (Muramoto and Dwinell, 1990), South Africa (Viljoen and Windfield, 1994), Chile (Wingfield *et al.*, 2002) and Europe. Particularly in Europe, *F. circinatum* was first reported in Spain affecting forest plantations and nurseries of *Pinus radiata* and *P. pinaster* (Landeras *et al.*, 2005; Pérez-Sierra *et al.*, 2007). Actually, the pathogen is also present in Italy (Carlucci *et al.*, 2007) and Portugal (Bragança *et al.*, 2009).

Taxonomic classification of *Fusarium circinatum*

Since 1946, the taxonomic status and scientific names of *F. circinatum* has been revised several times (Figure 1). The actual taxonomic classification of the pathogen fungus was established by Nirenberg and O'Donnell (1998) based on morphology, biochemical and genetic analysis.

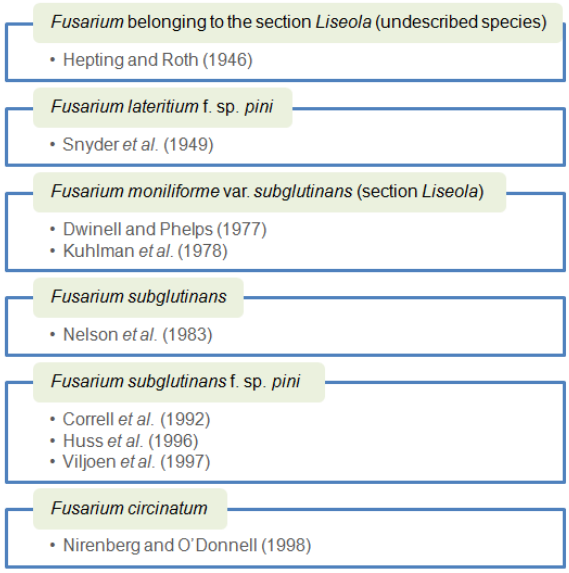


Figure 1. Evolution scheme of *Fusarium circinatum* taxonomy since its first report in 1946.

Infection biology and dispersal capacity of *Fusarium circinatum*

F. circinatum is an ascomycete and reproduces both asexually and sexually (Wingfield *et al.*, 2008). The asexual stage of the fungus (*F. circinatum*) results in clonal propagation. Their asexual spores (conidia) (Figure 2) develops in to small fruiting bodies salmon to purple (sporodochia), which usually occur in dry branches and stems of seedlings, but are difficult to observe. On the other hand, the sexual stage of the fungus receives the name of *Gibberella circinata* and its cycle results in recombination leading to new genotypes. However, the low frequency of sexual spores of the fungus (sporangia) observed, in areas where it has been recently introduced the pathogen, suggests that the asexual cycle occurs more frequently than the sexual cycle (Britz *et al.*, 2005).

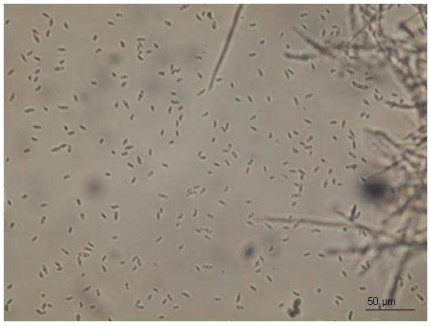


Figure 2. Conidia of *Fusarium circinatum*.

The inoculum of *F. circinatum* is available during all seasons of the year, and spore dispersion may occur through wind, water splash, insect vectors, soil and movement of infected materials (Blackeslee *et al.*, 1979; Viljoen and Windfield, 1994; Hoover *et al.*, 1996; Storer *et al.*, 1998; Gordon *et al.*, 2001). However, the subsequently infection of the tree will depend on the existence of a natural or artificial wound, because this fungus is not able to penetrate in the intact tissue of the plant (Dwinell and Barrows-Broaddus, 1981). *F. circinatum* do not develop resistance spores, anyway, its spores are able of surviving e.g. in soil and wood debris for a certain time (Gordon *et al.*, 2001). Regardless, we must be cautious since any of these aspects may suffer modifications depending on hosts species and geographical regions.

Climatic conditions also influence disease outbreaks. Spore germination and host infection by *F. circinatum* is influenced by temperature. Inman *et al.* (2008) showed that, in culture, the optimum temperatures for fungal growth and spore germination were 25ºC and 20ºC respectively. This fact may explain why *F. circinatum* seems to be most successful in some regions during warmer spring months. However, humidity is even more important that temperature. If

temperatures are within the optimum range but there is not enough humidity, the infection of the tree may not occur (Wingfield *et al.*, 2008). So, trees in an environment with an optimum range of temperatures for the fungus and high humidity may be more favorable to infection.

Overview, *F. circinatum* may be detected on infected trees in the field, when an infected tree die the fungus remain on the tree until is scattered and penetrate in other tree through a wound. In nursery, the pathogen fungus is transmitted from an infected plant to another through the air and, also, by the introduction of infected seeds, the use of contaminated tools and other human activities (Figure 3).

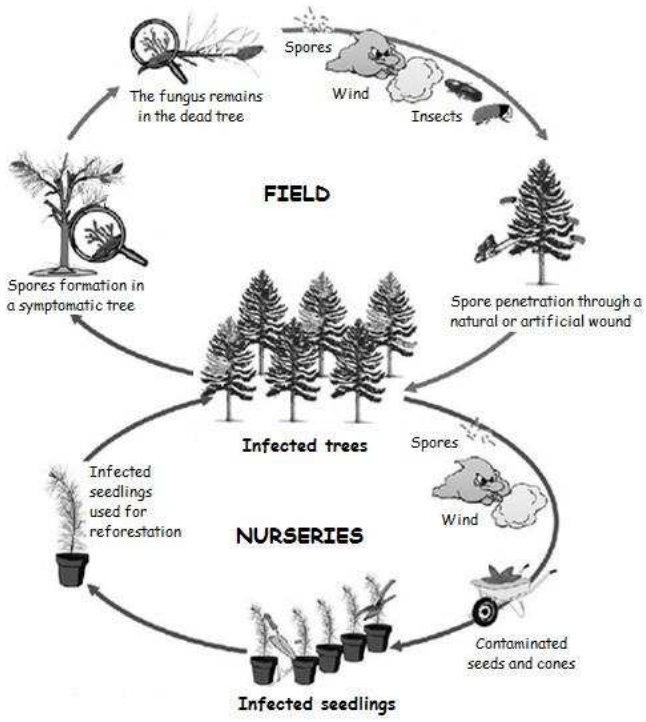


Figure 3. Biological cycle of *Fusarium circinatum* in the field and in the nursery. Source: Pintos *et al.* (2005) modified.

Symptoms of pitch canker disease

F. circinatum can affect all stages of the development and all vegetative and reproductive plant structures. Once the disease has been established in the host, the pathogen grows in the tissue and kills it. However, susceptibility to the pathogen can vary among and within species.

Most representative symptoms of pitch canker disease are (i) wilting and discoloration of needles, which eventually turn red and fall off (Figure 4a, 4b); (ii) dieback from the tips of branches to the infection site, due to the obstruction of water flow by girdling cankers that develop at the site of infection (Figure 4c); and (iii) resinous cankers in the trunk and/or branches associated with the infection sites (Figure 4d) (Dwinell *et al.*, 1985; Gordon *et al.*, 2001). Moreover, the fungus can deform the stem (Figure 4e), reduce growth rate or cause mortality. In nurseries, infected seedlings show wilting and chlorosis or reddening of needles and pre- and post-emergence damping-off; mortality of established seedlings also occur (Figure 4f) (Dwinell *et al.*, 1985; Carey and Kelley, 1994).

Reproductive plant structures (flowers, cones and seeds) are also infected by *F. circinatum* causing deterioration, growth depression and mortality (Barnard and Blackeslee, 2006). Moreover, infected seeds can significantly reduce seedling emergence or produce asymptomatic seedlings with no apparent symptoms but in which the fungus can be isolated (Correll *et al.*, 1991; Storer *et al.*, 2002). The problem of these asymptomatic seedlings arises because the latent fungus may switch to an active form of infection, at any moment, and facilitates its spread into new areas.

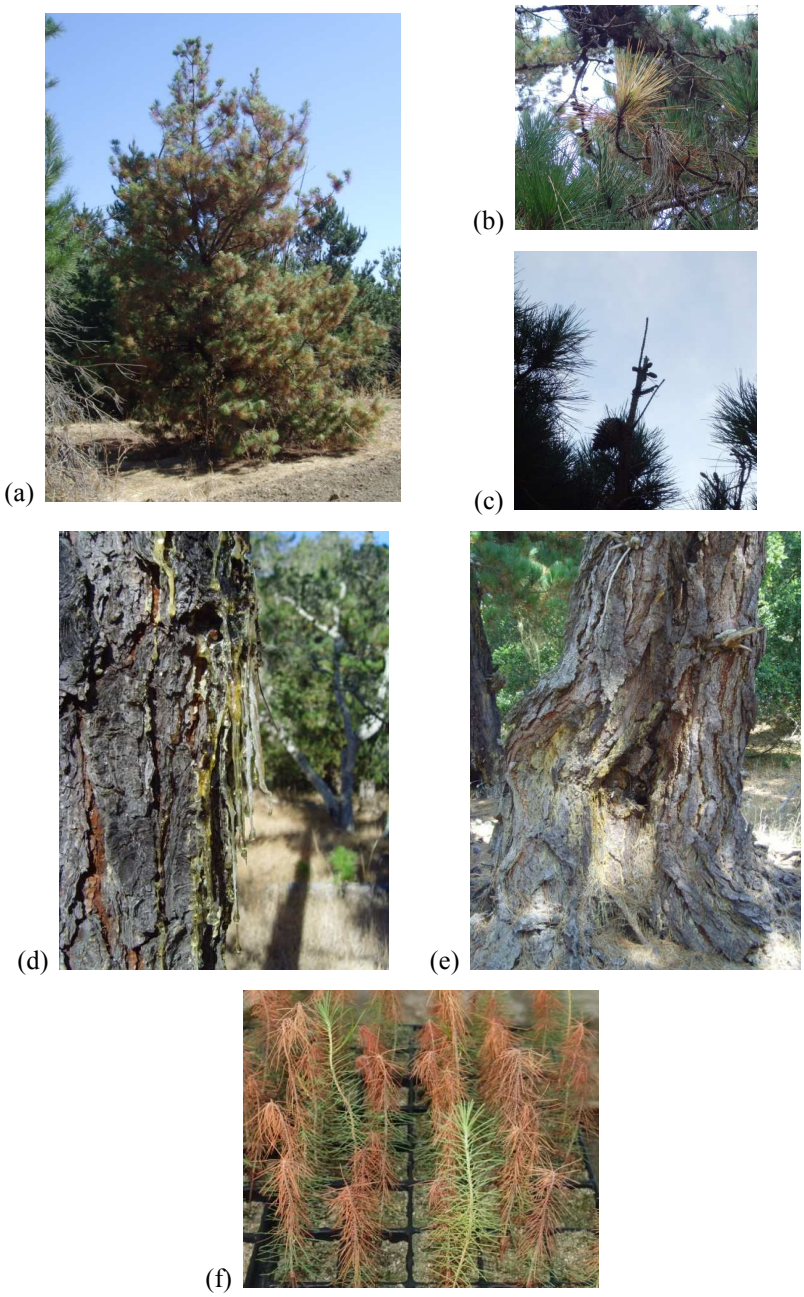


Figure 4. Pitch canker symptoms on pine trees: (a, b) yellowing of infected needles, (c) shoot tip dieback, (d) resinous canker on the trunk, (e) stem deformation and (f) seedling mortality.

Susceptible species. Brief description of *Pinus pinaster*

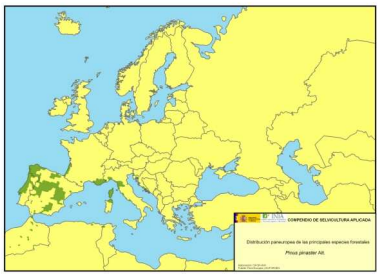
As far as we know, there are over 60 pine species susceptible to pitch canker (Hepting and Roth, 1953; Muramoto *et al.*, 1993; Storer *et al.*, 1994; Enebak and Stanosz, 2003; Hodge and Dvorak, 2000, 2007; Wingfield *et al.*, 2008) and a susceptible non-pine host, *Pseudotsuga menziesii* (Gordon *et al.*, 2006). Among these species, *Pinus radiata* could be the most susceptible species nowadays (Gordon *et al.*, 2001). This thesis was, however, focus in *P. pinaster* (Maritime pine) as a susceptible host to pitch canker (Figure 5a).

Maritime pine (*P. pinaster* Aiton) is a western Mediterranean species extending naturally from Spain, Portugal, continental France and Corsica, Italy (including Sicily and Sardinia Island), Malta, North of Morocco and some coastal points of the former Yugoslavia (Alía *et al.*, 1996; Serrada *et al.*, 2008) (Figure 5b). Moreover, *P. pinaster* presents important reforestations areas in many countries and continents. The Iberian Peninsula distribution of *P. pinaster* is patchy and comprises a broad spectrum of substrates, topographies and climates (López-Sáez *et al.*, 2010). Specifically in Spain, Maritime pine is the widest spread conifer species (near 1,200,00 ha) (DGCN, 2002) (Figure 5c).

P. pinaster usually ranges in height from 20-30 m. It has a pyramidal crown, a thick blackish bark and deeply fissured at the base of the trunk, and a deep and well developed radical system (Serrada *et al.*, 2008) (Figure 5a). The needles are in pairs, present a dark green color and their length are about 15-25 cm. The cones are conic, 8-22 cm long and ripen when 24 months old. The seeds are 8-10 mm long with a wide and dark wing which facilitates its anemophilous dispersion (Richardson, 1998).

(a)

(b)



(c)

Figure 5. (a) *Pinus pinaster* and its natural distribution area (b) in Europe and Africa and (c) in Spain. Maps source: Serrada *et al.* (2008)

Preventive management of pitch canker disease

Control methods to reduce the effects of pitch canker have been unsuccessfully investigated (Wingfield *et al.*, 2008). At present, the best mean of control pitch canker in forest plantations or nurseries is by an integrated management. An appropriate silvicultural and nursery management, including quarantine measures, e.g. BOE (2010), should be place to prevent the establishment of the pathogen in new free areas. Specifically, wounds cause by pruning practices in *P. radiata* plantations have shown to increased the chance of disease outbreak (Bezos *et al.*, 2012). Moreover, environmental stress, nitrogen contamination and high levels of fertilization increase the susceptibility of *Pinus* spp. to *F. circinatum* (Fisher *et al.*, 1981; Blakeslee *et al.*, 1999; Lopez-Zamora *et al.*, 2007), so it should be

avoid planting susceptible hosts on these sites. In nurseries, it should be used disease free seeds; even, some authors have suggested that hot water treatment of *P. radiata* seeds can reduced pitch canker contamination (Agustí-Brisach *et al.*, 2012).

Other possible mean to control pitch canker, may be based on the induction of resistance of the susceptible hosts. Plants possess constitutive defenses which are permanent structural or chemical compounds, present in the tree without any challenge, that represent the first lines of defence. In addition, plants also possess a second line of defenses, induce defenses, which are activated by plants generated upon perception of a foreign challenge (Franceschi *et al.*, 2005). It has been, already, reported the induction of resistance against pitch canker by repeated fungal inoculations of trees and seedlings (Bonello *et al.*, 2001; Gordon *et al.*, 2006; Sweet and Gordon, 2011). In other hand, some elicitor are also able to trigger induce defenses; for example, there is growing evidence that exogenous applications of Methyl Jasmonate (MeJA), Benzothiadiazole (BTH) or DL-β-aminobutyric acid (BABA) increase de level of defenses compounds to be used as defense mechanisms by plants (Cohen, 2002; Zeneli *et al.*, 2006; Moreira *et al.*, 2009; Barilli *et al.*, 2010; Eyles *et al.*, 2010). So, these elicitors may provide opportunities to enhance the resistance of *P. pinaster* to pitch canker disease.

Also, genetic selection for clones of species that are less susceptible to the pitch canker pathogen is a feasible way to reduce its long-term impact. Different families of *Pinus* inoculated with *F. circinatum* have consistently shown significant differences in susceptibility (Barrows-Broaddus and Dwinell, 1984; Dwinell and Barrows-Broaddus, 1979; 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*, a conifer characterized by high across- and within-population variation in adaptive traits (González-Martínez *et al.*, 2004).

In addition to genetic selection, there is now increasing evidence that effects of maternal environment contribute to the offspring phenotype without any changing in DNA sequence or any inducing signal in the environment (Donohue, 2009). The maternal environment by transgenerational plasticity may influence e.g. seed size, germination, seedling performance, resistance against herbivory or pathogens attacks, of conifers including *P. pinaster* (Schmidtling, 1987; Agrawal, 2002; Johnsen *et al.*, 2005; Besnard *et al.*, 2008; Cendán *et al.*, 2011; Rasmann *et al.*, 2012). Even so, we still know very little about how relevant effects of maternal environment are to the susceptibility of *P. pinaster* to *F. circinatum* and to what extend changes in the metabolites of the seedlings are occurring.

OBJECTIVES

The overall aim of this thesis was to evaluate the variability of *P. pinaster* susceptibility to *F. circinatum* and to test the influence of environmental maternal effects in such susceptibility. Particularly, the main objectives of this thesis (Roman numerals refer to the original articles) were to:

- Test if exogenous applications of Methyl Jasmonate (MeJA) (Article I), Benzothiadiazole (BTH) and DL-β-aminobutyric acid (BABA) (II) elicitors can induced resistance in *P. pinaster* seedlings to *F. circinatum*.

- Test the tolerance of *P. pinaster* seedlings to *F. circinatum* and check if the species can be improved against the pathogen through selection and breeding (III).

- Evaluate if environmental maternal effects may influence *P. pinaster* seedlings performance traits and seedlings resistance to *F. circinatum* and identify if the environmental maternal effects are seed mass-dependent or independent (IV).

- Quantify possible changes in the carbohydrate content (V) and in the antioxidant activity (VI) of *P. pinaster* seedlings inoculated with *F. circinatum,* and evaluate to what extend environmental maternal effects influence these responses.

MATERIALS AND METHODS

A brief explanation about the materials and methods used is exposed, but detailed information can be found in the original articles (Roman numerals).

Plant material

The studies were carried out on Maritime pine seedlings (*P. pinaster* Ait.) of about 6 months-old (Articles I, II & III) and 18 months-old (IV, V & VI). Seed material was obtained from *P. pinaster* of North-west Spain, specifically from: a single tree located in Cangas del Morrazo, Pontevedra (42.27ºN, 8.78ºW) (I); 39 genotypes selected for superior growth and form in mature plantations in Sergude seed orchard (42.82ºN, 8.45ºW) (II & III); 10 genotypes included in two *P. pinaster* seed orchards having identical genetic material and design but contrasting site qualities for *P. pinaster* in terms of growth and reproduction rate: Monfero (43.52ºN, 7.93ºW) and Sergude (42.82ºN, 8.45ºW), unfavourable and favourable seed orchard respectively (IV); and the genotype 1020 originating from the two contrasting maternal environments used in the study IV (V & VI). Monfero and Sergude seed orchards belong to the Galician Tree Breeding Program (Consellería do Medio Rural, Xunta de Galicia).

Fungal isolates

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), two different isolates were used indiscriminately: *F. circinatum* MAT-1, code Fc7-1, isolated in 2005

from a stem canker of a *P. pinaster* tree in Asturias, northern Spain (I, II & III); and *F. circinatum* MAT-2 isolated in 2011 from a stem canker on a *P. radiata* tree in Cantabria, northern Spain (IV, V & VI).

Methods

Elicitor treatments (I & II)

To study the effects of elicitors: an aqueous solution of MeJA (25 mM) was sprayed onto the seedling (I); and different aqueous solution of BTH (1 mM) and BABA (25 mM) were brushed onto the stem of the plant (II).

Inoculation (I, II, III, IV, V & IV)

Inoculum (I, II & III) was prepared by growing *F. circinatum* on potato dextrose agar. To inoculation, mycelium and conidia were scraped off the agar surface with a sterile scalpel, and immediately used to make a 1-mm-long slit wound into the succulent stem tissue, 5-10 cm above the ground level (Correll *et al.*, 1991).

Liquid inoculum (IV, V & VI) was prepared as a spore suspension of *F. circinatum* on 0.5% KCl. Inoculations were performed by placing 5 µl of the spore suspension of *F. circinatum* ($5 \cdot 10^3$ spores mL^{-1}) in a wound made in the stem with a drill bit at the junction of lignified and succulent tissue (Schmale and Gordon, 2003).

Susceptibility Test (I, II, III, IV, V & IV)

Plant susceptibility was recorded once a week during 8 weeks after inoculation (I, II & III). At each assessment, seedlings were assigned to one of the five categories proposed by Correll *et al.* (1991).

Seedlings were examined 4 weeks after inoculation and necrosis lengths were measured as described by Gordon *et al.* (1998b) (IV, V & VI).

Re-isolation of the fungus (I, II, III, IV, V & IV)

To assed seedlings infestation by *F. circinatum*, stems from inoculated seedlings were surface disinfested and cultured in Komada medium (Komada, 1975) (II) or *Fusarium* selective medium (Aegerter and Gordon, 2006) (I, III, IV, V & VI).

Tree assessment (I, III, IV, V & IV)

Seeds were individually weighed before sowing, in order to check the influence of seed weight on susceptibility (III, IV, V & IV). Non-inoculated seedlings were assessed for height growth, the number of resin ducts, root parameters and dry weight (I). Moreover, the germination status and plant height of each seed was examined (III, IV, V & VI) and individual stem diameter at ground level was also measured (IV, V & VI).

Carbohydrate analysis (V)

Stem samples were treated with 80% ethanol at 100ºC to obtain the alcohol insoluble residues (AIR). AIR was hydrolysed to obtained neutral sugars and uronic aids (Coimbra *et al.*, 1996; Oliveira *et al.*, 2009). Neutral sugars were derivatised as their alditol acetates (Selvendran and O'Neill, 1987) and separated by gas chromatography using a HP 5890 Series II chromatograph with a FID detector. And, uronic acids were quantified colorimetrically with *m*-phenylphenol

according to the method described by Blumenkrantz and Asboe-Hansen (1973) modified by Coimbra *et al.* (1996).

FT-IR spectroscopy (V)

FT-IR spectra of *P. pinaster* stem samples were obtained with a GoldenGate single-reflectance ATR accessory of a Bruker (Billerica, MA, USA) IFS-55 FT-IR spectrometer. The FT-IR spectral regions used were 1200-800 cm^{-1} coinciding with the 'fingerprint' region of carbohydrates (Filippov, 1992).

Antioxidant activity measurement (IV)

Antioxidant activity of *P. pinaster* stem samples was measured according to the method described by Re *et al.* (1999).

Statistical analysis

A General Linear Model (GLM) was used to analyze significant differences between normal variables, and individual means were separated by Fisher's least significant difference test (LSD; $P = 0.05$) (I & V). However, a Generalized Logit Model (GLZ) was used to analyze significant differences between non-normal variables (I & II).

A mixed model was also used to analyze significant differences between the data, and the statistical significance of the variance components for each random factor was assessed using likelihood ratio tests (III, IV).

The heritability was calculated to identified how much genetic was playing a role in different variables (III).

Survival analysis techniques were used to further described and model time to death data (III).

Pearson correlation coefficients (III) and beta regression model (VI) were used to analyze the relationships between parameters.

Principal component analysis (PCA) was applied to reduce the dimensionality of the data preserving most of the variance. Subsequently, discriminant function analysis (DFA) was applied (V).

RESULTS AND DISCUSSION

The main results and principal ideas of discussion are exposed below, but detailed information can be found in the original article (Roman numerals).

Evaluating elicitors in *Pinus pinaster* for induction of resistance against *Fusarium circinatum* (I & II)

P. pinaster seedlings treated with MeJA showed apical resinosis and reduction of height growth in comparison with control seedlings. However, seedlings treated with MeJA and subsequently inoculated with *F. circinatum* showed similar mortality in comparison with inoculated non-treated seedlings ($P > 0.05$) (I). In general, *P. pinaster* seedlings treated with BABA or BTH and subsequently inoculated with *F. circinatum* showed higher mortality rates than inoculated seedlings ($P < 0.001$) (II). In particular, some families showed induced resistant with both compounds, others only with BABA and another only with BTH, although most of them did not showed induced resistance with either of the two compounds (II). These results showed that MeJA treatment did not show any positive effect against *F. circinatum* (I) and BABA and BTH treatments showed different responses depending on *P. pinaster* family (II). We postulate four non-exclusive hypothesis to explain these results: (i) the protective and lasting effect of the elicitors on pines would depend on the host's age (Heijari *et al.*, 2005; Zeneli *et al.*, 2006; Moreira *et al.*, 2009), (ii) the dose and the timing were probably not appropriate to protect the seedlings accordingly (Bonello *et al.*, 2006), (iii) the inoculation method was probably too severe to allow the treatment to be effective, and/or (iv) the high virulence nature of the pathogen used allowed the resistance threshold of the plants to be easily surpassed.

Testing *Pinus pinaster* against *Fusarium circinatum* (III)

P. pinaster seedling inoculated with *F. circinatum* showed that disease incidence, time-to-death and seedling mortality varied significantly among 40 different families. Cumulative proportions of survival were also significantly different among families ($P < 0.01$). By the end of the experiment, 15 of 40 families showed a high disease incidence, with more than 70% of mortality, and seedling mortality among families ranged between 33 and 81%. Moreover, heritability estimates was high for mortality. Thus, screening of selected *P. pinaster* families showed that genetic variation in response to *F. circinatum* does exist. Heritability of mortality, in our study for *P. pinaster*, was high enough to allow screening for resistance and 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 that selection of resistance is possible.

Evaluating environmental maternal effects in *Pinus pinaster* performance and susceptibility against *Fusarium circinatum* (IV)

Effects of maternal environment on early seedlings performance (IV)

Our results indicated that, seedling height was significantly influenced by maternal environment (IV) and maternal genotype (III & IV). Seedlings in the favourable maternal environment were larger than in the unfavourable maternal environment ($P < 0.001$). Stem diameter was also higher in seedling from the favourable maternal environment ($P < 0.01$). However, the effect of the maternal environment on seedling height and stem diameter was negligible after including the covariation with the seed mass of each individual seedling in the

mixed model ($P > 0.05$). Particularly, our results agree with those of Zas *et al.* (2012) and suggest that most of the transgenerational maternal effects in early stages of *P. pinaster* offspring development were mediated by effects related to seed provisioning.

Effects of maternal environment on seedlings susceptibility (IV)

The maternal environment (IV) and maternal genotype (III & IV) significantly affected the evolution of the necrosis length on inoculated seedlings. The length of the necrosis was significantly shorter in seedlings coming from the favourable maternal environment than in seedlings from the unfavourable one ($P < 0.01$). On the contrary than with the maternal imprint on growth traits, the effect of the maternal environment on resistance to the pitch canker disease was not removed when individual seed mass was considered as a covariate in the mixed model. These results suggests that contrasting abiotic maternal environments of *P. pinaster* induced transgenerational defensive plasticity in their offspring against pitch canker pathogen. And this effect was not explained by seed quantitative provisioning, in contrast to other studies that has suggested positive and negative correlations between seed size and different levels of plant resistance (Agrawal, 2001; Hodge and Dvorak, 2000, 2007). Thus, other mechanisms, e.g. epigenetic changes (Jablonka and Raz, 2009), must be involved in this form of transgenerational plasticity, where the maternal environment influences in the susceptibility of *P. Pinaster* progenies against *F. circinatum*.

Maternal environmental effects on metabolites of *Pinus pinaster* when infected by *Fusarium circinatum* (V & VI)

Carbohydrate content (V)

Analysis of neutral sugar and uronic acids showed that total carbohydrate content of the *P. pinaster* stems was not influenced by the maternal environment and the inoculation treatment. However, higher relative content of glucose and lower content of uronic acid on inoculated *vs.* control seedlings ($P < 0.01$) were observed. Further, carbohydrate changes were not showed with equal intensity in seedlings from the contrasting maternal environments. These changes were only significant for seedlings from the favourable maternal environment, which significantly increased their glucose content and reduced their uronic acids content due to inoculation ($P < 0.05$). It is supposed that changes of glucose and uronic acid relative content of inoculated seedlings was due to the development of infection caused by the pathogen (Azevedo *et al.*, 2006). The significant effect of inoculation on carbohydrate mobilization in seedlings from the favourable maternal environment, not shown by seedlings from the unfavourable seed orchard, could be a reason of why seedlings from the favourable maternal environment presented shorter lesions length by the pathogen (IV). Vigorous trees generally accumulate enough carbohydrates to heal injuries, synthesize defensive chemicals, and maintain physiological processes at levels necessary to sustain life when exposed to a stresses (Chapin *et al.*, 1987). Also, in a manner similar to elicitors (I & II), carbohydrate could also act as signalling molecules in the induction of resistance (Bishop *et al.*, 2002).

The resulting FT-IR spectra of seedlings showed a clear separation of inoculated and non-inoculated seedlings when we

applied chemometric techniques. So, results of the FT-IR spectra were in accordance with carbohydrate analysis. However, chemometrics analyses were not able to distinguish between seedlings of both maternal environments. May be the carbohydrate changes between maternal environments are so light that this technique was not able to detect the differences found in the carbohydrate analysis.

Antioxidant activity (VI)

Inoculated seedlings from the favourable maternal environment showed lower antioxidant activity than inoculated seedlings from the unfavourable environment ($P < 0.001$). If little necrosis occurred, seedlings from the unfavourable environment showed higher antioxidant activity; on the contrary, if large necrosis occurred, seedlings form the favourable environment showed higher antioxidant activity ($P < 0.001$). It seems that seedlings from the favourable environment were able to develop a more efficient antioxidant system, down-regulated if facing low damage by the pathogen and up-regulated if extensive damage has been caused. Physical environmental factors may influence plant metabolism in such a way that antioxidant activity changes (Smirnoff, 1993). Our results confirm that environmental maternal effects significantly regulated the antioxidant activity of *P. pinaster* seedlings challenged with *F. circinatum*, in the way that low antioxidant activity if the damage is little, and high activity if the damage is large benefits the tree against the pitch canker disease.

CONCLUSIONS

1. Based on the morphological changes observed in the treated 6-months-old *P. pinaster* seedlings, there is evidence that methyl jasmonate activated the mechanisms of resistance. However, methyl jasmonate treatments at the doses tested did not reduce plant mortality cause by *F. circinatum*. The use of methyl jasmonate to prevent pitch canker disease of *P. pinaster* in nurseries should be discarded.

2. DL-3-amino-n-butanoic (DL-β-aminobutyric) acid (BABA) and benzo [1,2,3]thiadiazole-7-carbothionic acid-S-methyl ester (BTH) treatments on *P. pinaster* seedlings showed different induced resistance responses against *F. circinatum* depending on the family tested, though most families did not show any disease reduction with any of the two compounds tested. At the doses tested, the use of BABA and BTH to prevent pitch canker disease of *P. pinaster* in nurseries should be discarded. Despite these negative results on a general level, the different responses should be studied in order to identify chemical compounds that could trigger plant resistance induction in some families.

3. Rapid screening of *P. pinaster* seedlings for resistance to *F. circinatum* 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 pitch canker disease. The substantial levels of resistance among these *P. pinaster* families may provide an alternative species to landowners currently using the more susceptible *P. radiata*.

4. Abiotic environmental differences experienced by maternal *P. pinaster* trees affected the expression of the early phenotype in the subsequent generation, involving growth and resistance traits, up to one year and a half after germination. Progenies of *P. pinaster* from a favourable maternal environment (in terms of growth and reproduction) showed greater growth and improved resistance to *F. circinatum* than progenies from an unfavorable maternal environment.

5. The quantitative maternal investment of *P. pinaster* trees in seed mass explained the transgenerational plasticity of seedlings growth but not of seedlings resistance against *F. circinatum*, for which other mechanisms, probably epigenetic ones, may be involved.

6. Changes in *P. pinaster* carbohydrate compounds influenced by *F. circinatum* inoculation were mediated by the maternal environment. Inoculated *vs.* control seedlings of *P. pinaster* from a favourable maternal environment showed increased proportions in glucose and reduced proportions of uronic acids, probably related with an increase of pectic and a decrease of hemicellulosic polysaccharides on inoculated seedlings, respectively.

7. Carbohydrate changes of *P. pinaster* due to *F. circinatum* inoculation can be distinguished by gas chromatography, spectroscopy and FT-IR in association with chemometrics.

8. Maternal effects significantly regulated the antioxidant activity of *P. pinaster* seedlings challenged with *F. circinatum*, in the way that low activity if the damage is little and high activity if the damage is large benefits the pine seedlings against the pitch canker disease.

CONCLUSIONES

1. En base a los cambios morfológicos observados en plántulas de *P. pinaster* de 6 meses de edad, existen evidencias de que el metil jasmonato activó los mecanismos de resistencia de las plántulas. Sin embargo, las dosis empleadas en los tratamientos de metil jasmonato no redujeron la mortalidad de las plántulas frente a *F. circinatum*. La utilización del metil jasmonato como inductor de resistencia frente al chancro resinoso en viveros de *P. pinaster* no es eficaz.

2. Los tratamientos en brinzales de *P. pinaster* con ácido DL-3-amino-n-butanoico (DL-β-aminobutirico) (BABA) y con el compuesto Benzo [1,2,3]tiadiazol-7-carbotionico ácido-S-methyl ester (BTH) mostraron diferentes respuestas de inducción de resistencia contra *F. circinatum* dependiendo de la familia evaluada, aunque la mayoría de las familias no mostraron disminución de la enfermedad con ninguno de los compuestos. En las dosis estudiadas, la utilización de BABA o BTH para prevenir la enfermedad del chancro resinoso en brinzales de *P. pinaster* no es eficaz. A pesar de los resultados negativos a nivel general, las diferentes respuestas deben ser estudiadas para identificar los compuestos que pudieran desencadenar la inducción de resistencia en determinadas familias.

3. Evaluar la resistencia frente a *F. circinatum* en plántulas de *P. pinaster* en condiciones de invernadero es posible. Los clones mejorados 1020, 1049, 2002 y 2070 de *P. pinaster*, con niveles moderados de tolerancia a la enfermedad del chancro resinoso, pueden ser utilizados como material de mejora o como fuente de producción de semillas. Los niveles de resistencia de estas familias

ante *F. circinatum* pueden proporcionar una alternativa a propietarios de plantaciones de *P. radiata*, especie más susceptible.

4. Las diferencias abióticas en el ambiente materno experimentadas por árboles de *P. pinaster* afectaron al crecimiento y a la resistencia de la siguiente generación hasta un año y medio después de la germinación. Progenies de *P. pinaster* procedentes de un ambiente materno favorable (en términos de crecimiento y reproducción) mostraron un mayor crecimiento y una mayor resistencia frente a *F. circinatum* que las progenies de ambientes desfavorables.

5. La inversión materna realizada por árboles de *P. pinaster* en la masa de la semilla explicó la plasticidad generacional mostrada en el crecimiento de las plántulas pero no la resistencia de las plántulas ante *F. circinatum*, por lo que otros mecanismos, posiblemente los epigenéticos, podrían estar involucrados.

6. Los cambios en los carbohidratos de plántulas de *P. pinaster* debidos a la inoculación con *F. circinatum* dependieron del ambiente materno. Las plántulas inoculadas procedentes de un ambiente materno favorable frente a las no inoculadas mostraron un incremento del porcentaje de glucosa y una disminución de los ácidos urónicos, posiblemente relacionados con un incremento de polisacáridos pépticos y una disminución de polisacáridos hemicelulósicos, respectivamente.

7. Los cambios en los carbohidratos en *P. pinaster* debidos a la inoculación con *F. circinatum* se pueden distinguir por cromatografía de gases, espectroscopía y FT-IR asociado con quimiometría.

8. Los efectos maternos regularon de forma significativa la actividad antioxidante de las plántulas de *P. pinaster* inoculadas con *F. circinatum*, de forma que una baja actividad antioxidante si el daño es pequeño y una alta actividad si el daño es mayor beneficia a la plántula frente a la enfermedad del chancro resinoso.

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ORIGINAL ARTICLES

Article I

Evaluating methyl jasmonate for induction of resistance to *Fusarium oxysporum, F. circinatum* and *Ophiostoma novo-ulmi*

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Abstract

Evaluating methyl jasmonate for induction of resistance to *Fusarium oxysporum, F. circinatum* and *Ophiostoma novo-ulmi*

Damping off is probably the most common disease affecting seedlings in forest nurseries. In south-western Europe, the pitch canker and the Dutch elm disease cause relevant economic looses in forests, mostly in adult trees. The ability of the chemical plant elicitor methyl jasmonate (MeJA) to induce resistance in *Pinus pinaster* against *Fusarium oxysporum* and *F. circinatum*, and in *Ulmus minor* against *Ophiostoma novo-ulmi* was examined. In a first experiment, an aqueous solution of MeJA 5 mM was applied to *P. pinaster* seeds by immersion or spray, and different concentrations of MeJA (0, 0.1, 0.5, 1, 5 and 10 mM) were tested in seedlings before inoculations with *F. oxysporum* (10^5 and 10^7 spores mL^{-1}). In a second experiment, 6-months-old *P. pinaster* seedlings were sprayed with 0 and 25 mM of MeJA, and later challenged with mycelium of *F. circinatum*. Finally, 4-year-old *U. minor* trees were sprayed with 0, 50 and 100 mM of MeJA and subsequently inoculated with *O. novo-ulmi* (10^6 spores mL^{-1}). MeJA did not protect *P. pinaster* seeds and seedlings against *F. oxysporum*, probably because plants were too young for the physiological mechanisms responsible for resistance to be induced. Based on the morphological changes observed in the treated 6-months-old *P. pinaster* seedlings (reduction of growth and increased resin duct density), there is evidence that MeJA could have activated the mechanisms of resistance. However, 25 mM MeJA did not reduce plant mortality, probably because the spread of the virulent *F. circinatum* strain within the tree tissues was faster than the formation of effective defense responses. Based on the lack of phenological

changes observed in the treated elms, there is no evidence that MeJA would cause induction of resistance. These results suggest that the use of MeJA to prevent *F. oxysporum* and *F. circinatum* in *P. pinaster* seedlings in nurseries and *O. novo-ulmi* in *U. minor* trees should be discarded.

Keywords: *Pinus pinaster*, *Ulmus minor*, damping off, pitch canker, Dutch elm disease, traumatic resin ducts.

Resumen

Utilización de metil jasmonato para la inducción de resistencia ante *Fusarium oxysporum, F. circinatum* y *Ophiostoma novo-ulmi*

El "damping off" es una de las enfermedades más comunes en los viveros forestales. En árboles adultos del suroeste de Europa, el chancro resinoso y la grafiosis del olmo son enfermedades que están causando importantes pérdidas económicas en los bosques. Se ha estudiado la capacidad del metil jasmonato (MeJA), un elicitador químico de plantas, para inducir resistencia en *Pinus pinaster* ante *Fusarium oxysporum* y *F. circinatum*, y en *Ulmus minor* ante *Ophiostoma novo-ulmi*. En un primer experimento se aplicó una solución acuosa de MeJA 5 mM a semillas de *P. pinaster* mediante inmersión o pulverización de las mismas, y diferentes concentraciones de MeJA (0, 0.1, 0.5, 1, 5 and 10 mM) fueron pulverizadas en plántulas de *P. pinaster* antes de las inoculaciones con *F. oxysporum* (10^5 y 10^7 esporas mL^{-1}). En un segundo experimento, plántulas de *P. pinaster* de 6 meses de edad fueron pulverizadas con MeJA 0 y 25 mM, y posteriormente inoculadas con micelio de *F. circinatum*. Por

último, brinzales de *U. minor* de 4 años de edad fueron pulverizados con MeJA a 0, 50 y 100 mM e inmediatamente inoculados con *O. novo-ulmi* (10^6 esporas mL^{-1}). El MeJA no protegió a las semillas ni a las plántulas de *P. pinaster* ante *F. oxysporum*, quizá debido a que las plántulas eran demasiado jóvenes para inducir los mecanismos fisiológicos responsables de la resistencia. Basándonos en los cambios morfológicos observados en las plántulas de 6 meses de *P. pinaster* (reducción del crecimiento e incremento de la densidad de los canales resiníferos), hay evidencia de que el MeJA pudo haber activado los mecanismos de resistencia. El MeJA a 25 mM no consiguió reducir la mortalidad probablemente porque la dispersión de *F. circinatum* en el interior de los tejidos fue más rápida que la formación de respuestas defensivas efectivas. Basándonos en la falta de cambios fenológicos de los olmos tratados, no hay evidencias de que el MeJA pueda haber causado una inducción de resistencia. Los resultados sugieren que el uso del MeJA para prevenir los patógenos *F. oxysporum* y *F. circinatum* en plántulas de *P. pinaster* en viveros y *O. novo-ulmi* en brinzales de *U. minor* debe ser descartado.

Palabras clave: *Pinus pinaster*, *Ulmus minor*, "damping off", chancro resinoso, grafiosis del olmo, canales resiníferos traumáticos.

Introduction

Plants protect themselves against a diversity of attackers through constitutive and inducible defense strategies. Constitutive defenses are structural or chemical compounds permanently present in the tree and represent the first lines of protection. Inducible defenses are activated by plants upon perception of a foreign challenge, and occur at the site of the initial attack (local defence), in distant parts of the plant or throughout the entire plant (systemic defence) (Eyles *et al.*, 2010). Several types of systemic induced resistance have been characterized in detail, such as pathogen-induced systemic acquired resistance (SAR), systemic induced resistance by plant growth-promoting rhizobacteria or fungi (SIR), and wound or herbivore induced resistance (Pieterse and Van Loon, 2007; Eyles *et al.*, 2010). These types of resistance are initiated by different elicitors and partially controlled by distinct signaling pathways, but all share the characteristic of having a broad spectrum of effectiveness (Pieterse and Van Loon, 2007).

It is of practical interest to determine if elicitor molecules released, during the early stages of the plant–pathogen interaction could be directly applied to plants in order to suppress the effects of fungal diseases of plants. Exogenous applications of salicylic acid (SA) and carvacol to *Ulmus minor* successfully enhanced the resistance of trees to the fungal pathogen *Ophiostoma novo-ulmi* (Martín *et al.*, 2008b, 2010). Also, foliar sprays of SA or of benzothiadiazole to *Pinus radiata* significantly decreased plant infections by *Diplodia pinea* or by *Phytophthora cinnamomi*, respectively (Reglinski *et al.*, 1998; Ali *et al.*, 2000). Within forestry, both elm and pine trees are appropriate hosts for testing active elicitor

molecules, since previous research on these species reported several types of induced resistance to be operative (Solla and Gil, 2003; Bonello *et al.*, 2006; Kim *et al.*, 2010; Martín *et al.*, 2010; Gordon *et al.*, 2011). Worldwide, damping off caused by *Fusarium oxysporum* is probably one the most severe disease affecting seedlings in forest nurseries (Machón *et al.*, 2006). In south-western Europe, the pitch canker and the Dutch elm disease, caused by *F. circinatum* and *O. novo-ulmi* respectively, are amongst the problems causing higher impact in forests (Martín *et al.*, 2008a, 2008b; Vivas *et al.*, 2012) and none of the suggested control strategies have been effective, either for technical, economical, or environmental limitations. In view of this, the study of disease control methods based on the direct application of natural molecules on trees is gaining interest by researchers and foresters (Holopainen *et al.*, 2009).

There is growing evidence that exogenous applications of methyl jasmonate (MeJA) can enhance the levels of certain defensive compounds of plants and in consequence be used to trigger the defense mechanisms of trees (Moreira *et al.*, 2009; Eyles *et al.*, 2010). MeJA or (*Z,E*)-methyl 3-oxo-2-(2-pentyl) cyclopentane acetate is one of the mayor physiological active forms of jasmonates, and the most commonly studied elicitor in conifer species (Holopainen *et al.*, 2009). This compound is usually mixed with the surfactant Tween 20 at 0.1% (Huber *et al.*, 2005; Zeneli *et al.*, 2006; Moreira *et al.*, 2009) and directly applied by spraying or by brushing the plant. An aqueous solution of MeJA has been used to artificially induce defense responses of trees, through increasing the synthesis of terpenoid, phenolic and alkaloid compounds (Heijari *et al.*, 2005; Zeneli *et al.*, 2006) or by promoting the formation of traumatic resin ducts (Martin

et al., 2002; Hudgins *et al.*, 2004). Exogenous MeJA has been successfully used to enhance the resistance of trees against several insects and pathogens, i.e. of *Picea abies* against *Pythium ultimum* and *Ceratocystis polonica* (Kozlowski *et al.*, 1999; Zeneli *et al.*, 2006; Krokene *et al.*, 2008), or of *P. sylvestris* and *P. pinaster* against *Hylobius abietis* (Heijari *et al.*, 2005; Moreira *et al.*, 2009). The purpose of this study was to test if exogenous applications of MeJA can induce resistance in (i) *P. pinaster* seeds and seedlings against *F. oxysporum*, (ii) *P. pinaster* seedlings against *F. circinatum*, and (iii) *U. minor* trees against *O. novo-ulmi*.

Materials and methods

Fungal pathogens and inoculum preparation

The pathogen *F. oxysporum* (Fo-4P) used for the first experiment was isolated in 2005 from a diseased seedling growing in a commercial nursery located in Soria, central Spain. The isolate was selected because previous research confirmed its high virulence on *Pinus sylvestris* (Machón *et al.*, 2006). The isolate was long-term maintained on Komada (K) medium (Komada, 1975). Inoculum was prepared by subculturing the fungus in PDA and then placing four pieces of mycelium in sterile flasks containing potato dextrose broth (PDB) liquid medium under the dark. The flasks were shaken for 7 days at room temperature, and the suspensions filtered and adjusted to 10^5 and 10^7 spores mL^{-1} water.

The *F. circinatum* isolate (Fc7-1) used for the second experiment was isolated in 2005 from a stem canker of a *P. pinaster* tree in Asturias, northern Spain. Information about its virulence on *P. pinaster* seedlings is available (Vivas *et al.*, 2012). Long term storage

of the strain was carried out on PDA in the fridge, for periods no longer than 3 months. Inoculum was prepared by growing during 7 days the fungus into Petri dishes containing PDA, at 25 ± 1 °C under the dark.

The *O. novo-ulmi* ssp. *americana* isolate PM-SP used for the third experiment was selected because of its rapid in vitro growth rate (4.7 mm per day on 2% malt extract agar at 20 °C). The pathogen was isolated in 2002 from an *U. minor* tree growing in Majorca island (Spain), maintained on 2% Oxoid malt extract agar (MEA) in Petri dishes at 4 °C in the dark, and was subcultured at 3-month intervals. The inoculum consisted of a spore suspension prepared in Tchernoff's liquid medium, adjusted with water to 10^6 spores mL^{-1} (Martín *et al.*, 2008a).

Experiment 1

Plant material consisted of *P. pinaster* seeds (assay 1) and seedlings (assay 2), which originated from a single tree located in Cangas del Morrazo, north-west Spain. All seeds were surface sterilized in 30% H_2O_2 for 30 min, and then 10-times rinsed with sterile distilled water. In a first assay using seeds, these were divided into three groups and the following treatments were performed: (i) immersion of seeds during 10 minutes in an aqueous solution of MeJA; (ii) spraying of seeds with an aqueous solution of MeJA; and (iii) immersion during 10 minutes in water and subsequent spraying with water (untreated control). The aqueous solution of MeJA (Sigma-Aldrich, Germany) was adjusted to 5 mM and contained 0.1% (v/v) of Tween 20 (Panreac, Spain). One hundred fifty seeds per treatment were individually sown in 250 mL cylindrical pots containing sterilized soil

(peat and sand, 1:1, v/v), and again subdivided into three groups; seeds were (i) inoculated by pipeting 5 mL of a spore suspension of *F. oxysporum* (10^7 spores mL^{-1}) onto the ground, (ii) inoculated by pipeting 5 mL of *F. oxysporum* (10^5 spores mL^{-1}) onto the ground, or (iii) irrigated with 5 mL of sterile water (control). Pots were daily watered (~2 mL) and kept at room temperature. The germination of each seed was daily assessed, and mortality of seedlings was recorded once a week during 5 weeks.

In a second assay using seedlings, about a thousand seeds were sown and maintained as previously described. Four weeks after sowing, half of the germinated seedlings were treated with aqueous solutions of MeJA, at concentrations of 0 (control), 0.1, 0.5, 1, 5 and 10 mM. All solutions contained 0.1% (v/v) Tween 20 and were applied by spraying the whole plant. Five weeks after sowing, the other half of the seedlings was treated in the same manner. Four and five weeks after sowing, seedlings were about 1-2 and 2-3 weeks old, respectively, coinciding in time with the susceptibility window of *P. pinaster* to *F. oxysporum* (Figure 1). The susceptibility curve of *P. pinaster* to *F. oxysporum* was obtained previously, with seedlings (n = 30) being inoculated during 6 weeks at 10^7 spores mL^{-1} (Figure 1). For each of the MeJA concentrations, spraying was performed in separate rooms in order to avoid MeJA evaporation and a possible contamination of the control plants. One day after treatments, all seedlings were placed in the same room. Inoculations with *F. oxysporum* were performed by pipeting 5 mL of a spore suspension onto the stems (Alves-Santos *et al.*, 2007) at two spore concentrations (10^5 and 10^7 spores mL^{-1}) and at two inoculation dates (1 and 7 days after treatments). In consequence, the assay consisted of a complete

factorial design including 6 MeJA solutions × 2 plant ages × 2 *F. oxysporum* spore concentrations × 2 inoculation dates, i.e. 48 treatments. Each treatment included 20 seedlings as replicates. Plant death and growth height was weekly recorded during 8 weeks. After this period, fungal re-isolations were carried out onto Komada medium to confirm the presence of the pathogen. Samples from the non-inoculated seedlings were cut from the centre of the stem using a manual microtome and immediately photographed. Transverse sections were approximately 20 μm in thickness, and in each section the number of resin ducts was counted. The resin canal system was characterised through the *resin duct density* (# mm^{-2}), i.e. resin ducts per unit area, and the *relative duct area* (%), obtained by dividing the area occupied by the ducts in the section by the total area of the section (Moreira *et al.*, 2008). The *root length* (cm) and the *root surface* (cm^2) of non-inoculated seedlings were obtained using WinRhizo Pro v.2007d (Régent Instruments Inc., Quebec, Canada) software (Solla *et al.*, 2011). Plant tissues were separately placed inside paper bags, oven-dried at 65 °C for 48 hours and weighed.

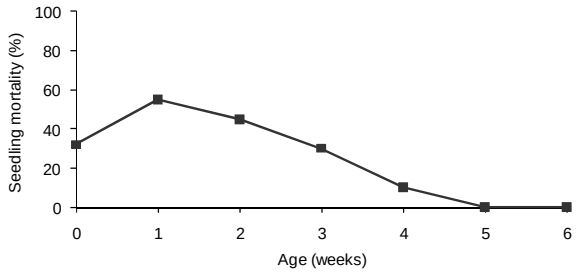

Figure 1. Susceptibility curve of *Pinus pinaster* seedlings to *Fusarium oxysporum* depending on plant age. Each value represents plant mortality 5 weeks post inoculation, if inoculations (10^7 spores mL^{-1}) were performed when the seedlings had the indicated age in weeks (n = 30 seedlings).

Experiment 2

Plant material consisted of *P. pinaster* seedlings obtained from the same tree used in experiment 1. Seeds were surface sterilized and individually sown as previously described. When seedlings were about 6 months old (25th of April), the pots were divided into four groups, and the following treatments were applied: (i) spraying of seedlings with water and, 1 month later, inoculation with *F. circinatum*; (ii) spraying with an aqueous solution of MeJA (25 mM) and, 1 month later, inoculation with *F. circinatum*; (iii) spraying with MeJA (25 mM); and (iv) spraying water (control treatment). The MeJA dose was selected according to Moreira *et al.* (2009). Each treatment consisted of 45 plants distributed among two blocks. The aqueous solutions contained Tween 20 at 0.1% (v/v), and inoculations consisted of placing mycelium of *F. circinatum* into a wound made in the stem. Mycelium was scraped off the PDA agar surface with a sterile scalpel, and immediately used to make a 1-mm-long slit wound into the succulent stem tissue, 5 cm above the ground level (Correll *et al.*, 1991; Vivas *et al.*, 2012). Plants from treatments iii and iv were wounded without placing any mycelium of the pathogen. Plant death was recorded once a week during eight weeks. Dead seedlings were removed weekly and fungal re-isolations were carried out onto FSM medium (Aegerter and Gordon, 2006). Eight weeks after inoculation, all remaining seedlings were harvested and cultured, and non-inoculated seedlings were assessed for height growth, the number of resin ducts, root parameters and dry weight as described before.

Experiment 3

The experiment included 42 ramets of the *U. minor* clone UPM171, used because of its high susceptibility to *O. novo-ulmi* (Martín *et al.*, 2008a). The clone was propagated in 2004 by root cuttings at the Forest Breeding Centre in Puerta de Hierro (Madrid, Spain) and the ramets were grown in 30 L pots containing perlite and peat (1:1, v/v), and irrigated to field capacity when required. Trees were placed outside under a shading mesh providing 25% of full sunlight throughout the experiment. When the first treatments were applied, the trees were 4 years old and 1.1-2.6 m in height. On 17 April 2008, ramets were divided into three groups, and the main trunk of the 14 trees per group were then sprayed with an aqueous solution of MeJA at concentrations of 0 (control), 50, and 100 mM, respectively. All solutions contained 0.1% (v/v) Tween 20. Two months later, seven trees per group were inoculated with *O. novo-ulmi* into the sap stream through a blade wound made at the base of the trunk and seven trees per group were inoculated with water. Dieback symptoms shown by the trees were evaluated at 120 days and at one year after inoculations. Bud break of trees was studied from March to May 2009 following Martín *et al.* (2008b). Bud break date was defined as the day when half of the buds had their scales open. Plant height was measured on dormant trees before the treatments and at the end of the 2008 and 2009 growing seasons, thus obtaining the apical growth of the trees.

Statistical analysis

Data were analyzed using Statistica v7.0 (Stat Software Inc., Tulsa, OK, USA). To compare *germination*, *incidence* and *mortality* of pines (dependent binomial variables) among *MeJA treatments* and *pathogen*

inoculations (factors), a Generalized Logit Model (GLZ) was used. In the second assay of the first experiment, mortality of pines was analyzed by two steps; first among dates of MeJA treatments and dates of inoculation, and then among MeJA concentrations and *F. oxysporum* spore suspensions. The *time to germination* was used as a covariate (continuous predictor). To compare *growth height* and *morphological parameters* of pines and *dieback* of elms (dependent continuous variables) among *MeJA treatments* and *pathogen inoculations* (factors), a General Linear Model was used. Individual means were separated by Fisher´s least significant difference (LSD) test ($P = 0.05$).

Results

Experiment 1

Germination rates of *P. pinaster* seeds immersed or sprayed with MeJA (first assay) were significantly increased (~10%) if compared to those of controls. Inoculations with *F. oxysporum* significantly reduced, in about 20%, the germination rates of untreated seeds for both spore concentrations tested ($P < 0.01$). Germination rates of inoculated seeds immersed in MeJA, inoculated seeds sprayed in MeJA and inoculated untreated seeds were 64, 51 and 41%, respectively, the first and third rates differing significantly ($P < 0.05$). Five weeks post inoculation, conditioning treatments with MeJA did not protect seeds against challenging inoculations with *F. oxysporum*, and mortality of seedlings was significantly higher if seeds were immersed in MeJA than if seeds were not treated ($P < 0.05$) (Table 1). Some other non-inoculated seeds especially those immersed in MeJA

resulted in plant chlorosis, tip necrosis, closure of cotyledons and plant mortality (Figure 2a).

In the second assay, final mortality of *P. pinaster* seedling sprayed with MeJA significantly varied depending on the date in which treatments were performed (Table 2). If MeJA treatments were performed 4 or 5 weeks after sowing, overall plant mortalities were 59 and 45% respectively. The date at which *F. oxysporum* was inoculated did not cause significantly different mortality rates of plants (50 or 57% if plants were inoculated 1 or 7 days after MeJA treatments; Table 2). Final mortality of seedlings depended of the concentration of MeJA and the dose of *F. oxysporum* used, but the conditioning treatments did not significantly protect the seedlings against the pathogen (Figure 3). Moreover, seedlings treated with MeJA at 5 and 10 mM and subsequently inoculated with *F. oxysporum* showed higher mortality values than seedlings treated with the aqueous solution and subsequently inoculated with *F. oxysporum* (Figure 3). No mortality was observed in the non-inoculated MeJA treated seedlings, but treatments at doses above 1 mM showed clear phytotoxicity, similar as the one described for the first assay. The percentage of infected seedlings (incidence, data not shown) showed the same trend as mortality, and re-isolation of the pathogen was possible in every inoculated seedlings. The number of constitutive resin ducts per transversal section of plant stems ranged from 4 to 6, and similar values of resin duct densities and relative duct areas among treated and untreated seedlings were obtained (~13 ducts mm^{-2} and ~2.5%, respectively). Any of the above or belowground plant parameters were affected by the MeJA treatments ($P > 0.05$; data not shown).

Table 1. Mortality of *Pinus pinaster* seedlings (%) being their seeds treated with 5 mM methyl jasmonate (MeJA) through immersion or spray, or with water (control) and subsequently inoculated with spore suspensions of *Fusarium oxysporum* or water. Different letters indicate significant differences of mortality values within lines (abc) and within columns (xy) ($P < 0.05$).

		Conditioning treatments		
		MeJA immersion	MeJA spray	Water (control)
Challenging inoculations	10^7 spores mL^{-1}	93 a x	69 b x	32 c x
	10^5 spores mL^{-1}	70 a xy	50 ab x	39 b x
	Water (control)	55 a y	33 ab x	0 b y

Table 2. Test of all effects to compare mortalities of 10-weeksold *Pinus pinaster* seedlings among two dates of methyl jasmonate treatments (4 and 5 weeks after sowing) and two dates of *Fusarium oxysporum* inoculations (1 and 7 days after treatments). A Generalized Logit Model was performed, and time to germination was used as a continuous predictor.

Effect	d.f.	Wald statistic	*P*-value
Date of methyl jasmonate treatment (DMeJA)	1	4.81	0.02
Date of *F. oxysporum* inoculation (DFO)	1	1.10	0.29
DMeJA × DFO	1	0.12	0.72
Time to germination	1	1.59	0.20

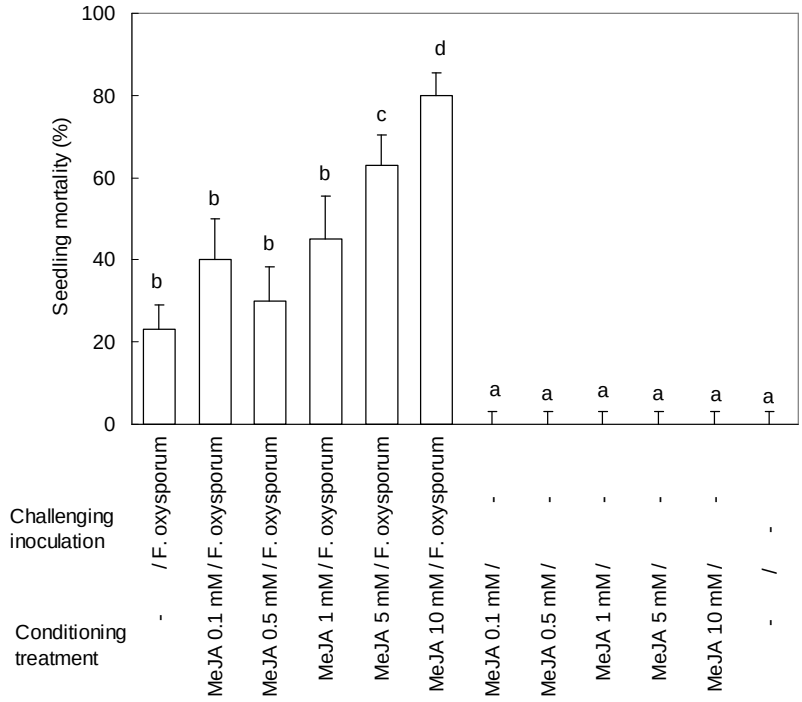

Figure 3. Mortality of *Pinus pinaster* seedlings sprayed with different concentrations of methyl jasmonate (MeJA) and subsequently inoculated with a spore suspension of *Fusarium oxysporum* (10^7 spores mL^{-1}). Vertical bars indicate standard errors and different letters show significant differences at $P < 0.001$.

(a)

(b)

(c)

Figure 2. Side-effects caused by methyl jasmonate (MeJA) in a 5-weeks-old *Pinus pinaster* seedling (a, plant on the left), after immersing its seed in MeJA 5 mM. Note the difference with the non treated 5-weeks-old seedling, on the right. Apical resinosis and reduction of height growth of a 8-month-old *P. pinaster* seedling (b, plant on the left), 8 weeks after spraying the plant with MeJA 25 mM. Note the difference with the non treated seedling, on the right. Leaf necrosis in a 4-year-old *Ulmus minor*, 5 weeks after spraying the tree with MeJA 100 mM (c).

Experiment 2

Eight weeks post inoculation, the *P. pinaster* seedlings inoculated with *F. circinatum* showed higher mortality rates than the non-inoculated control seedlings (58 *vs.* 0%; $P < 0.01$). Mortality of seedlings treated with MeJA was 0%, and mortality of seedlings treated with MeJA and subsequently inoculated with *F. circinatum* was 60%, thus the challenging treatment did not show any positive effect against the pathogen tested. By the end of the experiment, all inoculated seedlings that had survived showed leaf symptoms (incidence of 100%), and re-isolation of the pathogen was always possible. The exogenous application of MeJA in non-inoculated seedlings produced apical resinosis (Figure 2b) and significantly reduced above and belowground plant growth ($P < 0.05$; Table 3). Internally, exogenous application of MeJA significantly increased the average number of resin ducts per transverse section ($P < 0.01$) and marginally increased the relative conductive area of resin ducts ($P = 0.056$) in relation to the control plants (Table 3).

Table 3. Allometric parameters of 8-months-old *Pinus pinaster* seedlings treated with 25 mM methyl jasmonate (MeJA) in comparison with untreated control seedlings. Within lines, different letters indicate significant differences among values ($P < 0.05$).

Treatment	MeJA (25 mM)		Water (control)	
Total height growth (cm)	0.7	a	1.5	b
Root length (cm)	252.2	a	365.3	b
Root surface (cm^2)	56.2	a	77.6	b
Total dry weight (g)	1.0	a	1.4	b
Resin duct density (# mm^{-2})	15.1	a	14.3	b
Relative duct area (%)	0.19	a	0.18	a

Experiment 3

At day 120 post inoculation, the plants inoculated with *O. novo-ulmi* showed higher dieback symptoms (20.3 ± 6.7 %; mean ± SE) than water-inoculated plants (3.4 ± 2.3 %) ($P < 0.05$). Again, the MeJA treatments did not show any positive effect against *O. novo-ulmi* inoculation with respect to the control plants (Table 4). Furthermore, the treatment 100 mM of MeJA was slightly toxic to the trees, causing leaf necrosis and some wilting (Figure 2c). One year after inoculation, the trees inoculated with *O. novo-ulmi* and treated with MeJA 0 mM showed a reduction of dieback symptoms with respect to the previous year (Table 4). On the contrary, the trees inoculated with *O. novo-ulmi* and treated with MeJA 50 and 100 mM notably increased their dieback symptoms ($P = 0.03$; Table 4). No significant effects of the MeJA treatments on the time to bud burst and tree growth was observed ($P > 0.15$).

Table 4. Dieback symptoms (% of the total crown) shown by *Ulmus minor* trees treated with methyl jasmonate (MeJA) at different concentrations and subsequently inoculated with *Ophiostoma novo-ulmi*. Within lines, different letters indicate significant differences among values ($P < 0.05$).

Days after inoculation	MeJA (100 mM)	MeJA (50 mM)	Water (control)
120	17.5 a	16.3 a	20.3 a
365	61.4 a	40.3 a	8.7 b

Discussion

Exogenous applications of MeJA did not protect *P. pinaster* seed and seedlings against *F. oxysporum* probably because plants were too young for the physiological mechanisms responsible for resistance to be operative. Plant resistance can be described on several mechanistic levels, and ontogenetic disease resistance, also known as age-related resistance (Panter and Jones, 2002; Develey-Rivière and Galiana, 2007), refers to resistance to a pathogen that changes with the developmental stage of the host, with resistance usually increasing with age. Differences in resistance to the same pathogen among young seedlings and mature trees have been reported previously (Solla *et al.*, 2005; Aegerter and Gordon, 2006). From an ecological point of view, resistance to pathogens is a strong selective force and a competitive ability of young trees; as a result, there could be a trade-off between growth and expression of quantitative defences (Bonello *et al.*, 2006; Walters and Heil, 2007). Such interaction may be especially evident in young seedlings where growth during an early establishment phase is likely to be an important component of competitive ability. Resin production is the most familiar and visible component of pine defense (Davis *et al.*, 2002; Kim *et al.*, 2010). It was recently observed that the relative resin production of *P. nigra* was much lower in 1- than in 2-year-old seedlings, suggesting that the younger trees allocated a lower proportion of the carbon budget to resin synthesis (Wainhouse *et al.*, 2009). Traumatic resin ducts, easily induced in conifers in response to MeJA (Martin *et al.*, 2002; Hudgins *et al.*, 2004; Huber *et al.*, 2005) have never been reported in less than 1-year-old seedlings, in accordance to our observations.

In the first assay, although germination rates of seeds were higher if previously immersed in MeJA, the conditioning treatments did not finally protect seeds against *F. oxysporum*. Elicitor-induced changes in plant resistance can occur within hours or days after treatments, but their lasting effect could be also short. In the second assay, phytotoxicity was observed in seedlings treated at doses above 1 mM MeJA, and no protection occurred at lower doses. Phytotoxicity and plant mortality after exogenous application of 100 mM MeJA has been previously reported for older *P. sylvestris* and *P. pinaster* seedlings (Heijari *et al.*, 2005; Moreira *et al.*, 2009). Our findings do not give support to the idea of adding MeJA to irrigation water in nurseries as a method for protection against pests and pathogens, as suggested by Huber *et al.* (2005).

We postulate four non-exclusive hypotheses to explain why exogenous applications of MeJA did not protect *P. pinaster* seedlings against *F. circinatum*. First and as mentioned before, the protective and lasting effect of MeJA on pines would depend on the host's age. Positive results using MeJA were only reported using seedlings above one year old (Heijari *et al.*, 2005; Huber *et al.*, 2005; Moreira *et al.*, 2009) or mature trees (Zeneli *et al.*, 2006). Second, the dose and the timing were probably not appropriate to protect the seedlings accordingly. SIR is contingent on the type of treatment and dose to which a tree is subjected (Bonello *et al.*, 2006). In other words, the expression of SIR can be sustained or transiently expressed depending on the damage level resulting from the induction event. Moreover, changes involving cell division and differentiation such as traumatic resin duct formation are slow processes (Bonello *et al.*, 2006) and probably need more than one month to occur. As a third hypothesis,

the inoculation method was probably too severe to allow the treatment to be effective. Initial stages of fungal infection usually include the deposition and attachment of spores to aerial parts of the host plants, spore germination and subsequent formation of germ tubes that direct their growth to natural openings or wounds of plants. The inoculation method used here created an optimal infection court, allowing direct infection of the plant through a wound practiced deep into the xylem. At the time of inoculation, the seedlings were rather succulent, and the inoculum density of the pathogen high, while in nature the pitch canker disease starts at low pathogen inoculum density. Finally, we postulate that the high virulence nature of the pathogen used allowed the resistance threshold of the plants to be easily surpassed. In the same way that constitutive defences are not always enough to protect trees against attack by microbes or herbivores, in many circumstances inducible defences are not enough too. At the earliest stages of pathogen infection, SIR responses are predicted to rapidly and systemically increase concentrations of compounds involved in defence. However, if the pathogen is able to grow despite the deployment of localized defensive responses, the infection will progress, and the plant will become increasingly diseased. Elicitor compounds affect the synthesis of chemical compounds in plants, but this will result in constraints in carbon allocation and ultimately with reduce plant growth or even stop the shoot elongation after the elicitor treatment (Heijari *et al.*, 2005), as observed here. MeJA increased the resin duct density of our seedlings, but despite the general effectiveness of traumatic ducts to contain and reduce damages caused by insects and pathogens (Phillips and Croteau, 1999), *F. circinatum* is able to tolerate the resin and even stimulate its production on pine

trees (Davis *et al.*, 2002; Kim *et al.*, 2010). Thus, even if an increase of resin duct density was observed, *F. circinatum* would be able to surpass this inducible defense strategy. Among the families of *P. virginiana* examined in Barrows-Broaddus and Dwinell (1984), the high-to-moderately susceptible family had the largest ducts, and the least susceptible family had the smallest. The pitch canker fungus appears to frequently use the resin ducts as portals for vertical spread of the pathogen beyond the inoculation point (Barrows-Broaddus and Dwinell, 1984), thus large and numerous ducts seems to be a disadvantage to the host.

In addition to inherent genetic resistance to *F. circinatum*, systemic induced resistance has been reported to occur in *P. radiata* in California (Gordon *et al.*, 2011). However, the year-round susceptibility of pines to *F. circinatum* (Kuhlman *et al.*, 1982), together with the erratic results obtained here and the rapid spread of the pathogen within the host (Barrows-Broaddus and Dwinell, 1984) suggest that the use of MeJA for its control is not practical.

Concerning the biotroph *O. novo-ulmi*, a number of investigations explored the possibility of inducing resistance in elm trees threatened by Dutch elm disease, with variable results in terms of inducing agents (bacteria and fungi), range of effects, and applicability to disease management (Solla and Gil, 2003; Scheffer *et al.*, 2008). Normally, any stress factor causing a reduction of the normal growth of elms, a delayed budbreak, or an alteration of earlywood or latewood formation will generate resistance (Brener and Beckman, 1968; Martín *et al.*, 2008b). Delayed budbreak or decreases of plant growth resulting from differential allocations of carbon to defence rather than growth were not observed in the MeJA-treated elms. Based

on the lack of phenological changes and on the increased mortality observed in our treated plants, there is no evidence that MeJA had cause SIR on elms. The expression of induced plant defenses is mediated by complex signaling networks in which the plant jasmonates (MeJA) and salicylates (SA) play key roles. In general, JA-mediated signaling pathways are implicated in the regulation of defences against herbivores and necrotroph pathogens, while the SA pathway is associated with defences against biotrophic pathogens (Glazebrook, 2005). There are many exceptions to this basic framework, but signaling pathways controlled by jasmonates are required for host resistance to some pathogens, but not to all of them (Glazebrook, 2005; Kusumoto *et al.*, 2007). Thus, it could be expected that the role of the MeJA molecule would greatly vary among different pathosystems, e.g. foliar application of MeJA failed to enhance host resistance against *Phytophthora cinnamomi* in several *Eucalyptus* spp. (McComb *et al.*, 2008).

Conclusion

There is a real need for careful, long-term experiments on the use of induced resistance with trees to provide robust information, not just on understanding the systemic mechanisms of resistance, but also on effectiveness of disease control. While extensive research has examined plant and conifer SIR responses to attack by herbivores and pathogens, equivalent information for angiospermous tree species is lacking. This is the first work reporting the effect of MeJA on *U. minor* and *P. pinaster* seeds, and the first approach to test MeJA against three asomicetes previously not used for this purpose. Based in our results the use of MeJA to prevent damping-off and pitch canker

in nurseries of *P. pinaster* or Dutch elm disease on elm trees should be discarded.

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Artículo II

Aplicaciones de BABA y BTH en brinzales de *Pinus pinaster para la inducción de resistencia ante Fusarium circinatum*

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Abstract

Evaluating BABA and BTH in *Pinus pinaster* seedlings for induction of resistance to *Fusarium circinatum*

Fusarium circinatum is a pathogenic fungus that cause pitch canker disease in nurseries and matures plantation of pines. It is hypothesized that, DL-β-aminobutiric acid (BABA) and benzothiadiazole (BTH), compounds of induce resistance in plants may act as elicitors of *Pinus pinaster* defenses mechanism against the pathogen. The experiment was carried out with seeds from 39 *Pinus pinaster* selected clones and one unimproved seed source (control). Seven months after sowing, plants were treated with (i) BABA 25 mM, (ii) BTH 1 mM and (iii) sterile distilled water (n = 60). One day after treatments, seedlings were inoculated with *F. circinatum*. Generally, BABA or BTH treatments increased mortality caused by *F. circinatum* ($P < 0.001$). However, results changed significantly according to the family ($P < 0.001$). Some families showed induced resistance with both compounds, others only with BABA and another with BTH, although most of them did not show induced resistance with either of them. Despite the negative results obtained on a general level, of little use in nurseries, it is interested to continue studying the four contrasted groups of response in order to identify chemical compounds that trigger induce resistance in plants.

Keywords: Maritime pine, pitch canker, family variation, DL-3-amino-n-butanoic (DL-β-aminobutyric) acid, benzo [1,2,3]thiadiazole-7-carbothionic acid-S-methyl ester.

Resumen

Aplicaciones de BABA y BTH en brinzales de *Pinus pinaster para la inducción de resistencia ante Fusarium circinatum*

Fusarium circinatum es un hongo patógeno causante del chancro resinoso del pino que provoca mortalidad en viveros y en plantaciones adultas. Se establece la hipótesis de que los inductores de resistencia en plantas, el ácido DL-β-aminobutírico (BABA) y el benzotiadiazol (BTH), puedan actuar como elicitadores de los mecanismos defensivos de *P. pinaster* ante este patógeno. El experimento se llevó a cabo con semillas de 39 clones de *P. pinaster* mejorados y de una progenie no mejorada (control). A los 7 meses de la siembra, las plántulas fueron tratadas con (i) BABA a 25 mM, (ii) BTH a 1 mM y (iii) agua destilada estéril (n = 60). Un día después de los tratamientos, las plantas fueron inoculadas con *F. circinatum*. En general, los tratamientos con BABA o BTH incrementaron la mortalidad causada por *F. circinatum* ($P < 0.001$). Sin embargo, este resultado varió significativamente según la familia ($P < 0.001$). Algunas familias mostraron resistencia inducida con ambos compuestos, otras sólo con BABA y otra sólo con BTH, aunque la mayoría no lo hicieron con ninguno de los dos compuestos. A pesar de los resultados negativos obtenidos a nivel general, de poca utilidad en viveros, interesa seguir estudiando los cuatro grupos de respuesta contrastada, a fin de identificar en planta compuestos químicos desencadenantes de la inducción de resistencia.

Palabras clave: Pino marítimo, chancro resinoso, variación familiar, ácido DL-3-amino-n-butanoico (DL-β-aminobutirico), Benzo [1,2,3] tiadiazol-7-carbotionico ácido-S-methyl ester.

Introducción

Las plantas poseen estrategias de defensa constitutivas e inducidas para resistir el ataque de insectos y patógenos. Las defensas constitutivas se encuentran de forma permanente en el árbol y representan las primeras barreras de protección de la planta. Las defensas inducidas son activadas por la planta una vez se ha producido el ataque del insecto o patógeno, la finalidad de estas defensas es responder de forma eficaz a futuros peligros (Franceschi *et al.*, 2005). La activación de las defensas inducidas durante etapas tempranas a la interacción planta-patógeno podría disminuir o eliminar los daños causados en la planta debido al ataque de organismos nocivos. Se han descrito sustancias químicas y biológicas capaces de aumentar ciertos compuestos defensivos en plantas. Estas sustancias, conocidas como inductores, son capaces de desencadenar los mecanismos de defensa de las plantas sin mostrar un efecto antibiótico directo. El ácido DL-3-amino-n-butanoico (DL-β-aminobutirico) (BABA) y el compuesto Benzo [1,2,3]tiadiazol-7-carbotionico ácido-S-methyl ester (Bion®) (BTH) son dos conocidos inductores químicos de resistencia en plantas, que actúan contra un amplio espectro de patógenos (Cohen, 2002; Barilli *et al.*, 2010).

Fusarium circinatum Nirenberg y O'Donnell (teleomorfo *Gibberella circinata*) es un hongo causante del chancro resinoso del pino. Originario del sureste de Estados Unidos, su primera detección en la UE tuvo lugar en un vivero del País Vasco, sobre *Pinus radiata* y *P. pinaster* (Landeras *et al.*, 2005). Recientemente, *F. circinatum* también se ha detectado en *P. halepensis* y *P. pinea* en Italia (Carlucci *et al.*, 2007) y en brinzales de *P. radiata* y de *P. pinaster* en Portugal (Bragança *et al.*, 2009). Los síntomas que causa en patógeno incluyen

marchitamientos de plántulas en pre- y postemergencia, clorosis o enrojecimiento de acículas, torsión y puntisecado de brotes, aparición de chancros resinosos, aborto de piñas y mortalidad del árbol (Carey y Kelley, 1994).

El pino marítimo (*P. pinaster* Ait.) es una conífera natural de la cuenca mediterránea occidental. En España es la especie más utilizada en plantaciones, con una gran importancia en la economía forestal. La detección de *F. circinatum* en el norte de la Península Ibérica representa un problema fitosanitario, pues se trata de un patógeno de cuarentena, y una amenaza para las plantaciones de *P. radiata* y posiblemente también para *P. pinaster*. Actualmente no existe ningún medio para controlar la enfermedad. A pesar de ello, para paliar los daños se sugiere un adecuado manejo selvícola de las plantaciones, prácticas de higiene en viveros, la implantación de medidas de cuarentena y la selección de genotipos menos susceptibles (Wingfield *et al.*, 2008).

En este trabajo se parte de la hipótesis de que los compuestos BABA y el BTH puedan actuar como inductores de los mecanismos defensivos de *P. pinaster* ante *F. circinatum*.

Material y métodos

El experimento se llevó a cabo con semillas de 39 clones de *P. pinaster* mejorados (Huerto semillero de Sergude, Xunta de Galicia, Consellería de Medio Rural, 42°49′N, 8°27′O) y de una progenie de polinización abierta no mejorada (Cangas de Morrazo, Pontevedra 42º16´N, 8º47´O) que sirvió de control. El ensayo se realizó en un invernadero cerrado de metacrilato ubicado en el Centro Universitario de Plasencia (Cáceres). La siembra se hizo en diciembre de 2008,

utilizándose alveolos de 250 ml agrupados en bandejas de 40 alveolos, y una semilla por alveolo. Como sustrato se utilizó arena estéril y turba (3:1). Se siguió un diseño completo de bloques al azar con 60 bandejas repartidas en 2 bloques, de modo que cada bandeja incluía una semilla de cada una de las 40 familias. Se utilizaron un total de 2.400 semillas.

A los 7 meses de la germinación, las plántulas fueron tratadas con BABA y BTH e inoculadas con *F. circinatum*. Para decidir las dosis de BABA y BTH a utilizar, y el intervalo de tiempo entre los tratamientos y la inoculación, se realizó un ensayo previo con menor número de plantas (n = 10). Se testaron aplicaciones de BABA a 10, 25 y 50 mM, y aplicaciones de BTH a 1, 5 y 10 mM, en intervalos de 1, 3, y 5 días de separación frente a la inoculación con *F. circinatum*. Las dosis de BABA y de BTH que mayor protección ofrecieron a la planta ante *F. circinatum* fueron 25 y 1 mM, respectivamente, aplicadas un día antes de las inoculaciones. No se observaron síntomas de toxicidad.

Los tratamientos aplicados fueron (i) BABA a 25 mM, (ii) BTH a 1 mM, y (iii) agua destilada estéril. La aplicación se realizó pincelando el tallo de cada plántula con las distintas soluciones (Figura 1). Un día después de los tratamientos, todas las plantas fueron inoculadas con *F. circinatum*. La cepa de *F. circinatum* utilizada (Fc7-1, MAT-1, aislada en 2005 en Asturias) fue suministrada por el Laboratorio de Patología de la EUIT Forestal de Madrid (UPM). El inóculo se preparó mediante el cultivo del patógeno en PDA a oscuridad y temperatura de laboratorio. *F. circinatum* normalmente no penetra en tejidos de pino intactos, y para la infección es necesaria la existencia de heridas (Dwinell y Barrows-

Broaddus, 1981). Por ello la inoculación se realizó poniendo en contacto micelio de *F. circinatum* sobre incisiones longitudinales de 1 mm practicadas con bisturí a 10 cm del suelo (Muñoz y Ampudia, 2005) (Figura 2).

Figura 1. Aplicación de tratamientos mediante el pincelado del tallo de una planta de *Pinus pinaster*.

Figura 2. Inoculación de *Fusarium circinatum* poniendo en contacto micelio del hongo sobre incisiones longitudinales de 1 mm practicadas con bisturí a 10 cm del suelo, en el tallo verde de *Pinus pinaster* (Muñoz y Ampudia, 2005).

Después de la inoculación se evaluó semanalmente la aparición de síntomas, y como referencia se utilizó la clasificación propuesta por Correll *et al.* (1991): (0) sano, sin necrosis; (1), acículas verdes y necrosis sólo en el punto de inoculación; (2) acículas verdes y necrosis superior a 2 cm en las proximidades del punto de inoculación; (3) acículas y/o brotes marchitos, y necrosis anillando el brote; y (4) brote anillado y follaje muerto desde el extremo distal al punto de inoculación. Debido al reducido tamaño de los tallos no se pudo cuantificar la necrosis de las plántulas. A las 8 semanas de la inoculación se realizó una evaluación final de los daños y posteriormente se constataron los postulados de Koch reaislando el patógeno *F. circinatum* en medio selectivo K (Komada, 1975). Al final del experimento todo el material utilizado (sustrato incluido) fue esterilizado y el interior del invernadero reiteradamente pulverizado con Captan (Ramón-Albalat *et al.*, 2010).

Los datos fueron analizados con el programa estadístico Statistica v7.0 (Stat Software Inc., Tulsa, OK, USA). Para comparar la incidencia (porcentaje de plantas afectadas), la intensidad de los daños (grado medio de daño de las plantas afectadas) y la mortalidad de las plántulas (variables dependientes) entre los tratamientos y las familias (factores), se utilizó un modelo GLZ (Generalized Logit Model).

Resultados y discusión

Las plantas tratadas con BABA o BTH mostraron mayores síntomas y mayor mortalidad causada por *F. circinatum* que las plantas tratadas con agua (Figura 3; $P < 0.001$). Los resultados, sin embargo, variaron según la familia utilizada ($P < 0.001$). Las familias 2031, 2054 y 2082 mostraron menor mortalidad ante tratamientos de BABA o de BTH

que ante el tratamiento control de agua (grupo 1), la familia 1003 mostró menor mortalidad ante tratamientos de BABA que ante el tratamiento de agua (grupo 2), y las familias 1059, 2041, 2050, 2053, y 2077 mostraron menor mortalidad ante BTH que ante agua (grupo 3, Figura 4). El resto de las familias, incluida la control, presentó una mayor mortalidad ante *F. circinatum* si sus plantas fueron tratadas tanto con BABA como con BTH (grupo 4, Figura 4). Los resultados sugieren claramente que la activación de defensas inducidas en *P. pinaster* ante *F. circinatum* depende de la progenie seleccionada. Estudios recientes, realizados con el mismo material vegetal, muestran una significativa variación interfamiliar en cuanto al peso medio de la semilla, el momento de germinación, el crecimiento y la respuesta ante *F. circinatum* (Vivas *et al.*, 2012b).

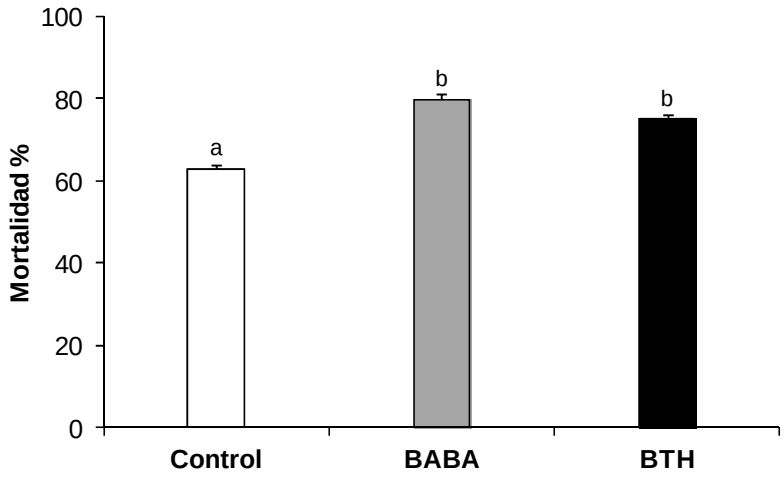

Figura 3. Mortalidad (%) causada por *Fusarium circinatum* en plántulas de *Pinus pinaster* tratadas con agua (control), BABA y BTH. Las barras son errores estándar, y letras distintas indican diferencias significativas ($P < 0.05$).

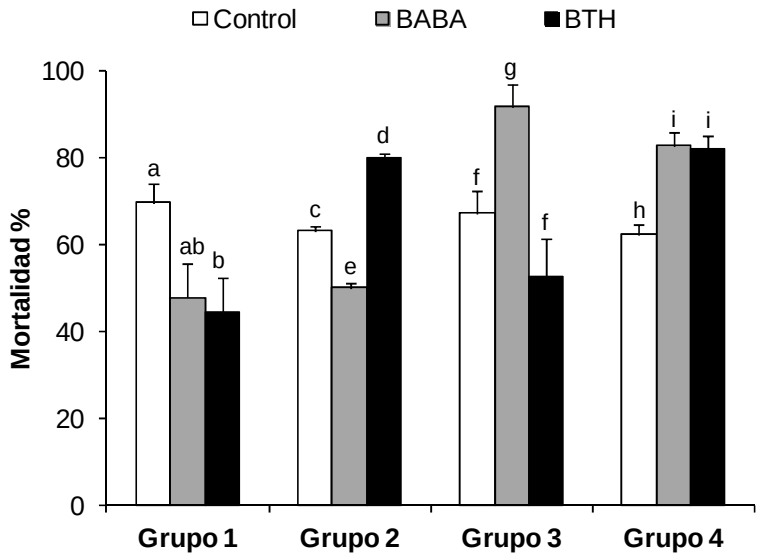

Figura 4. Mortalidad (%) causada por *Fusarium circinatum* en grupos de familias de *Pinus pinaster* tratados con agua (control), BABA y BTH. Las barras son errores estándar, y letras distintas indican diferencias significativas ($P < 0.05$).

Además de la influencia genética, hay que tener en cuenta que el efecto de los inductores de resistencia puede variar en función de la concentración utilizada, el momento de aplicación y el patosistema considerado (Bonello *et al.*, 2006). El ensayo previo realizado para calcular las dosis y el momento de aplicación de los tratamientos se realizó a los 5 meses de la germinación de las plántulas. Esta diferencia pudo haber provocado que las dosis de BABA y BTH utilizadas ante plántulas de 7 meses no hayan sido las adecuadas o suficientes para activar defensas. Además, los cambios desencadenados por los inductores de resistencia en las plantas tienen lugar en horas o a los pocos días tras los tratamientos (Van Loon *et*

al., 2006), por lo que no se descarta que el plazo utilizado entre tratamiento e inoculación fuera incorrecto para evitar los daños del patógeno.

Es de destacar la gran virulencia del hongo utilizado. Estudios realizados con metil jasmonato, otro inductor de resistencia, no consiguieron reducir la mortalidad en plántulas de *P. pinaster* ante *F. circinatum* (Vivas *et al.*, 2012a). Probablemente la dispersión del patógeno en el interior de los tejidos fue más rápida que la formación de respuestas defensivas efectivas, siendo rápidamente superado el umbral de resistencia de las plántulas por el patógeno.

Se ha observado que la resistencia inducida ante *F. circinatum* tiene lugar en *P. radiata* si se utiliza como inductor el propio patógeno (Gordon *et al.*, 2011), pero este hecho nunca ha sido demostrado en *P. pinaster*. Las coníferas representan un grupo de especies apropiado para avanzar en el conocimiento de los inductores de defensa (Bonello *et al.*, 2006; Gordon *et al.*, 2011). A pesar de ello, la mayoría de los estudios basados en el BABA y el BTH se concentran en el campo de la agricultura. El único informe existente sobre los efectos del BTH en la resistencia a patógenos en coníferas concluyó que la pulverización foliar con BTH redujo significativamente las infecciones por *Phytophthora cinnamomi* en *P. radiata* (Ali *et al.*, 2000). A pesar de nuestros resultados, poco convincentes a nivel práctico, se debe seguir estudiando la influencia de la variación familiar ante la distinta respuesta inductora ocasionada por los tratamientos de BABA y de BTH. Un análisis químico e histoquímico de los cuatro grupos de respuesta (Figura 4) tal vez permita dilucidar e identificar cuáles son los compuestos desencadenantes de la inducción de resistencia.

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Article III

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

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Abstract

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

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*.

Keywords: susceptibility, screening, genetic resistance, genetic variation, heritability, survival analysis.

Resumen

Evaluación de la resistencia del pino Marítimo (*Pinus pinaster*) ante *Fusarium circinatum*, el causante del Chancro Resinoso

El chancro resinoso, causado por el hongo *Fusarium circinatum*, es una enfermedad introducida en Europa que ataca a plantaciones y extensiones naturales de pino. Las investigaciones realizadas no han testado si alguna especie de pino Europeo muestra variación genética en la susceptibilidad a esta enfermedad. En este ensayo se evaluó la resistencia de familias de medios hermanos procedentes de 39 clones de *Pinus pinaster* mejorados y una progenie no mejorada (control). La resistencia al chancro resinoso no estuvo genéticamente relacionada con el crecimiento de la plántula, pero el peso de la semilla y los porcentajes de germinación pudieron predecir el tiempo de supervivencia. Las heredabilidades y las correspondientes ganancias genéticas calculadas con el ensayo de invernadero fueron consistentes, h_i^2= 0.18 y 0.45 para la supervivencia y la mortalidad de la plántula, respectivamente. Estas heredabilidades son lo suficientemente altas para conseguir la disminución del chancro resinoso a través de la utilización de las estrategias genéticas apropiadas. Los resultados indican que la selección de árboles de *P. pinaster* con mayor crecimiento dentro de los programas de mejora, no implica necesariamente un incremento en la susceptibilidad a *F. circinatum*. El estudio sugiere el uso de determinados individuos de pinos autóctonos con niveles de tolerancia moderados a *F. circinatum* como individuos reproductores o fuentes de producción de semillas.

Palabras clave: susceptibilidad, evaluación, resistencia genética, variación genética, heredabilidad, análisis de supervivencia.

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.

0 1 2 3 4

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).

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.

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 $\chi 2$, 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).

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).

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 ***$P < 0.001$.

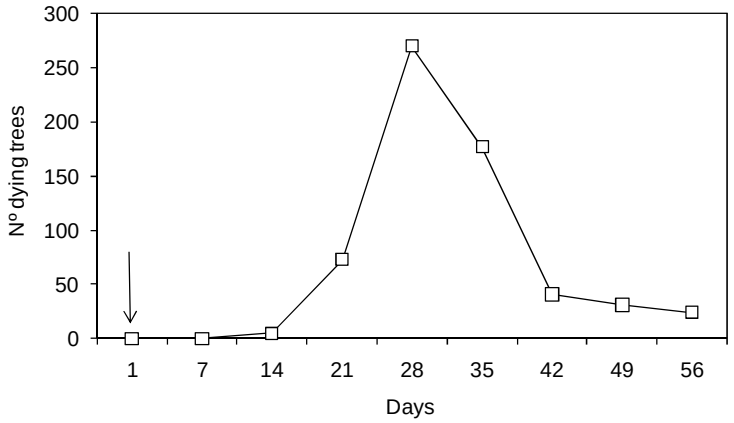

Figure 2. Mortality of *Pinus pinaster* seedlings after inoculation with *Fusarium circinatum* (*n* = 984). Arrow indicates inoculation on 24 June 2009.

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.

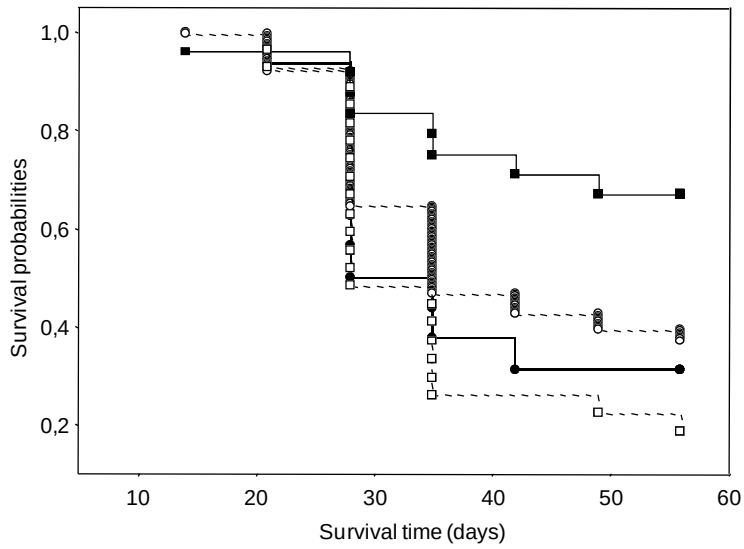

Figure 3. Plot of survival probabilities using the Kaplan–Meier estimate (P = 0.0069) of the survival function for *Pinus pinaster* inoculated with *Fusarium circinatum* at time 0. Lines correspond to all genetic entries (open circles), control family (filled circles), highly susceptible 2051 family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

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).

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

	Family			Tray / Block			Residual	
Variable	σ2	*F*/ χ2	*P*-value	σ2	*F*/ χ2	*P*-value	σ2	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 (σ2) and corresponding likelihood ratio significance tests (χ2) 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.

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 behavior supports previous concerns that infected seedlings can remain symptomless and thereby serve as vectors for long distance transport of the pathogen (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 vigor 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 vigor 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 favor 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 vigor. 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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Article IV

Environmental maternal effects in a pine tree: early performance and susceptibility against *Fusarium circinatum*

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Abstract

Environmental maternal effects in a pine tree: early performance and susceptibility against *Fusarium circinatum*

Environmental maternal effects can affect progeny performance by seed provisioning. It is suspected that these effects may be also relevant in the resistance of the progeny against subsequent biotic stress. We analyzed the influence of maternal environment, maternal genotype and their interaction on the growth and resistance to *Fusarium circinatum* pathogen of *Pinus pinaster* seedlings collected from ten genotypes clonally replicated in two contrasting maternal environments; and, at what extent differences in performance and resistance between maternal environments were mediated by seed provisioning. Height and stem diameter of seedlings were significantly influenced by the maternal environment and the maternal genotype. Seedlings from the favourable maternal environment were 10.6% higher than those from the unfavourable one. In this case, maternal effects were mediated by seed provisioning. Necrosis length cause by *F. circinatum* was also influenced by the maternal environment and the maternal genotype. Infected seedlings showed necrosis lengths 16% shorter in seedlings coming from the favourable maternal environment than in those coming from the unfavourable one. But seed mass did not have any relationship with the seedling ability to resist the pathogen, thus other mechanisms must be involved in this form of transgenerational plasticity. These results suggest that environmental maternal effects are detectable in *P. pinaster*.

Keywords: adaptative traits, transgenerational plasticity, seed mass, Maritime pine, maternal effects, pitch canker disease.

Resumen

Efectos del ambiente materno en *Pinus pinaster*: desarrollo temprano y susceptibilidad frente a *Fusarium circinatum*

El ambiente materno puede afectar al desarrollo de la progenie mediante el aprovisionamiento de las semillas. Se sospecha que estos efectos también pueden influir en la resistencia de la progenie ante un estrés biótico. Se analizó la influencia del ambiente materno, el genotipo materno y su interacción en el crecimiento y resistencia ante el patógeno *Fusarium circinatum*, en plántulas de *Pinus pinaster* procedentes de diez clones replicados en dos contrastados ambientes maternos; y, en qué medida las diferencias en desarrollo y resistencia entre los ambientes maternos, fueron debidas al aprovisionamiento de las semillas. El ambiente materno y el genotipo materno influyeron significativamente en la altura y diámetro de las plantas. Las plántulas procedentes de un ambiente materno favorable fueron un 10.6% más altas que las del ambiente desfavorable. En este caso, los efectos maternos fueron mediados por el aprovisionamiento de las semillas. El ambiente materno y el genotipo materno también influyeron significativamente en la longitud de la necrosis provocada por *F. circinatum*. Las plántulas infectadas procedentes del ambiente favorable mostraron necrosis un 16% menor que las del desfavorable. Pero, el aprovisionamiento de las semillas no estuvo relacionado con la capacidad de las plántulas de resistir al patógeno, otros mecanismos deben dirigir esta plasticidad transgeneracional. Estos resultados sugieren que los efectos maternos en *P. pinaster* son detectables.

Palabras clave: masa de la semilla, rasgos adaptativos, plasticidad transgeneracional, pino marítimo, efectos maternos, chancro resinoso.

Introduction

The phenotype of a plant may not only depend on its genotype and the environmental conditions where it grows, as conventionally thought, but also it can be determined by the environment that the parents (mainly the mother) experienced (Roach and Wulff, 1987). Transgenerational responses to the maternal environment are transmitted to the offspring phenotype without any change in the DNA sequence (Donohue, 2009). Being a relevant source of phenotypic variation, maternal environmental effects can influence evolutionary process and population dynamic of many plant species (Galloway, 2005; Herman and Sultan, 2011). Further, there is now increasing evidence that transgenerational plasticity could be adaptive, enhancing offspring fitness under environments similar to the maternal environment (Galloway and Etterson, 2007). Although much less studing, maternal environmental effects could also be potentially exploited to improve the performance of man-made plantations by exposing the mother plants to the appropriate environmental cues (Whittle *et al.*, 2009; Yakovlev *et al.*, 2012).

The maternal environment is known to influence many different traits, e.g. seed traits (Violle *et al.*, 2009), germination (Donohue, 2009), and seedling performance (Elwell *et al.*, 2011) in many different plant species, including long-lived plants such as conifers (Stoehr *et al.*, 1998; Webber *et al.*, 2005; Cendán *et al.*, 2011; Yakovlev *et al.*, 2012). One of the better known examples of transgenerational plasticity in conifers is the epigenetic memory reported for Norway spruce, in which the temperature and photoperiod experience by the mother tree during embryo development modulates offspring tolerance to frost through growth phenology adjustments

(reviewed in Yakovlev *et al.*, 2012). The effect is long lasting (up to 20 years), quantitatively important, and has been recognized as a relevant mechanism of rapid adaptation to environmental changes (Skrøppa *et al.*, 2010). Similar transgenerational responses to climate cues have been reported in several other conifer species (see references in Yakovlev *et al.*, 2012).

Transgenerational responses to maternal environments are not restricted to abiotic cues; biotic stresses are also known drivers of transgenerational phenotypic changes (Holeski *et al.*, 2012). For example, maternal wild radish plants exposed to caterpillar herbivore damage produce offspring seedlings more resistant (Agrawal, 2002) or seedlings able to respond stronger or faster to herbivory damage (i.e. primed seedlings, Rasmann *et al.*, 2012) than the offspring of unthreatened parents. Most studies of transgenerational induction of defenses to pests and pathogens in plants have been focused, however, on short-living annuals, and up to date, whether this type of transgenerational plasticity also occur in long-living trees remains largely unknown (Holeski *et al.*, 2012).

Environmental maternal effects can be transmitted to the next generation through different mechanisms that have different ecological and evolutionary implications (Herman and Sultan, 2011). On one hand, the amount of resources that the mother plants allocate to seeds is environmentally dependent, in fact the amount and quality of the resources stored within the seeds largely affects germination and early development of plants (Castro *et al.*, 2006; Metz *et al.*, 2010). Seed provisioning is an important transmission vehicle of environmental maternal effects (Herman and Sultan, 2011), its influence is restricted to one generation and normally diminishes with

age (Boyko and Kovalchuk, 2011). On other hand, epigenetic mechanisms, i.e. a set of molecular processes that modulate the phenotype by modifying gene expression, can be transmitted through the seed, and contribute to transmit heritable plastic responses to environmental cues (Jablonka and Raz, 2009). Transgenerational epigenetic changes include DNA methylation, histone modification, and small RNA interference (Boyko and Kovalchuk, 2011; Holeski *et al.*, 2012), and may persists over the whole life cycle, even across multiple generations (Herman *et al.*, 2012). Quantifying the relative contribution of resource-dependent and resource-independent mechanisms in the transmission of specific transgenerational plastic responses will help to understand their relevance and impact in the ecology and evolution of natural populations (Bossdorf *et al.*, 2010; Elwell *et al.*, 2011; Zas *et al.*, 2012).

It has been well studied that abiotic stressors in the maternal environment may elicit transgenerational plasticity affecting the performance of the progeny when challenging environmental harshness (Herman and Sultan, 2011). Similarly, evidence exists that biotic stresses exerted by herbivores or pathogens on the mother plants can induce transgenerational defences in the progeny (Holeski *et al.*, 2012). Little is still known, however, about whether abiotic stress in the maternal environment can be also associated to transgenerational defensive plasticity. Here we aim to elucidate whether maternal environments strongly differing in abiotic characteristics have any influence in the early resistance of *Pinus pinaster* seedlings to the major pathogen *Fusarium circinatum*, and to what extent differences in performance and resistance between maternal environments are mediated by seed provisioning. *P. pinaster* is a native conifer of the

Western Mediterranean basin with great importance to the economy of this area. The *F. circinatum* fungus causes the pitch canker disease on pines. This invasive forest pathogen is native from North America but has been recorded in various countries outside its natural range (Santini *et al.*, 2012), affecting with great virulence most of the *Pinus* species all over the world (Wingfield *et al.*, 2008). In Spain, it was first isolated in nurseries and forest plantations of *P. radiata* and *P. pinaster* (Landeras *et al.*, 2005) and nowadays is apparently well established in the north and northwest of the country, representing the main threat for *Pinus* in this area (Berra and Urkola, 2010, Vivas *et al.*, 2012a). It was recently shown (Vivas *et al.*, 2012b) that genetic selection of *P. pinaster* genotypes less susceptible to the pathogen could be an adequate measure to reduce the impact of the disease. Besides, Cendán *et al.*, (2011) and Zas *et al.* (2012) have recently shown that the maternal abiotic environment can deeply determine seed mass, germination and early performance of the progeny in this pine species. Following a logical rationale it is hypothesized that environmental maternal effects may also influence seedling resistance to plant pathogens.

Materials and Methods

Plant and fungal material

Seed material was obtained from two twin clonal *P. pinaster* seed orchards that were established within the Galician Maritime Pine Breeding Program (Consellería do Medio Rural, Xunta de Galicia) to provide seeds of high genetic quality for reforestation in the area. One hundred and sixteen unrelated genotypes selected for superior growth and stem form within the Atlantic Coast of Galicia are included in

each plantation. The selected genotypes were clonally replicated by grafting, and 10 copies of each genotype were planted in each site following a randomized complete block design with 10 blocks and single-tree plots. The two plantations include exactly the same genetic material, followed identical experimental designs, and were planted far away from other *P. pinaster* stands to minimize pollen introgression. Both seed orchards strongly differed, however, in their site qualities. One seed orchard is sited at Sergude (42.82ºN, 8.45ºW) in a favourable maternal environment for *P. pinaster* in terms of growth and reproduction rate, with mild temperatures, adequate moisture all over the year and well drained and deep soils. On the other hand, the Monfero seed orchard (43.52ºN, 7.93ºW) is sited in an unfavourable maternal environment for *P. pinaster*, with low winter and spring temperatures, extreme wind exposure, and thin soils exposed to waterlogging (see Supplementary Material Table S1). Seeds were collected from 10 out of the 116 genotypes included in both seed orchards. In January 2009, two cones from each of three individual trees per genotype (ramets) and environment were sampled. Cones were oven-dried at 35 ºC and all seeds were removed. For the present study 12 randomly-selected filled seeds per cone were used. Seeds were individually weighted (± 0.0001 g) and stored at 4ºC until sowing. A total of 1440 seeds (2 maternal environments × 10 genotypes × 3 ramets × 2 cones × 12 seeds) were sown.

The *F. circinatum* strain used (MAT-2) was isolated in May 2011 from a stem canker on a *P. radiata* tree in Cantabria, northern Spain. Species identification was confirmed by cultural and morphological features and by PCR-RFLP of the histone H3 gene. And the virulence of the strain was previously tested. Since the

virulence of *F. circinatum* in Spain is fairly homogeneous (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), a single isolate was used.

Greenhouse experiment design and seedling assessment

In April 2010, preweighted seeds were sown in 2 L pots containing a commercial substrate composed of sandy soil and peat (4:1 v/v, pH 5.8-6.8) and covered with a thin layer of sterilized white sand to promote germination. Pots were set in a factorial design with 12 blocks, each including one plant of each of the 120 cones, and with both the maternal environment and the maternal genotype randomly distributed within each block. Seedlings were grown in a greenhouse at Universidad de Extremadura (Plasencia, 40.03ºN, 6.08ºW) with temperatures fluctuating in the range of 23 ± 5 °C, under 70 per cent full sunlight and watered every 2-3 days as needed. Individual seedling height was measured monthly from February to October 2011 and individual stem diameter at ground level was measured on October 6th 2011, when plant material was 18 months old.

Fungal inoculation and symptom assessments

On October 17 2011, when plant material was 18 months old and 47.0 ± 0.3 cm tall, half of the blocks were inoculated with *F. circinatum* and the other half with distilled sterile water as control. The day of inoculation, a spore suspension was prepared by flooding *F. circinatum* cultures growing on PDA plates with a sterilized aqueous solution of 0.5 g KCl L^{-1} and gently scraping the fungus from the

surface with a sterile glass slide. The suspended fungal biomass was filtered through a double layer of sterile cheesecloth that retained mycelium and allowed most of the spores to pass through. The concentration of spores in the suspension was estimated using a hematocytometer and adjusted to a final concentration of $5 \cdot 10^3$ spores mL^{-1} (Schmale and Gordon, 2003). The inoculation was performed according to (Gordon *et al.*, 1998a), small wounds deep enough to reach the sapwood were made, at the junction of lignified and succulent tissue, using a drill bit (1.5 mm diameter) and 5 µL of the spore suspension (equivalent to approximately 25 spores) were placed in the wound site (Matheson *et al.*, 2006). Control inoculations were performed by depositing distilled sterile water in the wound site instead of the spore suspension. Each tree was inoculated only once.

Four weeks after inoculation seedlings were examined. Bark was removed from the area surrounding the inoculation point and the length of the lesion on the sapwood measured to the nearest mm. When necrosis girdled the stem, to estimate the lesion length only necrosis proximal to the inoculation site was measured and this value was doubled as suggested by (Gordon *et al.*, 2011). Seedlings were considered dead only if the entire stem developed a canker and needles turned brown. On the other hand, seedlings with lesion length less or equal to that observed in control plants (~ 0.3 cm) were considered not susceptible. Seedlings that had not been inoculated with the pathogen were similarly analyzed.

In order to confirm the presence of the fungus in the inoculated seedlings, re-isolations of *F. circinatum* from a random subsample of 25% of the harvested seedlings were performed. A 5-cm-long segment near the point of inoculation was cut from each

stem, the needles removed and the stem portion surface disinfested in an aqueous solution of 0.1% (v/v) Tween® 20 (Panreac, Spain), then 70% (v/v) ethanol for 30 s and finally 20% (v/v) bleach for 1 minute. Each segment was placed onto FSM agar (Aegerter and Gordon, 2006) and incubated at 22ºC for 5-10 days. Colonies of *F. circinatum* were identified morphologically (Leslie and Summerell, 2006).

Statistical analysis

The effects of design factors on seedlings growth (monthly seedlings height and stem diameter) were analyzed with a general linear mixed model with the PROC-MIXED procedure of the SAS System. For the analysis of height growth, we first tried to fit a repeated measures mixed model, but it failed to converge, so we then analyzed each monthly height independently, using a hierarchical model similar to those used to solve a split-split design with three levels of nested experimental units (ramets, cones and seeds) (Littell *et al.*, 2006). Values within a given cone, and values from different cones of the same ramet were assumed to be dependent measures within the same subject (cone or ramet, respectively), and were considered random factors. The general mixed model included the fixed effects of the maternal environment (ME) and the block of the field experimental design, nested within each seed orchard (B(ME)). The random effects of the mixed model included the maternal genotypes (G), the interaction between maternal genotypes and maternal environments (G × ME, which represents the genetic variation of the plastic responses to the maternal environment), and the random effects of ramets (G × B(ME)) and cones within ramets (cone(B×ME×G)), accounting for micro-environmental variation at scales lower than the

block size and other phenotypic effects associated to the individual ramets and cones (Zas *et al.* 2012). The mixed model also included the fixed effect of the greenhouse blocks and the covariation with germination time, in order to account for greenhouse heterogeneity and for variation in ontogenic development among seedlings, respectively.

For the analysis of necrosis length we used a general mixed model similar to the one described above, but including the fixed effect of the inoculation treatment (I), and its interaction with all the previous fixed and random factors. The interaction I × ME denotes whether the maternal environment influences the necrosis length after inoculation, i.e. whether there are significant maternal effects on seedling resistance to the pathogen. Similarly, the I × G interaction random term accounts for the genetic variation in susceptibility to the pathogen, whereas the I × G × ME interaction was interpreted as the genetic variation in the transmission of environmental maternal effects. Germination time was included again as a fixed covariate, but it was removed as it was not significant and did not improve the resolution of the model.

Seed weight was strongly influenced by the contrasting site qualities of the maternal environment and the maternal genotypes (Table S2; Figure S1). So, in order to quantify to what extent the observed maternal environmental effects were mediated by seed provisioning, all the previous models were run including and excluding the covariation with individual seed weight.

The statistical significance of the variance components for each random factor in all statistical models was assessed using likelihood ratio tests, where the differences in two times the log-

likelihood of the models including and excluding that random factor were distributed as one tailed χ^2, with one degree of freedom (Fry, 2004).

Results

Environmental maternal effects on seedlings performance

Without adjusting for seed mass covariation, seedling height was significantly influenced by both the maternal environment and the maternal genotype (Table 1, left). Both factors significantly affected seedling height over all the studied period (Figure 1a), although the magnitude of the effects tended to diminish with seedling age as revealed by the reductions of the *F* ratios and the relative genetic variances (Table S3). At age 18 months, seedlings from the favourable maternal environment were 10.6% higher than seedlings from the unfavourable maternal environment (Figure 1a), and seedling height among maternal genotypes ranged from 49.4 to 43.4 cm (Figure S2). Stem diameter was also higher in seedlings from the favourable maternal environment ($F_{1,9}$ = 15.3; $P < 0.01$), but no differences among genotypes were observed ($\chi^2 = 1.4$; $P > 0.05$).

The effect of the maternal environment on seedlings height and diameter became negligible after including the covariation with the seed mass of each individual seedling in the mixed model (Table 1, right; Figure 1b) and this result was consistently observed over the entire studied period (Table S3). When adjusting for seed mass covariation, the genetic variance for height growth was reduced, although it remained significant in all assessment dates (Table 1, right; Table S3). The interaction between maternal environments and

maternal genotypes for height growth was not significant irrespective of accounting or not for seed weight covariation (Table 1).

Table 1. Results of the general lineal mixed models for the height of 18 months old pine seedlings from ten maternal genotypes clonally replicated in two contrasting maternal environments, one favourable and one unfavourable for pine growth. Analyses excluding and including the individual seed mass as a covariate are shown. Degrees of freedom (DF) and *F*-ratios of fixed factors, and variance components (VarComp) and associated χ^2 values of random factors are shown. Significance *P* values are given in bold ($P < 0.05$).

Effects	Without accounting for seed mass covariation			Accounting for seed mass covariation		
	DF/VarComp	*F*-ratio/χ^2	*P* value	DF/VarComp	*F*-ratio/χ^2	*P* value
Fixed factors						
Maternal environment [ME]	1,9	20.2	**0.001**	1,9	0.8	0.387
Block(ME) [a]	10,30	0.7	0.731	10,30	0.7	0.747
Tray	11,1206	27.7	**< 0.001**	11,1205	28.3	**< 0.001**
Germination time	1,1206	19.2	**< 0.001**	1,1205	16.1	**< 0.001**
Seed mass				1,1205	30.8	**< 0.001**
Random factors						
Maternal genotype [G]	1.4 ± 1.4	4.1	**0.021**	0.9 ± 1.5	3.9	**0.024**
G × ME	0.5 ± 1.4	0.1	0.376	1.8 ± 1.6	2.5	0.057
Ramet [G × B(ME)]	2.1 ± 1.5	2.9	**0.044**	0.6 ± 1.1	0.3	0.292
Cone(B × ME × G)	1.2 ± 1.1	1.4	0.118	1.2 ± 1.1	1.7	0.096
Residual	53.7 ± 2.2			52.8 ± 2.2		

[a] Block was nested within maternal environments [B(ME)], and cones were nested within ramets [G × B(ME)].

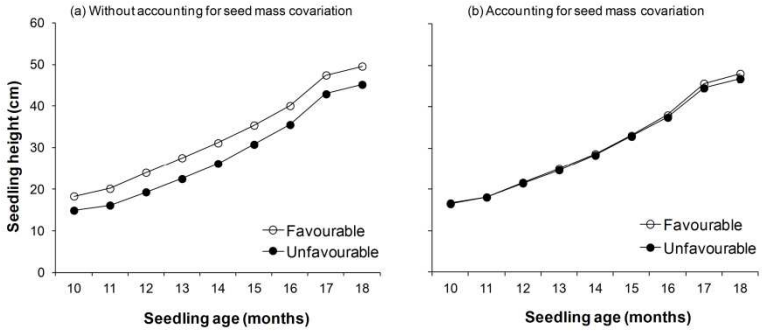

Figure 1. Height evolution of the offspring of ten maternal genotypes clonally replicated in two contrasting maternal environments, favourable (open circles) and unfavourable (black circles) for pine growth. Least square means obtained from the mixed models excluding (a) and including (b) the seed mass as a covariate are shown. The effect of the maternal environment was statistically significant ($P < 0.01$) in every monthly assessment in (a) but in none in (b).

Environmental maternal effects on early susceptibility to Pitch canker

Without accounting for seed weight covariation, the maternal environment and the maternal genotype significantly affected the necrosis length, as shown by Figure 2a and by the significant I × ME and I × G interactions, respectively (Table 2-left). The I × G × ME interaction was not significant (Table 2-left), suggesting that the effect of the maternal environment on the progression of the damage was similar across the ten maternal genotypes included in this study. The length of the necrosis of infected seedlings was 16% shorter in seedlings coming from seeds of the favourable maternal environment than in seedlings coming from the unfavourable environment (Figure 2a), and ranged from 1.7 ± 0.2 to 2.4 ± 0.2 cm among maternal genotypes (Figure 3). Only 4 and 2.4% of the inoculated seedlings from the favourable and the unfavourable maternal environment had

lesion lengths less or equal to 0.3 cm and were considered non-susceptible. During the study, no seedlings died because of *F. circinatum* and no control seedlings showed necrosis lengths caused by the drill bit higher than 0.3 cm.

Contrary to what occurred with the maternal imprint on growth traits, the effect of the maternal environment on resistance to the pitch canker was not removed when the individual seed mass was considered as a covariate in the mixed model (Table 2-right). Necrosis length remained larger for those seedlings derived from seeds of the unfavourable environment (Figure 2b). The variation of necrosis lengths among maternal genotypes also remained significant after adjusting for seed mass covariation (I × G interaction in Table 2-right), and no genetic variation was observed in the sensibility to the maternal environment (non-significant I × G × ME interaction; Table 2-right).

Table 2. Results of the general lineal mixed models for the length of necrosis of 18 months old pine seedlings, four weeks after seedling inoculation with distilled sterile water or *Fusarium circinatum* pathogen, from ten maternal genotypes clonally replicated in two contrasting maternal environments, one favourable and one unfavourable for pine growth. Analyses excluding and including the individual seed mass as a covariate are shown. Degrees of freedom (DF) and *F*-ratios of fixed factors, and variance components (VarComp) and associated χ^2 values of random factors are shown. Significance *P* values are given in bold ($P < 0.05$).

Effects	Without accounting for seed mass covariation			Accounting for seed mass covariation		
	DF/VarComp	*F-ratio*/χ^2	*P* value	DF/VarComp	*F-ratio*/χ^2	*P* value
Fixed factors						
Inoculation [I]	1,1175	493.5	**< 0.001**	1,1175	501.2	**< 0.001**
Maternal environment [ME]	1,9	3.5	0.095	1,9	0.4	0.561
I × ME	1,1175	7.0	**0.008**	1,1175	7.0	**0.010**
Block(ME) [a]	10,30	0.8	0.627	10,30	0.7	0.694
Trays(I) [b]	1,1175	32.0	**< 0.001**	10,1174	32.1	**< 0.001**
Seed mass				1,1174	2.21	0.137
Random factors						
Maternal genotype [G]	0.002±0.012	0.0	0.500	0	0.5	0.500
G × ME	0.001±0.008	0.9	0.171	0.001±0.007	1.1	0.240
Ramet [G × B(ME)]	0	0.0	0.500	0	0.0	0.500
I × G	0.02±0.015	17.1	**< 0.001**	0.019±0.011	17.1	**< 0.001**
I × G × ME	0.007±0.01	0.7	0.201	0.008±0.011	1.1	0.147
Cone (B × ME × G)	0	0.0	0.500	0	0.0	0.500
Residual	0.453±0.018			0.452±0.018		

[a] Block was nested within maternal environments [B(ME)], and cones were nested within ramets [G × B(ME)].

[b] Tray was nested within inoculation treatment [T(I)].

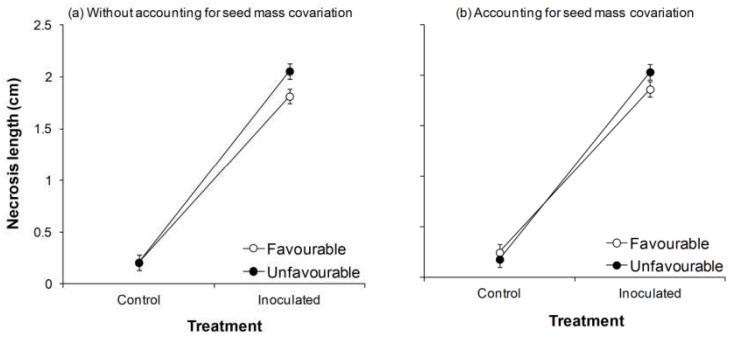

Figure 2. Necrosis length of pine seedlings four weeks after inoculation with distilled sterile water (Control) and the *Fusarium circinatum* pathogen (Inoculated). Seedlings derived from ten maternal genotypes clonally replicated in two contrasting maternal environments, favourable (open circles) and unfavourable (black circles) for pine growth. Least square means (± standard errors) were obtained from mixed models, excluding (a) and including (b) the seed mass as a covariate (N = 360).

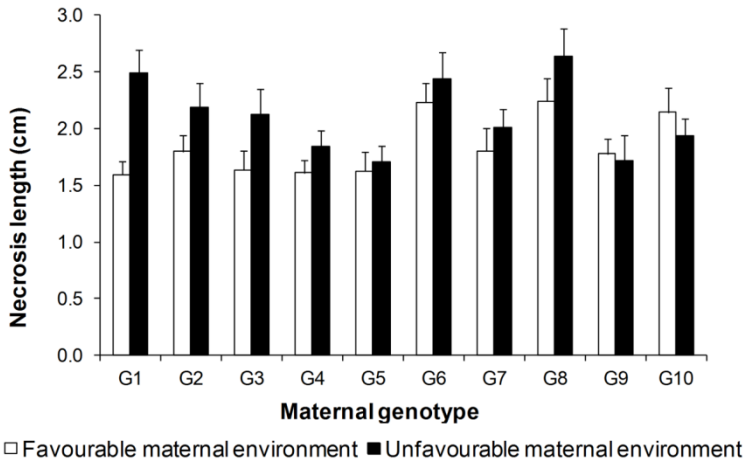

Figure 3. Mean necrosis length of pine seedlings caused by *Fusarium circinatum* four weeks after inoculation. Seedlings derived from ten maternal genotypes clonally replicated in two contrasting maternal environments, one favourable (white bars) and one unfavourable (black bars) for pine growth. Means ± standard errors are shown.

Discussion

Seedling performance variation

Results indicated that the maternal environment determins the height and stem diameter in the progenies of *P. pinaster*. Seedlings coming from seeds of the favourable maternal environment were 10 % taller and had 7% higher stem diameters than seedlings from the unfavourable maternal environment, and this trend was consistent among all the studied maternal genotypes (no G × ME interaction). These maternal environmental effects were seed mass dependent, and the effect of the maternal environment disappeared when the statistical model properly accounted for seed weight variation. Seeds from the favourable environment were 34 % heavier than those of the stressed environment and this resulted in bigger seedlings. Although maternal environmental effects explaining variations in seedling performance has been previously identified in conifers (e.g. Lindgren and Wei, 1994; Stoehr *et al.*, 1998), less attention has been paid to the interactive effects of the maternal environment and the maternal genotype (i.e. whether transgenerational responses are genetically variable) and to the mediation of seed provisioning in the transmission of maternal effects. Our results agree with those of Zas *et al.* (2012), who using seeds from the same seed orchards reported, in an independent experiment, significant maternal environmental effects on *P. pinaster* offspring phenotype, with seedlings from the favourable maternal environment being larger and with more root biomass than those from the stressed seed orchard. As observed here, the effect of the maternal environment on seedling size was mostly explained by seed mass variation among seed orchards, and this trend did not vary among the studied genotypes. Seed mass is a key trait for many

aspects of the ecology and evolution of plant species (Linkies *et al.*, 2010). The quantity and quality of the resources allocated to the seeds may strongly differ between maternal environments and this may result in large differences in mean seed weight among environments (Violle *et al.*, 2009), as observed here. Furthermore, there are numerous studies showing positive relationships between seed size and the probability and speed of germination, and subsequent seedling size (Weiner *et al.*, 1997; Moles and Westoby, 2006). Thus, our results showing large difference in seedling growth depending on the environment where the seeds are developed, appear to be a side effect of the resources that the mother trees allocated to seeds. This passive response to the maternal environment is expected to have little adaptive significance.

Pitch canker susceptibility variation

Results showed that the necrosis length caused by *F. circinatum* treatments was largely influenced by the maternal environment and the maternal genotype. Interestingly, the influence of both factors did not disappear when the model accounted for seed weight covariation. This suggests that transgenerational defensive plasticity in *P. pinaster* offspring to the pitch canker pathogen was not a passive response to resource availability on seed provisioning.

Most studies exploring transgenerational plastic responses of plants focused on determining whether seedlings derived from mother plants growing under a particular biotic or abiotic stress may be able to tolerate better that particular stressor (Agrawal, 2001, 2002; Latzel *et al.*, 2010). Transgenerational induction of defences in response to biotic stress has been reported in several plant species (Holeski *et al.*,

2012). Here, however, mother tress growing under contrasted abiotic conditions provided transgenerational cues to the progeny that elicited phenotypes with differential biotic resistance. This suggests that abiotic and biotic transgenerational defense plasticity could share at some extent stressor cues, or at least that the phenotypic transgenerational responses to abiotic cues influence seedling resistance. Growth and resistance are not independent features of an individual plant, and usually tradeoff among each other (Koricheva, 2002). Sharing the same resources, maximizing at a time growth and resistance is not possible, and plants tend to optimize the allocation of resources to each feature depending on the environmental conditions (Herms and Mattson, 1992). Different theoretical frameworks predict that plants growing in resource-limited environments should be better protected against natural enemies (reviewed by Stamp, 2003). Results are not consistent with this prediction and indicated not only that the bigger seedlings were more resistant but higher resistance of plants derived from mothers growing under resource deprivation.

Seed mass did not have any relationship with the seedling ability to resist the pathogen. Other mechanisms must be involved in this form of transgenerational plasticity, where the abiotic maternal environment influences the susceptibility of *P. pinaster* offspring to *F. circinatum*. Alternatively, epigenetic mechanisms, i.e. those processes that modulate gene expression without any variations in DNA base sequences (Jablonka and Raz, 2009), may be involved. Epigenetic mechanisms are known to be responsible of different forms of transgenerational plasticity (Herman and Sultan, 2011). Particularly, conifers have an excessive amount of genomic DNA without apparent duplications which could be a rich source of sites for epigenetic

regulation and modifications (Yakovlev *et al.*, 2012). We do not have information about seed composition or gene expression, to discriminate to what extent these mechanisms were involved in the transmission of the observed environmental maternal effects regarding seedling resistance. However, the fact that the transgenerational response was independent for the seed mass and the lack of a clear association between seedling growth and resistance suggest that some of these epigenetic mechanisms could be involved in the observed transgenerational plasticity. As transgenerational changes in epigenetic regulation of gene expression can be long lasting (Herman *et al.*, 2012), disentangling their role in the observed transgenerational responses would help to elucidate whether the observed differences in seedling resistance will be maintained as seedlings get older.

Previous studies provided evidence that *P. pinaster* families showed different levels of resistance to the pitch canker pathogen (Vivas *et al.*, 2012b). In the present study, seedlings performance and resistance seems to be plastic traits controlled by environmental maternal effects and maternal genotype. However, the lack of genetic variation in response to transgenerational effects in seedling performance and resistance observed (i.e. lack of G × ME interaction), would imply a potential constraint on the evolution of this traits. Genetic variation within the offspring population sensitive to the maternal environment does not exist. These results contrast with other experiments (e.g. Agrawal, 2001) and probably arise because the seedlings used here belonged to selected improved trees and not from the wild type.

In conclusion, it has been demonstrated that abiotic differences in the environment experienced by maternal trees affected

the expression of the early phenotype in the subsequent generation involving both growth and resistance traits up to one year and a half after germination. The quantitative maternal investment in seed mass explained the transgenerational plasticity of seedlings growth but not of seedling resistance to *F. circinatum*, for which other mechanisms should be involved. Results suggest that the *P. pinaster* progenies derived from mother trees growing in a favourable maternal environment (in terms of growth and reproduction) may show greater growth rates and improved resistance against the pathogen *F. circinatum*. From a practical point of view, further research is needed to explore the possibilities of transgenerational responses as a driver of seedling phenotypic performance in nurseries, breeding programs, forests plantations and management practices.

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Supplementary material

Table S1. Climatic, edaphic and dasometric characteristics of the two contrasting maternal environments, the favourable (Sergude) and the unfavourable (Monfero) for pine growth and reproduction. Both seed orchards have exactly the same genetic material and follow the same spatial design (Cendán *et al.*, 2012).

	Sergude	Monfero
Altitude (m)	258	615
Mean annual temperature (ºC)	13.2	10.6
Maximum temperature (ºC)	38.7	35.3
Minimum temperature (ºC)	-4.7	-8.0
Mean temperature of the warmest month (ºC)	19.2	15.5
Mean temperature of the coldest month (ºC)	7.8	5.9
Number of frost-free months [a]	5	3
Annual precipitation (l m^{-2})	1445	1435
Daily average wind speed (m s^{-1})	3.2	5.2
Number of windy days per year [b]	35	166
Soil pH (H_2O, 1:2.5)	5.1	4.5
Soil depth (cm)	>120 cm	45.1 ± 3.2
Tree age at sampling	27	20
Mean tree diameter at breast height (cm)	20.9 ± 0.6	6.1 ± 0.3
Annual individual tree growth in basal area ($cm^2 year^{-1}$)	13.6 ± 0.8	1.65 ± 0.15
Number of cones per tree at age 9	76.5 ± 11.0	3.8 ± 0.2
Reproductive allocation (cones dm^{-2}) [c]	47.8 ± 4.1	15.8 ± 2.8

[a] Sensu Emberger *et al.* (1963), i.e. the period during which the average minimum temperature is over 7ºC

[b] Average wind speed > 5 m s^{-1}

[c] Number of cones per unit of basal area at breast height (modified from Climent *et al.*, 2008)

Table S2. Results of the general lineal mixed model for the individual seed mass from ten maternal genotypes clonally replicated in two contrasting maternal environments, one favorable and one unfavourable for pine growth. Degrees of freedom (DF) and *F*-ratios of fixed factors, and variance components (VarComp) and associated χ^2 values of random factors are shown. Significance *P* values are given in bold ($P < 0.05$).

Effects	Seed mass		
	DF / VarComp	*F*-ratio / χ^2	*P* value
Fixed effects			
Maternal environment [ME]	1,9	67.7	< 0.001
Block(E) [a]	10,30	4.2	0.001
Random effects			
Maternal genotype [G]	138.6 ± 76.6	42.7	< 0.001
G × ME	30.9 ± 2.1	6.1	0.007
Ramet [G × B(ME)]	15.9 ± 8.9	4.8	0.014
Cone (B × ME × G)	29.9 ± 6.1	319.8	< 0.001
Residual	39.6 ± 1.5		

[a] Block was nested within maternal environments [B(ME)], and cones were nested within ramets [G × B(ME)].

Tabla S3. Results of the general mixed model for the analysis of the monthly seedling height from February to October 2011, from ten maternal genotypes clonally replicated in two contrasting maternal environments, one favourable and one unfavourable for pine growth. Analyses excluding and including the individual seed mass as a covariate are shown. Degrees of freedom (DF) and *F*-ratios of fixed factors, and variance components (VarComp) and associated χ^2 values of random factors are shown. Significance *P* values are given in bold ($P < 0.05$).

a) Without accounting for seed mass covariation

Effects		February		March		April		May		June		July		August		September		October	
	DF	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value
Fixed factors																			
Maternal environment [ME]	1,9	38.4	**< 0.001**	38.5	**< 0.001**	38.9	**< 0.001**	37.6	**< 0.001**	36.8	**< 0.001**	29.4	**< 0.001**	25.5	**< 0.001**	21.0	**0.001**	20.2	**0.001**
Random factors																			
Maternal genotype [G]		27.5	**< 0.001**	24.2	**< 0.001**	21.0	**< 0.001**	19.8	**< 0.001**	18.7	**< 0.001**	13.3	**< 0.001**	8.0	**0.002**	4.4	**0.018**	4.1	**0.021**
G × ME		0.0	0.500	0.0	0.500	0.0	0.500	0.2	0.327	0.3	0.292	0.3	0.292	0.3	0.292	0.3	0.292	0.1	0.376

b) Accounting for seed mass covariation

Effects		February		March		April		May		June		July		August		September		October	
	DF	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value	F/χ^2	*P* value
Fixed factors																			
Maternal environment [ME]	1,9	0.0	0.877	0.1	0.767	0.2	0.684	0.3	0.602	0.3	0.589	0.1	0.739	0.3	0.572	0.6	0.452	0.8	0.387
Seed mass	1,1205	98.3	**< 0.001**	103.7	**< 0.001**	99.9	**< 0.001**	97.7	**< 0.001**	90.0	**< 0.001**	75.8	**< 0.001**	58.7	**< 0.001**	37.7	**< 0.001**	30.8	**< 0.001**
Random factors																			
Maternal genotype [G]		12.3	**< 0.001**	8.7	**0.002**	7.1	**0.004**	8.7	**0.002**	9.4	**0.001**	5.9	**0.008**	2.4	0.061	3.1	**0.039**	3.9	**0.024**
G × ME		1.4	0.118	0.6	0.219	1.2	0.137	1.4	0.118	2.1	0.074	2.7	0.050	3.3	**0.035**	3.4	**0.033**	2.5	0.057

[a] Block was nested within maternal environments [B(ME)], and cones were nested within ramets [G × B(ME)].

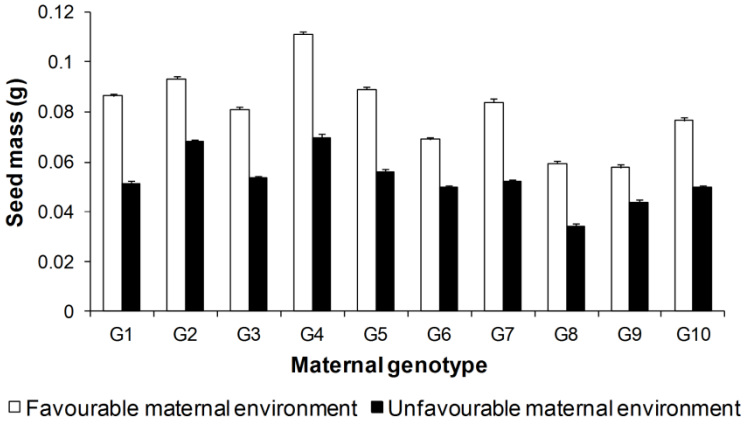

Figure S1. Seed mass of pine seedlings from ten maternal genotypes clonally replicated in two contrasting maternal environments, favourable (white bars) and unfavourable (black bars) for pine growth. Means ± standard errors are shown.

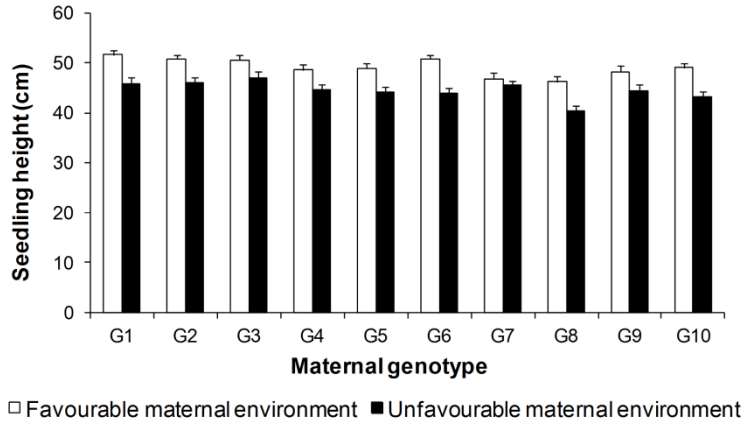

Figure S2. Seedling height of 18 months old pine seedlings from ten maternal genotypes clonally replicated in two contrasting maternal environments, favourable (white bars) and unfavourable (black bars) for pine growth. Means ± standard errors are shown.

Article V

Carbohydrates of *Pinus pinaster* seedlings originating from contrasting maternal environments: do they influence susceptibility to *Fusarium circinatum*?

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Preliminary manuscript

Abstract

Carbohydrates of *Pinus pinaster* seedlings originating from contrasting maternal environments: do they influence susceptibility to *Fusarium circinatum*?

The reserves of plant carbohydrates are important in plant-pathogen interactions. *Pinus pinaster* seedlings susceptibility to the fungal pathogen *Fusarium circinatum* was analyzed with respect to changes in carbohydrate compounds occurred in seedling stem. Besides, seeds were collected from the same clone replicated in two contrasting maternal environments to analyze the influence of maternal environment. Stem samples were characterized quantitatively by gas chromatography and spectrophotometry and qualitatively by FT-IR. A significant increase in glucose proportion occurred in inoculated seedlings from the favourable maternal environment which diagnosed an increase of pectic polysaccharides (wavenumbers 1145, 1100 cm^{-1} in FT-IR spectra), in comparison with control seedlings. Instead, a significant decreased in uronic acid proportions occurred in inoculated seedlings from the favourable maternal environment which diagnosed a decreased of hemicellulosic polysaccharides (wavenumbers 1050 and 1025 cm^{-1} in FT-IR spectra), in comparison with control seedlings. These results suggest that changes in carbohydrate compounds of *P. pinaster* seedlings after *F. circinatum* inoculation were mediated by the maternal environment.

Keywords: Maternal effects, Maritime pine, pitch canker disease, sugar composition, FT-IR, polysaccharides.

Resumen

Hidratos de carbono en plántulas de *Pinus pinaster* procedentes de contrastados ambientes maternos: ¿influyen en la susceptibilidad ante *Fusarium circinatum*?

Las reservas de carbohidratos de las plantas son importantes en las interacciones planta-patógeno. Se analiza la susceptibilidad de las plántulas de *Pinus pinaster* ante el hongo patógeno *Fusarium circinatum* con respecto a los cambios en los hidratos de carbono producidos en el tallo de las plántulas. Además, las semillas se obtuvieron de un mismo clon replicado en dos contrastados ambientes maternos para analizar la influencia del ambiente materno. Las muestras de tallo de las plántulas se caracterizaron cuantitativamente por cromatografía gaseosa y espectrofotometría y cualitativamente por FT-IR. La proporción de glucosa aumentó significativamente en las plántulas inoculadas que provenían del ambiente materno favorable, lo cual evidencia el aumento de polisacáridos pécticos (longitudes de onda 1145, 1100 cm^{-1} en los espectros del FT-IR), respecto a las control. En cambio, la proporción de ácidos urónicos disminuyó significativamente en las plántulas inoculadas que provenían del ambiente materno favorable, lo cual pone de manifiesto la disminución de polisacáridos hemicelulósicos (longitudes de onda 1050, 1025 cm^{-1} en los espectros del FT-IR), respecto a las control. Los resultados sugieren que los cambios en los carbohidratos de plántulas de *P. pinaster* después de la inoculación con *F. circinatum* estuvieron influidos por el ambiente materno.

Palabras clave: Efectos maternos, pino marítimo, enfermedad del chancro resinoso, composición de los azúcares, FT-IR, polisacáridos.

Introduction

Over time, plants have developed a complex set of defense mechanisms to be protected from a wide variety of plant pathogens. Particularly, plant carbohydrates, which are one of the most abundant groups of organic compounds in the plant kingdom (Avigad and Dey, 1997), play an essential role in the pathogenesis of fungal diseases. The reserves of carbohydrates are important in preventing invasion by certain plant pathogens (Kozlowski, 1992). Even, specific changes in cell wall associated monosaccharides were reported to be linked to plant´s ability to combat pathogens (Truernit *et al.*, 1996). Carbohydrates may be involved in the metabolic reactions associated with disease resistance by the production of defensive chemicals, but also may be used as substrates for some pathogens (Morkunas *et al.*, 2007, 2010). At present, the precise relationship between carbohydrate levels and resistance phenomena are not known, but it is clear that carbohydrate seem to be important in the challenge of a plant against a pathogen.

Maritime pine (*Pinus pinaster*) is a profitable conifer species of the Western Mediterranean basin. In particular, it is the forest species more extensively used in Spain in plantations with great importance to the economy. Susceptibility of *P. pinaster* to the fungus *Fusarium circinatum* (Nirenberg and O'Donnell), the causal agent of pitch canker disease, has reduced the profitability of *Pinus* spp. in northern Spain. Attempts to control *F. circinatum* which included sanitation or biological treatments (Wingfield *et al.*, 2008; Vivas *et al.*, 2012a) failed. Breeding strategies, aimed to reduce the impact of the disease, allowed the identification of some *P. pinaster* clones more resistant to *F. circinatum* than others (Vivas *et al.*, 2012b). Moreover,

depending on the site quality those clones were planted, the seeds obtained provided seedlings with different susceptibility to *F. circinatum* (Vivas *et al.*, unpublished). The reasons why the maternal environment significantly influences the resistance of the offspring to *F. circinatum* are ignored.

The aim of this study was evaluate to what extend carbohydrate content of *P. pinaster* seedlings originating from contrasting maternal environments could influence their susceptibility to *F. circinatum*. With the hypothesis that carbohydrate content could be mediated by the maternal environment of the seedlings and influence susceptibility. Changes in plant carbohydrates were assessed quantitatively by conventional techniques of gas chromatography and spectrophotometry and qualitatively by Fourier transform-infrared (FT-IR) spectroscopy in association with chemometrics.

Materials and Methods

Plant material and susceptibility test

Two seedlots originating from the same clone but collected from two different clonal orchards were used. Clone 1020 was selected among other 10 clones because it was the best *P. pinaster* candidate to be used as breeding stock for resistance (Vivas *et al.*, 2012b), and because it seedlings significantly differed in resistance to *F. circinatum* depending on the orchard from which the seed was collected ($P < 0.05$; Table 1) (Vivas *et al.*, unpublished). Orchards were established by the tree breeding program of Consellería do Medio Rural (Xunta de Galicia, Spain) and they have identical genetic material and experimental design but contrasting site qualities. Sergude (42.82ºN, 8.45ºW) is a favourable seed orchard because its

high quality environment for *P. pinaster* development and Monfero (43.52ºN, 7.93ºW) is an unfavourable seed orchard located in a harmful environment (Table 1). On January 2009, seedlots were obtained from three ramets per clone and site (Cendán *et al.*, 2011). Two cones per ramet were collected and 12 seeds per cone obtained. A total of 144 seeds, comprising 2 orchards × 3 ramets × 2 cones × 12 replicates were used.

Table 1. Plant material specifications of *Pinus pinaster* clone 1020. In the field, ramets (mother plants) were grown under two contrasting maternal environments, one unfavourable and one favourable for pine growth. In the greenhouse, seedlings were grown under the same controlled environment. Values are presented as averages ± standard errors.

		Maternal environment	
		Unfavourable	Favourable
Field	Tree diameter (cm)	7.3 ± 0.2	27.0 ± 0.3
	Vain seeds per cone (#)	19.5 ± 1.0	13.7 ± 0.9
	Healthy seeds per cone (#)	116.3 ± 3.0	148.6 ± 0.7
	Vain seed weight (mg)	32.0 ± 0.6	48.9 ± 0.5
	Healthy seed weight (mg)	58.6 ± 1.1	86.5 ± 1.0
Greenhouse	Seedling diameter (mm)	3.6 ± 0.1	3.9 ± 0.1
	Seedling height (cm)	46.4 ± 1.2	51.7 ± 0.9
	Needles weight (g)	1.7 ± 0.1	1.9 ± 0.1
	Root weight (g)	0.9 ± 0.1	1.3 ± 0.1
	Necrosis length (cm)	2.5 ± 0.2	1.6 ± 0.1

Seeds were individually set in 2 L pots in a randomized completed block design and germinated during spring 2010. Seedlings were grown 18 months in a greenhouse at temperatures fluctuating in the range of 23 ± 5°C and under 70% full sunlight. On October 2011, when plants were about 1.5 years old, half of the seedlings were inoculated with *F. circinatum* (MAT-2). The strain was isolated in May 2011 from a stem canker on a *P. radiata* tree in Cantabria, northern Spain, and its virulence was previously tested (Vivas *et al.*, unpublished). Inoculations were performed by placing 5 µl of a spore suspension of *F. circinatum* ($5 \cdot 10^3$ spores per mL in 0.5% KCl) in a wound made in the stem with a drill bit at the junction of lignified and succulent tissue (Schmale and Gordon, 2003). The other half of the seedlings (controls) were identically wounded using distilled sterile water. Four weeks after inoculation all seedlings were harvested and samples removal (Table 1). Samples per seedling consisted of a 3 cm long stem segment collected under the inoculated or wounded site. The samples were immediately frozen in liquid N_2 and stored at -80ºC until required for carbohydrate analysis and FT-IR spectroscopy.

Carbohydrate analysis

Stem samples were treated with 80% ethanol at 100ºC for 25 minutes to obtain the alcohol insoluble residues (AIR). About 3 mg of each AIR dry sample was hydrolysed in 0.2 mL of 72% H_2SO_4 for 3 h at room temperature followed by dilution to 1 M H_2SO_4 at 100ºC for 2.5 h; 0.5 mL of the hydrolyzed were removed after 1 h for uronic acid analysis (Coimbra *et al.*, 1996; Oliveira *et al.*, 2009). Neutral sugars released after 2.5 h were derivatised as their alditol acetates (Selvendran and O'Neill, 1987) and separated by gas chromatography

using a HP 5890 Series II chromatograph with a FID detector. A 30 m column DB-225 (J&W) with i.d. 0.25 mm and 0.15 μm film thickness was used. The injector and detector temperatures were 220 and 230ºC, respectively. The oven temperature program used was 220ºC for 7 min with a rate of 40ºC min^{-1}, followed by 230ºC for 1 min with a rate of 2ºC min^{-1}. The pressure of the carrier gas (N_2) on the head of the column was 110 kPa at 220ºC. Uronic acids were quantified colorimetrically with *m*-phenylphenol according to the method described by (Blumenkrantz and Asboe-Hansen, 1973) modified by (Coimbra *et al.*, 1996). The hydrolysate obtained in previous steps (0.5 mL) was added to 3 mL of ice cold concentrated sulphuric acid containing 50 mM boric acid. The solution was mixed and boiled for 10 min. After cooling, 100 μl of *m*-phenylphenol (0.15% in 0.5% NaOH) were added and the solution was allowed to stand in the dark for 30 min. The absorbance was read at 520 nm. Standards were made with galacturonic acid with linear correlation from 0 to 40 μg of uronic acids in the tube (Oliveira *et al.*, 2009).

FT-IR spectroscopy

FT-IR is a rapid and sensitive method that has been successfully used to evaluate carbohydrate changes and profiles of plants exposed to biotic and abiotic stresses (Johnson *et al.*, 2003; Martín *et al.*, 2005a, 2005b; Oliveira *et al.*, 2009). FT-IR spectra of *P. pinaster* stem samples were obtained with a GoldenGate single-reflectance ATR accessory of a Bruker (Billerica, MA, USA) IFS-55 FT-IR spectrometer. For each sample, the spectrum was recorded six times by accumulating 64 scans at a resolution of 8 cm^{-1} in the absorbance mode from 4000 to 600 cm^{-1}. The FT-IR spectral regions used were

1200-800 cm^{-1} coinciding with the 'fingerprint' region of carbohydrates (Filippov, 1992).

Statistical and chemometric analysis

To compare the amount of carbohydrates of seedlings between *maternal environments*, *inoculation treatments* and *types of carbohydrates* a General Linear Model was used. Three levels of interaction were included between the previous three fixed factors. *Seed weight* (g), *time to germination* (days), and *plant height* were considered as covariates but finally not included in the model because of lack of significance. Means were separated by Fisher´s least significant difference (LSD, P=0.05) tests. The FT-IR spectra obtained were exported in ASCII format from instrument manufactures' software into R software. All spectra, in the FT-IR region of interest (1200-800 cm^{-1}), were standard normal deviates before multivariate analysis. Principal component analysis (PCA) was applied to reduce the dimensionality of the spectral data while preserving most of the variance (Goodacre *et al.*, 2000). Subsequently, discriminant function analysis (DFA) was applied to discriminate between the four groups of samples (2 *inoculation treatments* × 2 *maternal environments*) on the basis of the retained PCs (Martín *et al.*, 2005b; Allwood *et al.*, 2006). All the statistical analysis was performed with R software version 2.14.1 (Wien, Austria).

Results

The maternal environment and the inoculation treatment did not significantly affect the total carbohydrate content of the *P. pinaster* stems (Table 2). The total carbohydrate content was similar in

seedlings from the unfavourable and favourable maternal environments (means ± SE were 186.7 ± 10.9 and 192.0 ± 10.5 mg per g^{-1}, respectively; $P > 0.05$) and in non-inoculated (control) and inoculated seedlings (188.1 ± 11.3 and 190.8 ± 9.2 mg per g^{-1}, respectively; $P > 0.05$). Glucose was significantly more abundant than uronic acids ($P < 0.001$) and represented about 40 and 22% of the total carbohydrate content, respectively. Arabinose, xylose, manose and galactose were also present but in less proportion than uronic acids ($P < 0.001$), and represented about 9, 11, 8 and 10% of the total carbohydrate content, respectively.

Table 2. Results of the general linear model for the analysis of carbohydrate content of *Pinus pinaster* stems seedlings (clone 1020) originating from seeds harvested from two contrasting maternal environments, one unfavourable and one favourable for pine growth, four weeks after seedling inoculation with distilled sterile water or *F. circinatum* pathogen. Degrees of freedom (DF) and *F* ratios are shown. Significant *P* values are given in bold ($P < 0.05$).

Effects	DF	*F* ratio	*P* value
Maternal environment (ME)	1	0.00	1.000
Inoculation treatment (I)	1	0.00	1.000
Carbohydrate type (C)	5	129.42	**< 0.001**
ME × I	1	0.00	1.000
ME × C	5	0.69	0.629
I × C	5	3.37	**0.005**
ME × I × C	5	2.31	**0.045**

Interestingly, higher relative content of glucose on inoculated *vs.* control seedlings (44 ± 3 *vs*. 37 ± 2 %, respectively; $P = 0.005$) and lower content of uronic acid on inoculated *vs*. control seedlings (19 ±

3 *vs.* 25 ± 2 %, respectively; $P = 0.005$) were observed (significant *inoculation treatment* × *carbohydrate type* interaction; Table 2). Seedlings from the favourable maternal environment increased about 13% their glucose content due to inoculation ($P = 0.045$) and reduced about 11% their uronic acids content due to inoculation ($P = 0.045$), while seedlings from the unfavourable maternal environment had their carbohydrate contents unaltered with the inoculation treatment (significant *maternal environment* × *inoculation treatment* × *carbohydrate type* interaction; Table 2 and Figure 1).

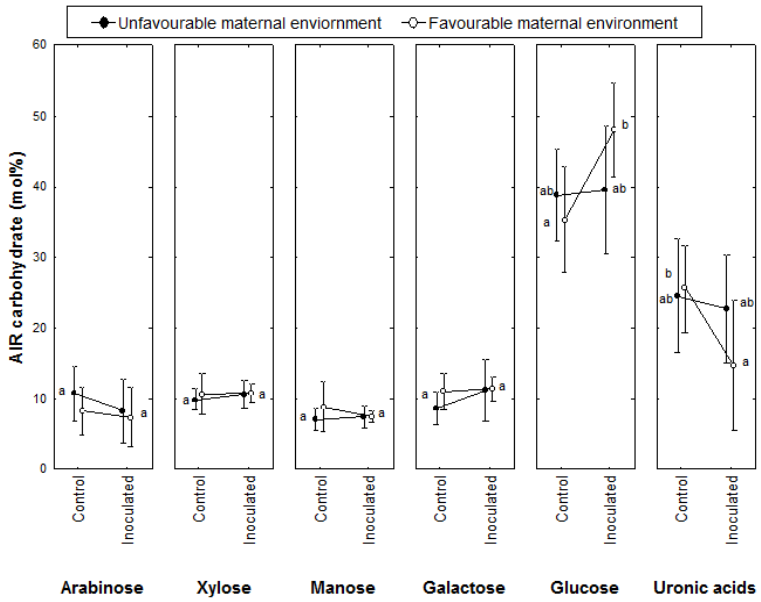

Figure 1. AIR carbohydrate composition expressed as mol (%) of *Pinus pinaster* stem seedlings (clone 1020) originating from seeds harvested from two contrasting maternal environments, one unfavourable and one favourable for pine growth, four weeks after seedling inoculation with distilled sterile water (Control) or *Fusarium circinatum* (Inoculated). Within compounds, different letters indicate significant differences among values ($P < 0.05$) and vertical bars are standard errors.

The resulting FT-IR spectra of seedlings from the unfavourable and favourable environments and of control and inoculated seedlings, although complex due to broad overlapping peaks, showed a well-defined pattern (Figure 2). Control seedlings from the unfavourable maternal environment showed higher absorbances than control seedlings of the favourable maternal environment (Figure 2). In the same way, inoculated seedlings from the unfavourable maternal environment showed higher absorbances than inoculated seedlings of the favourable maternal environment (Figure 2). Depending on the inoculation treatment, some peaks had higher absorbances for the control seedlings (e.g. 1050 and 1025 cm^{-1}) and some peaks had higher absorbances for the inoculated seedlings (e.g. 1145 and 1100 cm^{-1}).

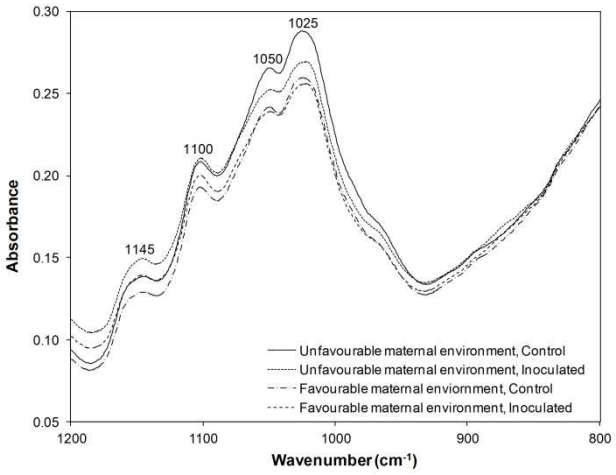

Figure 2. FT-IR average spectra of *Pinus pinaster* stem seedlings (clone 1020) originating from seeds harvested from two contrasting maternal environments, one unfavourable and one favourable for pine growth, four weeks after seedling inoculation with distilled sterile water (Control) or *F. circinatum* pathogen (Inoculated).

Classification of samples by DFA was based on 20 principal components from PCA, which explained 99.98% of the total variance. The first two discriminant functions were significant and control stem seedlings were clearly separated from inoculated samples (Figure 3). DFA did not allow discrimination of samples between maternal environments. The separation was mainly characterized by the discriminant function I, which showed a negative score gradient for control seedlings and a positive score gradient for inoculated seedlings (Figure 3).

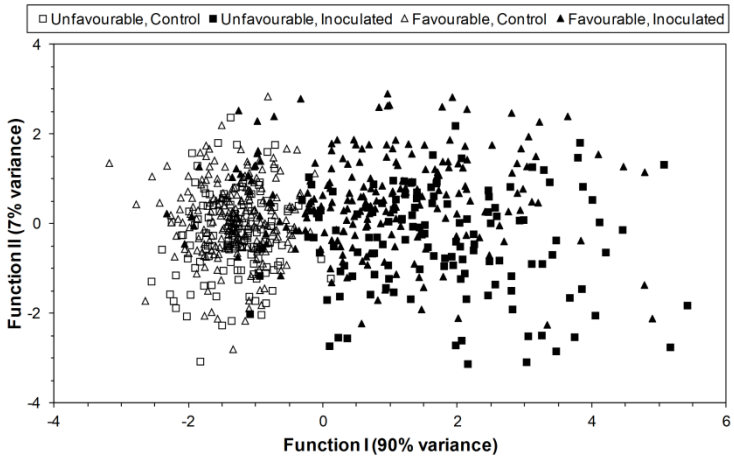

Figure 3. Discriminant function analysis plot of FT-IR spectra of *Pinus pinaster* stem seedlings (clone 1020) based on 20 principal components (explaining 99.98% of the total variance). The two discriminant functions and the relative percentage of variance explained by each function are shown in the axis. The classification factor used were the two contrasting maternal environments of *P. pinaster*, one unfavourable and one favourable for pine growth, and the two inoculation treatment, one with distilled sterile water (Control), and one with *F. circinatum* pathogen (Inoculated).

Discussion

The experiment showed for the first time changes in the amount of carbohydrates of *P. pinaster* stem tissues in response to *F. circinatum*. Specific cell wall monosaccharides changes have been shown as plant defense responses to pathogens (Reiter *et al.*, 1997). Carbohydrate changes were not the same in seedlings from the contrasting maternal environments. The cause of such changes may probably occur as a mechanism of plant defence.

Analysis of neutral sugar showed a higher proportion of glucose in inoculated than in control seedlings, and the opposite trend for the uronic acids. Furthermore, these changes were only significant for seedlings from the favourable maternal environment. So, in our study it is supposed that changes of glucose and uronic acids relative content of inoculated seedlings was due to the development of infection caused by the pathogen, as observed *in vitro* when challenging *P. pinaster* cells with the *Botrytis cinerea* pathogen (Azevedo *et al.*, 2006). Results are in accordance with those of (Oliveira *et al.*, 2009) who showed higher proportions of glucose and lower proportions of uronic acids in infected grapevines, relative to the controls. In contrast, lower concentrations of glucose in infected embryo axes, relative to the controls was observed elsewhere (Morkunas *et al.*, 2010).

Most studies about maternal environmental effects have been focused on the phenological development of seeds and seedlings (Donohue, 2009; Cendán *et al.*, 2011; Vivas *et al.*, unpublished), and not on chemical changes of the plant. Only, inoculated seedlings from the favourable maternal environment showed a significant change of carbohydrate mobilization, and this could be a reason of why

seedlings from the favourable maternal environment presented shorter lesions length by the pathogen than seedlings from the unfavourable maternal environment (Table 1). Vigorous trees, as those found in the favourable seed orchard, generally accumulate enough carbohydrates to heal injuries, synthesize defensive chemicals, and maintain physiological processes at levels necessary to sustain life when exposed to stresses (Chapin *et al.*, 1987).

During infection, both plant and pathogen compete for the same nutrients. In one hand the pathogen harness carbohydrates as its metabolic substrate, in other hand, the plant uses carbohydrates for the synthesis of chemical defenses (Azevedo *et al.*, 2006; Morkunas *et al.*, 2007, 2010). Carbohydrates could also act as signalling molecules in the induction of genes encoding pathogenesis-related proteins, cell wall structural proteins and/or hydrolytic enzyme (Bishop *et al.*, 2002). In particular, glucose has defined roles in the regulation of plant defense through independent signal transduction cascades, and is involved in cross talk, which has enabled the plant to respond to avirulent pathogens and elicit the hypersensitive response (Bishop *et al.*, 2002). Anyway, it has been shown that the use and control of host carbon sources are highly dependent on the pathogen (Azevedo *et al.*, 2006), and the speed of sugar mobilization will be a key factor to provide the host with the energy required for an effective resistance response (Allwood *et al.*, 2010). The study was focused on the carbohydrate levels 4 weeks after inoculation, and it was not analyze the first steps of the carbohydrate mobilization. However, it is possible to hypothesize that carbohydrate changes in our pines trees were promoted to fulfill the energy and carbon requirements for the resistance response.

FT-IR spectra of seedlings in the range of the carbohydrates (1200-800 cm^{-1}; Figure 2) defined peaks at 1145 and 1100 cm^{-1}, main absorbance regions for pectic polysaccharides. Pectic polysaccharides are involved in the complex fibrilar network of plant cell wall structures that defines the mechanical and functional properties of the cell wall (Barros *et al.*, 2002). In inoculated seedlings, higher proportions of glucose which diagnosed the relatively higher spectral peaks observed at 1145 and 1100 cm^{-1}, compared with the non-inoculated seedlings, reflected increased metabolic activity of pectic polysaccharides in the host trees. This metabolic increase was probably linked with a mayor reinforcement of the cell walls due the pathogen infection (Oliveira *et al.*, 2009). In other hand, the defined peaks at 1050 and 1025 cm^{-1} correspond to main absorbance regions for hemicellulosic polysaccharides (Coimbra *et al.*, 1998, 1999) which are to great extent structural carbohydrates (Schädel *et al.*, 2009). Inoculated seedlings showed lower proportions of uronic acids diagnose of lower spectral peaks at 1050 and 1025 cm^{-1} in comparison with control samples. Hemicellulosic polysaccharides, could probably serve as mobile carbon sources during the pathogenesis, period of challenge with strong carbon demand (Schädel *et al.*, 2009). Thus, FT-IR results were in accordance with the carbohydrate analysis. Moreover, a clear separation of inoculated and non-inoculated seedlings was obtained when chemometric techniques on the FT-IR spectrum of *P. pinaster* seedlings stems were applied.

Conclusion

The experiment supports the use of carbohydrate analysis and FT-IR in association with chemometrics, as methods for distinguishing

carbohydrate changes of *P. pinaster* due *F. circinatum* inoculation. Changes in carbohydrate compounds after *F. circinatum* inoculation were mediated by the maternal environment. The different fitness of plants from the favourable maternal environment in comparison with the unfavourable one induced different carbohydrates during the chemical defense reactions that took place during pathogenesis.

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Article VI

Maternal environments influence the antioxidant activity of *Pinus pinaster* when infected by *Fusarium circinatum*

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Preliminary short manuscript

Abstract

Maternal environments influence the antioxidant activity of *Pinus pinaster* when infected by *Fusarium circinatum*

The antioxidant activity of a plant consists in numerous compounds that control the production of reactive oxygen species caused by pathogens and other environmental stresses. This study examined the antioxidant activity of *Pinus pinaster* seedlings collected from two contrasting maternal environments, inoculated with the fungal pathogen *Fusarium circinatum*. Results showed that the antioxidant activity differed between maternal environments ($P < 0.001$) and increased with the necrosis length cause by the pathogen ($P = 0.005$). Moreover, the increase of antioxidant activity with the necrosis length differed between maternal environments ($P < 0.001$). Thus, maternal effects significantly regulated the antioxidant activity of *P. pinaster* challenged with *F. circinatum*.

Keywords: ABTS radical cations, reactive oxygen species, pitch canker disease, Maritime pine, maternal effects.

Resumen

El ambiente materno influye en la actividad antioxidante de brinzales de *Pinus pinaster* infectados por *Fusarium circinatum*

La actividad antioxidante de una planta consiste en numerosos compuestos que controlan la producción de especies reactivas del oxígeno generadas por agentes patógenos y otros estreses ambientales. Este estudio examinó la actividad antioxidante de plántulas de *Pinus pinaster* procedentes de dos contrastados ambientes maternos, inoculadas con el hongo patógeno *Fusarium circinatum*. Los resultados mostraron que la actividad antioxidante fue distinta entre plántulas procedentes de distintos ambientes maternos ($P < 0.001$) y aumentó con la longitud necrosis causada por el patógeno ($P = 0.005$). Además, el aumento de la actividad antioxidante con la longitud de la necrosis fue distinta entre ambientes maternos ($P < 0.001$). Por lo tanto, los efectos maternos influyeron significativamente en la actividad antioxidante de plántulas de *P. pinaster* inoculadas con *F. circinatum*.

Palabras clave: ABTS cationes radicales, especies reactivas del oxígeno, enfermedad del chancro resinoso, pino marítimo, efectos maternos.

Introduction

The oxidative stress has an important role in plant defense when plant-pathogen interactions occur (Polkowska-Kowalczyk *et al.*, 2007). The production and burst of reactive oxygen species (ROS) is one of the earliest and most rapid detectable responses in plant cell following invasion by pathogens (Bi and Felton, 1995; Deighton *et al.*, 1999). On the other hand, the plant antioxidant system consists of a number of enzymes and low molecular weight compounds that control ROS production during stress and maintain the correct levels of plant growth and signalling (Mittler *et al.*, 2004). Many studies have suggested that the more efficient is the antioxidative system of a plant, the more resistant is the plant to environmental abiotic or biotic stresses (Pukacka and Pukacki, 2000; Martínez and Araya, 2010).

Pitch canker disease is caused by *Fusarium circinatum*, a quarantine fungus in Europe with great virulence on several *Pinus* species (Wingfield *et al.*, 2008). Since 2003, *Pinus pinaster* and *P. radiata*, the most productive conifer species of south Western Europe, are suffering the destructive effects of pitch canker disease in some forests and nurseries of north western Spain (Landeras *et al.*, 2005). Big efforts are being invested to control the disease (Agustí-Brisach *et al.*, 2012; Vivas *et al.*, 2012a), being the selection of resistant *P. pinaster* clones a feasible way to reduce its long-term impact (Vivas *et al.*, 2012b). However, the environment in which the resistant clones grow significantly influences the resistance of their offspring to *F. circinatum*, i.e. seedlings obtained from ramets growing under a favourable environment resist better than seedlings obtained from ramets growing under an unfavourable environment (Vivas *et al.*, unpublished). The aims of the present study were to quantify the

antioxidant activity of *P. pinaster* seedlings inoculated with *F. circinatum* and to investigate if maternal effects influence this activity.

Materials and Methods

Plant material and inoculation test

Seeds were collected from two *P. pinaster* seed orchards located in Galicia (NW Spain) having identical genetic material and experimental design but contrasting site qualities (Zas *et al.*, 2004; Cendán *et al.*, 2011). Unfavourable maternal conditions of Monfero orchard (43.52ºN, 7.93ºW) were distinguished from favourable maternal conditions of Sergude orchard (42.82ºN, 8.45ºW). More information about the climatic, edaphic and dasometric characteristics of both sites can be obtained from Cendán *et al.*, (2011) and Vivas *et al.* (unpublished). Clone 1020 was selected because it was one of the best *P. pinaster* candidates to be used as breeding stock for resistance (Vivas *et al.*, 2012b), and because maternal environment significantly influenced the resistance of its offspring to *F. circinatum* (Vivas *et al.*, unpublished). Three ramets of this clone per orchard were included. In each ramet, two cones were randomly sampled and 12 seeds per cone were used (N=144). On February 2010, a randomized complete block design was established with seedlings growing in 2L pots at greenhouse conditions (23 ± 5°C and 70% full sunlight).

On October 2011, when plants were ~1.5 years old, inoculations with a single strain of *F. circinatum* (MAT-2) were performed. The virulence of the strain had been previously tested. Half of the seedlings were inoculated placing 5µl of a spore suspension of *F. circinatum* (5 x 10^3 spores mL^{-1} in 0.5% KCl) in a drill bit wound of 1.5 mm diameter at 5-10 cm from the tip of the

main stem (Schmale and Gordon, 2003). Control seedlings were identically wounded using distilled sterile water.

Necrosis length, sampling, and antioxidant activity assessment

Four weeks after inoculations, necrosis lengths were measured as described by Gordon *et al.* (1998). To take into account the influence of the wound, necrosis lengths of seedlings that had not been inoculated were similarly examined. From all 144 seedlings a 3 cm stem segment below the inoculation point was collected, immediately frozen in liquid N_2, and stored at -80ºC. Before analysis, stem segments were longitudinally cut into two pieces, and then transversally cut into 0.5 cm length pieces. Antioxidant activity was measured on three stem pieces (replicates) per seedling according to the method described by Re *et al.* (1999). First, ABTS [2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] solution was diluted in water (1:80) so that its absorbance was adjusted to 0.7-0.8 at 734 nm. Five mL of diluted ABTS solution was added to the stem piece and 15 minutes later the absorbance of the solution was measured at 734 nm, using the diluted solution as blank. The antioxidant activity of the sample was expressed as the inhibition percentage, by the equation (Ahn *et al.*, 2007):

$$\%\ \text{inhibition} = \frac{A_c - A_s}{A_c} \times 100$$

were A_c is the absorbance of the blank solution and A_s the absorbance of the stem piece solution.

Statistical analysis

To analyze the *antioxidant activity* data a beta regression model was used because the variable is continuous, assumes values in the standard interval (0, 1) and is related to other explanatory variables through a regression structure (Cribari-Neto and Zeileis, 2012). *Maternal environment*, *necrosis length* and its interaction were included in the model as explanatory variables. AIC criteria were used for model selection and the estimation of model parameters was calculated using likelihood ratio tests, using twice the difference between the log-likelihoods of a full model and a restricted model whose covariates are a subset of the full one (Smithson and Verkuilen, 2006). The best model was reached when all variables selected showed a significant influence on the response variable, together with an overall best fit of the model. Seed weight and germination time were first included as covariates but then removed because they were not significant and did not improve the model's resolution. The model was fitted with betareg package implemented in R software (Cribari-Neto and Zeileis, 2012).

Results and discussion

The antioxidant activity differed between maternal environments (Z = 5.12, $P < 0.001$). Inoculated seedlings from the favourable maternal environment showed lower antioxidant activity than inoculated seedlings from the unfavourable environment (67 ± 3 and 84 ± 2 %, respectively). In the same way, necrosis length of inoculated seedlings from the favourable environment was smaller than necrosis length of seedlings from the unfavourable environment (1.6 ± 0.1 and 2.5 ± 0.2 cm, respectively; Vivas *et al.*, unpublished). The antioxidant activity

increased with necrosis length ($Z = -2.78$, $P = 0.005$) but in non-inoculated (control) seedlings the antioxidant activity did not differ if seeds were collected from the favourable or the unfavourable maternal environments (27 ± 1 and 27 ± 2 %, respectively). A significant interaction between *maternal environment* × *necrosis length* was shown and it means that the increase of antioxidant activity with necrosis length differed between maternal environments ($Z = -5.96$, $P < 0.001$) (Figure 1).

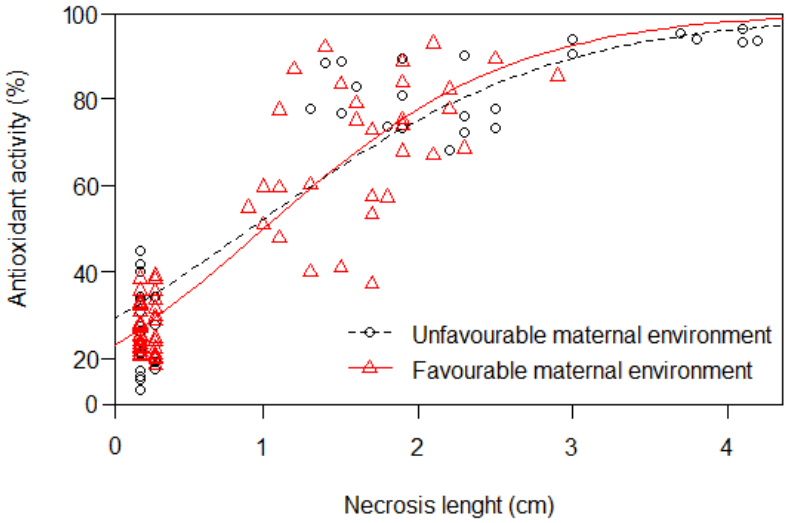

Figure 1. Antioxidant activity of *Pinus pinaster* seedlings from unfavourable (circles, dashed line) and favourable (triangles, continuous line) maternal environments as function of necrosis length caused by water (samples with a necrosis length between 0.2 and 0.3 cm) and by *Fusarium circinatum* (samples with a necrosis length higher than 0.5 cm) inoculations.

The beta regression model indicated that *maternal environment*, *necrosis length* and *maternal environment* × *necrosis length* interaction contributed significantly to the antioxidant activity response of the seedlings. If little necrosis occurred, seedlings from the unfavourable environment showed higher antioxidant activity than seedlings form the favourable environment; on the contrary, if large necrosis occurred, the opposite trend was observed. It seems that seedlings from the favourable environment were able to develop a more efficient antioxidant system, which is down-regulated facing low damage by the pathogen and up-regulated if extensive damage has been caused.

Physical environmental factors may influence plant metabolism in such a way that antioxidant activity changes (Smirnoff, 1993). Results confirm the increasing evidence that environmental maternal effects are involved in the transgenerational plastic responses of plants to stress conditions (Boyko and Kovalchuk, 2011). More maternal environments, clones and fungal isolates should be tested to strengthen the results obtained and to quantify genetic variation of plant responses.

In summary, maternal effects significantly regulated the antioxidant activity of *P. pinaster* challenged with *F. circinatum*, in the way that low activity if the damage is little, and high activity if the damage is large benefits the tree against the pitch canker disease. The antioxidant activity measured included compounds of the range of carotenoids, phenolics, and some other plasma antioxidants (Re *et al.*, 1999). Further studies may be conducted to measure individual antioxidants which may give more biologically relevant information than that obtained from measuring total concentrations.

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