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Fusarium circinatum – host interaction: ecological and molecular aspects of the
pathogenic and endophytic association

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A mi familia y amigos

“Every daring attempt to make a great change in existing conditions, every lofty vision of new possibilities for the human race, has been labeled Utopian”

“Cada intento de hacer un gran cambio en las condiciones existentes, cada noble visión de nuevas posibilidades para la raza humana, ha sido etiquetado como utopía”

Emma Goldman

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ABREVIATIONS

ABA: abscisic acid

ACC: 1-aminocyclopropane-1-carboxylic acid

ACO: 1-aminocyclopropane-1-carboxylic acid oxidase

ACO: allene oxidase cyclase

ACS: 1-aminocyclopropane-1-carboxylic acid synthase

AOS: allene oxidase synthase

APX: ascorbate peroxidases

ARF: auxin response factor

Aux/IAA: auxin/indole-3-acetic acid

BP: biological process

BR: biological replicate

BRs: brassinosteroids

CAT: catalase

CC: cellular compartment

CDPKs: calcium-dependent protein kinases

CDS: coding DNA sequences

CHS: chalcone synthase

CKs: cytokinins

COI1: coronatine Insensitive 1

CWDE: cell wall degrading enzymes

DE genes: differentially expressed genes

Dpi: days post-inoculation

EDS1: enhanced disease susceptibility 1

EIN2: ethylene insensitive 2

ET: ethylene

ETI: effector-triggered immunity

ETR: endoplasmic reticulum-associated receptors

ETS: effector-triggered susceptibility

FFC: Fusarium fujikuroi complex

FSM: Fusarium Selective Medium

GA: gibberellic acid

GA20ox: GA 20-oxidases
GA2ox: GA 2-oxidases
GFP: Green fluorescent protein
GH3: Gretchen Hagen 3 acyl acid amido synthetase
GO: gene ontology
GPX: glutathione peroxidases
HR: hypersensitivity response
IAA: auxin indol-3-acetic acid
IAM: indol-3-acetamide
ICS: isochorismate synthase
ICSH: isochorismatase hydrolase
ISR: induced systemic resistance
JA: jasmonic acid
JA-Ile: jasmonyl-isoleucine
JAZ: jasmonate ZIM
JMT: jasmonate methyl transferase
KO: ent-kaurene oxidase
KS: ent-kaurene synthase
LOX: lipoxygenases
LRR: leucin rich repeat
MAPKs: mitogen-activated protein kinases
MeIAA: methyl auxin indol-3-acetic acid
MeJA: methyl jasmonate
MeSA: methyl salicylate
MF: molecular function
NB: nucleotide binding
NCED: 9-cis-epoxycarotenoid dioxygenase
NGS: Next Generation Sequencing
NINJA: novel interactor of JAZ
NPR1: non-expressor of PR1
OPR: 12-oxo-PDA-reductase
PAL: phenylalanine ammonia lyase
PAMPs: pathogen-associated molecular patterns

PDA: Potato dextrose agar

PE: pair-end

PG: polygalacturonase

PK: protein kinase

PP2C: 2C protein phosphatases

PPC: Pine pitch canker

PR proteins: pathogenesis related proteins

PRRs: pattern recognition receptors

PTI: PAMP-triggered immunity

RLK: receptor-like kinases

RNA-seq: RNA sequencing

ROS: reactive oxygen species

RT-qPCR: reverse transcriptase-quantitative PCR

SA: salicylic acid

SAG: 2-O- β -D-glucoside

SAM: S-adenosyl-L-methionine

SAR: systemic acquired resistance

SAURs: small auxin up RNAs

SIR: systemic-induced resistance

SNA: Spezieller Nährstoffarmer agar

SOD: superoxide dismutase

SSR: microsatellite or simple sequence repeat

TIR1: transport inhibitor response 1

TLPs: thaumatin-like proteins

TPL: TOPLESS

UGT: UDP-glucosyltransferase

VCG: vegetative compatibility groups

ZEP: zeaxanthin epoxidase

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Resumen

Fusarium circinatum Nirenberg & O'Donnell es el agente causante de la enfermedad del chancro resinoso del pino, reconocida como una de las enfermedades de coníferas más graves, afectando a más de 57 especies de pino y causando importantes pérdidas económicas y medioambientales en viveros y plantaciones forestales. Tradicionalmente, *F. circinatum* ha sido considerado exclusivamente un patógeno de pinos, sin tener en cuenta la posibilidad de otras relaciones simbióticas con su huésped. Sin embargo, recientemente ha sido detectado colonizando como endófito especies pertenecientes a la familia de las gramíneas, sin causar daños aparentes. El objetivo principal de esta tesis es el estudio de los aspectos ecológicos y moleculares en la interacción de *F. circinatum* con sus huéspedes, explorando las distintas formas de vida del hongo, bien como endófito o como patógeno.

En el capítulo 4 de esta tesis exploramos el rango de huéspedes no sintomáticos de *Fusarium circinatum* en una parcela de *Pinus radiata* con síntomas de la enfermedad. También determinamos si estos huéspedes no sintomáticos pueden actuar como fuente de inóculo. *F. circinatum* fue detectado en 5 especies de familias pertenecientes al grupo de las dicotiledóneas (Asteraceae, Lamiaceae, Rosaceae), además de en dos especies de gramíneas (Poaceae). Sólo fue detectado en la parte aérea de estas plantas, por lo que describimos *F. circinatum* como un endófito de transmisión principalmente aérea. El hongo fue también detectado en semillas de la especie *Hypochaeris radicata*, por lo que la transmisión vertical podría ser posible, al menos en esta especie. Los hongos endófitos frecuentemente esporulan en el tejido senescente de plantas infectadas. En condiciones de laboratorio, hemos demostrado que las plántulas de pino pueden infectarse a partir de tejido senescente de plantas no sintomáticas pero colonizadas por el hongo.

También hemos explorado el endofitismo de *F. circinatum* en pinos adultos. Estudios previos han descrito que el hongo puede permanecer en estado de latencia en plántulas de pino. Además, el estudio de esta fase latente en el ciclo de la enfermedad ha estado centrado en la parte aérea del huésped, pero no en raíces. En el presente trabajo se detecta *F. circinatum* en raíces de árboles adultos no sintomáticos en plantaciones de *Pinus pinaster* y *P. radiata*, en las que el patógeno está presente y causa chancros en los árboles, síntoma característico de la enfermedad. El hongo fue aislado con una mayor frecuencia en raíces de árboles no sintomáticos de *P. radiata* que en árboles sintomáticos. Además, la presencia de *F. circinatum* en las raíces parece que estar influenciada por el grado de susceptibilidad de las especies, ya que no fue detectada en raíces de *P. pinaster*, especies con una moderada resistencia al patógeno.

Un análisis basado en marcadores moleculares de tipo microsatélite reflejó la presencia de un único haplotipo en toda la población de *F. circinatum*, independientemente de que el origen del aislado fuera chancros de pino, raíces de pinos asintomáticos o plantas herbáceas no sintomáticas. Por lo tanto, el mismo haplotipo del hongo puede adoptar una forma de vida endofítica o patogénica.

Además, todos los aislados resultaron ser patogénicos en plántulas de pino inoculadas artificialmente. Estos resultados evidencian el riesgo potencial de dispersión de *F. circinatum* a través de especies no sintomáticas que sirven como reservorio de inóculo y abren nuevos interrogantes para el estudio de la epidemiología de la enfermedad. Por otro lado, la colonización de raíces supone un factor importante en el ciclo de la enfermedad que debe ser tenido en cuenta para un correcto manejo de la enfermedad y, en particular, para las medidas de erradicación y control.

A pesar de la gravedad de la enfermedad del chancro resinoso del pino, el conocimiento de la base molecular de la interacción de *F. circinatum* con las distintas especies de pino es limitado. *P. pinaster* es una especie de gran importancia económica y medioambiental en la región mediterránea y, aunque han sido descritas infecciones naturales en plantaciones, la especie presenta una moderada resistencia al patógeno. Por este motivo, estudiamos la interacción *F. circinatum*-*P. pinaster* a nivel molecular (capítulo 6), tratando de identificar los procesos y rutas metabólicas que están involucrados en la resistencia y virulencia de planta y patógeno, respectivamente. Para ello, ante la ausencia de un genoma de referencia, creamos un transcriptoma *de novo* de buena calidad para *P. pinaster* (capítulo 5), que usaremos posteriormente como referencia para el análisis de expresión diferencial de genes. Mediante la técnica de secuenciación dual del ARN (dual RNA-seq) determinamos el perfil transcriptómico de ambos organismos, huésped y patógeno, durante el proceso de infección a los 3, 5 y 10 días post-inoculación (capítulo 6). La moderada resistencia que *P. pinaster* muestra puede ser explicada en parte por la rápida activación de la respuesta de defensa, basada en la inducción de proteínas PR (Pathogenesis Related proteins) y la activación de una compleja señalización basada en fitohormonas que incluye al ácido salicílico, ácido jasmónico y etileno. Además, indicamos los puntos claves en los que *F. circinatum* puede estar manipulando el balance hormonal de la planta a su favor, contribuyendo así a una mayor susceptibilidad del huésped. Tras examinar los transcritos del patógeno, proponemos que *F. circinatum* previene la biosíntesis del ácido salicílico a partir de la vía del corismato mediante la síntesis de la isocorismatasa hidrolasa (ICSH), perturba la homeostasis del etileno en el huésped mediante la expresión de genes relacionados con la biosíntesis del etileno y bloquea la señalización del ácido jasmónico mediante la supresión de COI1.

En resumen, este trabajo estudia diversos aspectos de la interacción de *F. circinatum* con sus huéspedes y evidencia la importancia de una fase endofítica en el ciclo de vida del patógeno, que consecuentemente tiene implicaciones en el manejo de la enfermedad hasta ahora no consideradas. Además, exploramos los mecanismos moleculares involucrados en la interacción patogénica de *F. circinatum* con *P. pinaster*, analizando qué procesos moleculares y rutas metabólicas son responsables de la defensa de la planta y de la virulencia del patógeno, aportando nuevos conocimientos sobre los mecanismos por los cuales se produce la enfermedad.

Summary

Fusarium circinatum Nirenberg & O'Donnell, the causal agent of pitch canker disease, is one of the most important pathogens of conifers worldwide, affecting at least 57 species of *Pinus*, and causing important economic and environmental losses in nurseries and forest plantations. The fungus has been traditionally considered exclusively a pathogen of conifers, not taking into account the possibility of other symbiotic relationships with the host. The fungus was recently detected living as an endophyte in grasses causing no apparent damage. The main objective of this dissertation is to study ecological and molecular aspects of *F. circinatum* and its host association, exploring both endophytic and pathogenic lifestyles.

In Chapter 4 we explored the non-symptomatic host range of the fungus in a plantation of *Pinus radiata* with symptoms of pine pitch canker (PPC) disease and we also tested whether this non-symptomatic host may act as a reservoir of inoculum. We detected the fungus in five species of dicot families (Asteraceae, Lamiaceae, Rosaceae), in addition to two species in the Poaceae. The fungus was found in the aerial part of non-symptomatic hosts, so we describe *F. circinatum* as an endophyte that is mainly transmitted by spores through the air. Vertical transmission may also occur at least in one species, *Hypochaeris radicata*, since fungus was detected in seeds of these plants. Endophytes frequently sporulate when infected host tissue dies, and we proved that pine seedlings can be infected from senescent tissue of non-symptomatic hosts colonized by the fungus under laboratory conditions.

This endophytic stage of *F. circinatum* is not exclusive of herbaceous plants, since the existence of a latent stage within pine host tissue has previously been cited. However, studies on this cryptic phase in the disease life cycle have only been focused on the host aerial part but not on the roots. We analysed the presence of the *F. circinatum* in roots of non-symptomatic mature trees in *Pinus pinaster* and *P. radiata* plantations, where the pathogen is known to be causing canker symptoms. The fungus was isolated from roots of non-symptomatic *P. radiata* trees in a higher frequency than from roots of symptomatic trees. Host susceptibility seems to be involved in the presence of the pathogen in roots because it was not isolated from any roots in the *P. pinaster* plantation, pine species reported to have moderate resistance to the pathogen.

An analysis of microsatellite markers showed a unique haplotype in *F. circinatum* population regardless of whether the isolates origin was pine cankers, symptomless pine roots or non-symptomatic herbaceous plants. Thus, the same haplotype can adopt a pathogenic or endophytic lifestyle. Indeed, all isolates resulted pathogenic to pines by artificial inoculations. These results show the potential risk of spread of *F. circinatum* across non-symptomatic species that serve as reservoirs of inoculum and provide insights into the epidemiology of pitch canker disease. In addition, root infection is a relevant factor in the disease cycle, and this finding needs to be considered hereafter in management of the PPC disease.

In spite of the importance of the disease, knowledge of the molecular basis of the pathogenic interaction of *F. circinatum* with *Pinus* trees is limited. *P. pinaster* is an ecological and economical important species in the Mediterranean area and, although natural infections have been reported, the species have shown a moderate resistance to the pathogen. For these reason we study *F. circinatum*-*P. pinaster* interaction at a molecular level, trying to determine which mechanism are strongly involved in plant resistance and pathogen virulence. For this purpose, in the absence of a reference genome, we generated a high quality *de novo* *P. pinaster* transcriptome assembly (Chapter 5), used for downstream gene expression analyses. By a dual RNA-sequencing approach, we determined the expression profiling of both organisms during the interaction at 3, 5 and 10 days post-inoculation (Chapter 6). The moderate resistance that *P. pinaster* showed at the early time points, may be explained, at least in part, by the early activation of defence response based on the induction of pathogenesis-related proteins and the activation of complex phytohormone signalling that involves crosstalk between salicylic acid, jasmonic acid and ethylene. Moreover, we hypothesize the key steps where the pathogen could be manipulating host phytohormone balance to its own benefit, contributing to host susceptibility. Upon examination of the pathogen transcripts, we propose that *F. circinatum* prevents salicylic acid biosynthesis from the chorismate pathway by the synthesis of isochorismatase family hydrolase (ICSH) genes, perturbs ethylene homeostasis in the host by expression of genes related to ethylene biosynthesis, and could be blocking jasmonic acid signalling by COI1 suppression.

In summary, this work present a broader repertoire of interaction of *F. circinatum* with its hosts and evidence the importance that an endophytic stage of the pathogen may have in the disease lifecycle, and therefore, for disease management. Indeed, we explore the molecular mechanism involved in the pathogenic interaction of *F. circinatum* with *P. pinaster*, analysing which processes are responsible for plant defence and pathogen virulence, providing new knowledge in mechanisms by which PPC is caused.

Chapter 1: GENERAL INTRODUCTION

1.1. Lifestyles in plant-fungal interactions

In plant-fungal interaction research, emphasis has been placed on plant pathogens and their outcome (Stergiopoulos and Gordon, 2014), as well as on arbuscular mycorrhizae and on endophytes (Zabalgogea, 2008; Rodriguez *et al.*, 2009; Hardoim *et al.*, 2015). Traditionally, a fungus that causes symptoms in the host was defined exclusively as a pathogen, not taking into account the possibility of an endophytic lifestyle in other hosts (i.e., colonizing tissue of living plants without causing symptoms). However, there are a growing number of examples in the literature where a fungal species can be pathogenic to some plants while only endophytic in others (Zabalgogea, 2008; Hardoim *et al.*, 2015), even in plants of non-related families (Redman, Dunigan and Rodriguez, 2001). It is also recognized that some fungi can infect endophytically a specific host for a period of time, and then cause symptoms (Bacon and Hinton, 1996; Photita *et al.*, 2004; Hardoim *et al.*, 2015). Consequently, many of the fungi described only as pathogens have a wider ecological interaction with their host, including the capacity to infect and colonize without causing any visible damage.

Endophytes are defined as organisms living inside plants that exhibit no visible symptoms as a result of this colonization (Bacon and White, 2000). However, this definition does not specify a functional relationship with their hosts, which has been categorized in mutualism (both partners benefit from the association) and commensalism (where only one partner benefits). The most common endophytes are commensals (Hardoim *et al.*, 2015) probably because they have unknown roles in plants. However, evidence showing that endophytes have a functional role in their hosts has been increasing in recent years.

Mutualistic fungi can confer fitness benefits to the host under abiotic and biotic stress conditions such as tolerance to drought and metals, disease resistance, enhanced nutrient acquisition, increased shoot and root biomass and growth promotion (Rodriguez *et al.*, 2009; Redman *et al.*, 2011; Hardoim *et al.*, 2015). At the same time the fungus receive protection and nutrients from the host plant (Bacon and Hill, 1996). An example is the case of *Fusarium culmorum* and the dune grass *Leymus mollis*; the plant is not able to survive under temperature and salt stress and the fungus growth is limited when is not living inside plant (Rodriguez *et al.*, 2008).

Some fungal endophytes are known to produce secondary metabolites with an inhibitory effect in plant pathogens and herbivores such as alkaloids, terpenoids, sterols, flavonoids, fenols and quinols (Siegel *et al.*, 1990; Higginbotham *et al.*, 2013; Tejesvi *et al.*, 2013). Tanaka *et al.*, (2005) demonstrated that the fungal metabolite peramine confers protection to plants from herbivores. Some endophytes are also able to induce disease systemic resistance, such as *Diplodia sapinea* and *Diplodia scrobiculata* when infecting *Pinus nigra* (Blodgett, Eyles and Bonello, 2007) or *Fusarium oxysporum* in banana against *Radopholus similis* (Vu, Hauschild and Sikora, 2006).

Historically, two endophytic groups (clavicipitaceous and nonclavicipitaceous) have been discriminated based on phylogeny, taxonomy, plant hosts, and ecological functions (Rodriguez *et al.*, 2009). The clavicipitaceous endophytes include *Neotyphodium* species and their *Epichloë* teleomorphs which constitute the most intensively studied group of endophytes. They produce chemicals that are toxic to

animals and decrease herbivory and increase drought tolerance (Schardl, Leuchtman and Spiering, 2004). Endophytes in this group colonize systemically the intercellular space of their hosts infecting leaves, stems and seeds, and thus, they are vertically transmitted to host progeny via seed infection.

The non-clavicipitaceous endophytes constitute a wider group that includes fungi recovered from asymptomatic tissues of non-vascular plants, ferns, conifers, and angiosperms (Rodriguez *et al.*, 2009). It contains fungi capable of infecting the plant extensively or limited either to the above-ground or below-ground tissues (Stone, Polishook and White Jr, 2004; Zabalgogazcoa, 2008). Endophytic fungi in this group can be transmitted horizontally, this means from plant to plant, and/or vertically. Sporulation within the life tissue is limited or absent whereas endophytes use to sporulate in senescent tissue (Sánchez Márquez, Bills and Zabalgogazcoa, 2007; Zabalgogazcoa, 2008), which can represent an important source of inoculum favouring the infection of surrounding plants and hence, horizontal transmission. Generally, horizontal transmission allows colonization by a variety of fungi that can adopt different symbiotic lifestyles, while vertically transmitted fungi are strongly linked to the host and mutualism is selected (Rodriguez *et al.*, 2009).

Categories in which plant-fungi interactions have been defined (parasitism, mutualism and commensalism) do not reflect the continuum of this interaction (Saikkonen *et al.*, 1998; Schulz and Boyle, 2005), which can result in different outcomes. Recent studies have shown that fungi may adopt different lifestyles depending on biotic and abiotic factors, including genotype of both host and fungus, the environmental conditions and the whole microbiome inside the plant (Saikkonen *et al.*, 2004; Rodriguez *et al.*, 2009; Rai and Agarkar, 2016). Consequently, interaction between plants and fungi may range from mutualism, commensalism to parasitism (Redman, Dunigan and Rodriguez, 2001). The factors by which a fungus switches from an endophytic lifestyle to a parasitic lifestyle, as well as mechanisms that regulate the response of plants to fungi require more research.

F. circinatum is a fungus that presents duality in its lifestyle, colonizing different hosts or even the same host, either as a pathogen or as an endophyte. The work presented in this dissertation try to elucidate the ecological and molecular aspects involved in the outcome of the interaction.

1.2. *Fusarium circinatum* Nirenberg and O'Donnell

Fusarium circinatum Nirenberg and O'Donnell, anamorph of *Gibberella circinata* Nirenberg and O'Donnell, belongs to the Ascomycota Filum, Subfilum Pezizomycotina, Class Sordariomycetes, Subclass Hypocreomycetidae, Order Hypocreales and Family Nectriaceae. The taxonomic background of the species has been complex and, therefore, the name has varied along the last years based on morphological and molecular studies. The fungus was originally named in 1949 as *Fusarium lateritium* f. sp. *pini* Hepting (Snyder, Toole and Hepting, 1949), renamed in the 1970s as *F. moniliforme* Sheldon var. *subglutinans* Wollenweber (Kuhlman, 1982) and then raised to the species level in 1983 *F. subglutinans* (Wollenweber & Reinking) Nelson (Nelson, Toussoun and Marasas, 1983). In 1992 the strain *F. subglutinans* f. sp. *pini* was

assigned, based on restriction fragment length polymorphism (RFLP) patterns of mitochondrial DNA and host specificity (Correll, Gordon and McCain, 1992). It was in 1998 when Nirenberg and O'Donnell finally described the asexual stage of the fungus as *Fusarium circinatum* and the sexual as *Gibberella circinata*.

Regarding its morphology, sporodochia with macroconidia are sparse and difficult to find. Macroconidia are relatively thin, with three or four septum, curved in the apical size and relatively poorly developed in the basal size. Microconidia are ovoid or oval, without septum, present in aerial mycelium, commonly aggregated in false heads, with branched mono- and polyphialidic conidiophores. Chlamydospores are absent. *F. circinatum* differs from other *Fusarium* species in coiled sterile hyphae, also known as 'circinate' hyphae, characteristics of the species (Figure 1).

Identification relies on isolation and culturing of the fungus. For species isolation, *F. circinatum* is cultured in a *Fusarium* semi-selective medium (FSM) which consists of peptone agar supplemented with streptomycin, neomycin and pentachloronitrobenzene (PCNB) (Aegerter and Gordon, 2006). Aerial mycelium is white cottony or slightly violet in the middle in Potato dextrose agar (PDA) medium. For morphological identification, the fungus is cultured in Spezieller Nährstoffarmer Agar (SNA) medium (Nirenberg, 1981), and then microscopically inspected for the formation of coiled sterile hyphae (circinate) (Figure 1.B) characteristic of *F. circinatum* (Nirenberg and O'Donnell, 1998). Molecular methods based on PCR are also available for a fast and reliable identification of the species. Amplification of a IGS (inter genic spacer) region using specific primers CIRC1A (CTTGGCTCGAGAAGGG) and CIRC4A (ACCTACCCTACACCTCTCACT), as described by Schweigkofler *et al.*, (2004), produces a 360 bp amplicon for *F. circinatum*. Same primers can be also used in a SYBR green real-time PCR reaction. Method for real-time PCR with primers and a dual-labelled probe has been also designed for fungal identification (Ioos *et al.*, 2009).

Figure 1. A: *Fusarium circinatum* growing in potato dextrose agar. B: 'Circinate', coiled and sterile hyphae. C: Sporodochia. D: Macro and microconidia.

F. circinatum can reproduce either asexually or sexually, although sexual reproduction has only been described under *in vitro* experiments and not in nature (Britz, Wingfield and Leslie, 1998; Steenkamp *et al.*, 2000). The fungus is heterothallic and mating is controlled by a single locus with two idiomorphs, MAT1 and MAT2 (Wallace and Covert, 2000). Sexual reproduction is also dependent on female fertility, capable of producing sexual fruiting structures upon fertilization (Correll, Gordon and McCain, 1992).

1.2.1. *Fusarium circinatum* as a pathogen: Pine pitch canker disease

As a pathogen, *F. circinatum* is the causal agent of pine pitch canker disease (PPC), which is recognized as one of the most important diseases of conifers worldwide, affecting at least 57 species of *Pinus* as well as *Pseudotsuga menziesii* (Wingfield *et al.*, 2008). Plants can be affected at any stage of development, from seedling to mature tree, causing important economic and environmental losses in forest plantations and nurseries. Most of the commercial pine species are especially susceptible to the pathogen, causing a high economic impact due to reduction of growth or tree mortality.

In mature trees, the most common symptom is the appearance of large cankers in the main trunk and lateral branches with abundant production of resin (Figure 2. B, G). Wilting and discolouration of needles at the branch tip also occurs due to the obstruction of the water flow by girdling cankers on branches and eventually results in branch dieback (Figure 2. A, C, D, E) (Wingfield *et al.*, 2008; Gordon, Swett and Wingfield, 2015). Wilted needles become necrotic and turn from green to red and brown colour, and abscission tends to occur leaving the branch tip naked (Figure 2. A, C). Multiple infections within the tree usually occur. Young tissue is more susceptible to the infection than old and lignified tissue. The fungus can also affect vegetative structures, since female strobili and mature cones can be infected (Figure 2. E) (Barrows-Broadus, 1990). Infected cones are misshaped and stunted and often abort before reaching maturity. Seeds can be infected either in the surface or the inner tissue, and are thought to be an important source of inoculum in nurseries.

Infected seeds can result in pre- and post-emergence damage, as well as mortality of seedlings. Infected seedlings show wilting and girdling at the collar region, which often results in damping-off (Figure 2. H). Roots of infected seedling are frequently underdeveloped and show necrosis at the cortex (Viljoen, Wingfield and Marasas, 1994). However, infected seedlings can grow, resulting in asymptomatic infection (Storer, Gordon and Clark, 1998). Colonization of *P. radiata* seedlings by a green fluorescence protein (GFP)-tagged strain of *F. circinatum* has been described (Martín-Rodrigues *et al.*, 2013). The pathogen grows in the intercellular spaces of the cortex and then it moves radially to the pith via medullary rays. It moves vertically through the inner stem by the xylem and via cortex and phloem through the stem periphery. *F. circinatum* is able to complete its cycle inside the host by producing conidiophores in the cavities of the pitch. The fungus is also able to colonize resin ducts, and it tolerates oleoresin and even induces its production. Disruption of water flow due to collapse of the xylem and resin production ultimately leads to plant death. When infecting roots, abundant sporulation happens in the surface of the root as well as inside tracheids, and from here the fungus can move to the aerial part of the plant (Martín-Rodrigues *et al.*, 2015).

Susceptibility to the pathogen varies widely between different *Pinus* species (Hodge and Dvorak, 2000; Wingfield *et al.*, 2008; Iturrutxa *et al.*, 2012). The most susceptible species are *Pinus patula* and *P. radiata*, whereas at the other end of the spectrum are *P. thunbergii*, *P. canariensis* and *P. pinea*. Even for species regarded as susceptible, the degree to which individuals support development of pitch canker can vary widely (Gordon, Swett and Wingfield, 2015). Furthermore, species known to be relatively resistant can develop a high incidence of disease under stress environmental conditions or under inoculum pressure (Lopez-Zamora *et al.*, 2007).

Figure 2. Pitch canker disease symptoms in a naturally infected *Pinus radiata* plantation (A-G). A: detail of discoloured tip of branches. B, G: detail of cankers with abundant resin in the main stem of the tree. C-D: affected trees with cankers in the main stem and discoloured needles in the branches. E: broken branch at the infection site; infected cone. F: main stem broken at the canker point H: Seedling with symptoms of disease in the shoot tip and needles.

The disease was first recorded in United States in 1946 (Hepting and Roth, 1946) and then spread through South-Eastern US and the coastal region of California (Dwinell, Barrows-Broadbent and Kuhlman, 1985; McCain, Koehler and Tjosvold, 1987). To date it has been reported in Haiti (Hepting and Roth, 1953), South Africa (Viljoen, Wingfield and Marasas, 1994), Japan (Kobayashi, 1989), Korea (Lee *et al.*, 2000), Mexico (Britz *et al.*, 2001), Chile (Wingfield *et al.*, 2002), Uruguay (Alonso and Bettucci, 2009) and Colombia (Steenkamp *et al.*, 2012) (Figure 3). In Europe, the disease was officially reported for the first time in Spain in 2005 (Landeras *et al.*, 2005) and thereafter in France ('France: EPPO', 2006), Italy (Carlucci, Colatruglio and Frisullo, 2007) and Portugal (Bragança *et al.*, 2009), although in Italy and France are now eradicated. *F. circinatum* was added to the European Plant Protection Organization (EPPO) A2 action list of pests (pests locally present in the EPPO region) and is regulated as a quarantine pest in the EU, as specified in the emergency measures (Commission Decision 2007/433/EC). In Spain, since the first appearance in Asturias in 2005, the fungus has been reported throughout the Atlantic coast (see for example occurrence in 2014, Figure 4) representing a serious threat to *P. radiata* plantations. Consequently, a national program for eradication and control of *F. circinatum* in Spain was defined (RD 637/2006 and modified in RD65/2010) which establishes legislative and administrative procedures to prevent spread within Spanish territory and contains essential elements of a contingency plan.

Figure 3. Map of global distribution of *Fusarium circinatum*.

Figure 4. Distribution of *Fusarium circinatum* in Spain in 2014. Brown: affected municipalities; green: municipalities where the pathogen has been eradicated. Source: Ministerio de Agricultura, Alimentación y Medio Ambiente; November 2014.

F. circinatum can be dispersed through spores by the wind, water splash, insect vectors and movement of infected plant material (Wingfield *et al.*, 2008). In plantations, mechanical damage caused by branch removal and harvested cones as well as weather related injuries such as wounds made by wind damage to the tree, can be the cause of natural infections by airborne spores deposition in the wound. The type and age of the wound have been shown to significantly influence in successful colonization, being fresh and deep wounds and the presence of water favourable factors for the infection (Dwinell, Barrows-Broadus and Kuhlman, 1985; Inman *et al.*, 2008). Although wounds constitute important sites of infection, *P. radiata* branches can also be infected without wounding when spores are deposited on either succulent or lignified tissue (Swett, Reynolds and Gordon, 2018). In this case, lesion occurs on branches at the point of needle attachment suggesting the site of pathogen entry. From tree to tree, the fungus can be moved by aerial dispersion of the spores or by insect vectors (Gordon, Storer and Wood, 2001; Schweigkofler, O'Donnell and Garbelotto, 2004). Insects can be both vector of *F. circinatum* or wounding agents on trees (Blakeslee *et al.*, 1978) and spread of pitch canker in California has been strongly correlated to insects. In Spain Romón *et al.*, (2008) reported the association of *F. circinatum* with several insects, suggesting a possible role in the epidemiology of the disease in Europe.

Pine infected seeds are also a potential source of inoculum in nurseries (Storer, Gordon and Clark, 1998; Wingfield *et al.*, 2008; Elvira-Recuenca, Iturrutxa and Raposo, 2015), causing pre- and post-emergence damping-off damage. Infected seeds are also considered one of the most important pathways for long distance dispersal of the fungus, and it is thought to be the major pathway of introduction into new counties. Infected seeds result in symptomatic or even symptomless seedlings, and these symptomless but infected seedlings can constitute a vehicle for disease dissemination into new forest plantations. The mechanism by which *F. circinatum* infects seed is unknown. In *P. radiata*, superficial infestation was found in seeds coming from symptomless branches, whereas internal colonization of the seed was found in seeds from healthy cones but infected branches (Storer, Gordon and Clark, 1998), suggesting

symptomless growth of the fungus through the cone into the seed. Several physical and chemical treatments have been applied to pine seeds trying to control seed infection (Agusti-Brisach *et al.*, 2012). However, due to the colonization of the fungus in the inner part of the seed, no absolute means for controlling the infection has been found (Storer, Gordon and Clark, 1998).

Figure 5. Dissemination pathways of *Fusarium circinatum*.

Although the infection can occur at any moment of the year, fungal infection is also influenced by climatic conditions, especially by ambient temperature and moisture availability. Germination and growth of the pathogen is optimal around 25°C and very slow under 10°C (Inman *et al.*, 2008). However, in infected plantations spores are detected throughout the whole year (Garbelotto, Smith and Schweigkofler, 2008; Dvořák *et al.*, 2017) and spores were found at a distance larger than 200 m, suggesting midrange aerial dispersal (Garbelotto, Smith and Schweigkofler, 2008).

Risk maps describe the potential distribution of a disease based on the probability of disease establishment. The potential global distribution of PPC disease on *Pinus* spp. was predicted (Ganley *et al.*, 2009) using the model CLIMEX (Sutherst and Maywald, 1985) as a function of climate using a global climate database (New, Hulme and Jones, 1999). Predictions of this model fit well with areas where the disease was present. According to CLIMEX, climatic conditions in northern Spain are ranged as suitable or optimal. It also predicts suitable climate for other regions where the disease has not been reported. A Species Distribution Model of PPC was developed with a higher resolution in the Spanish Iberian Peninsula (Serra-Varela *et al.*, 2017) based on the occurrence of PPC recorded in 159 municipalities and a risk map was defined (Figure 6). Occurrence of PPC was modelled as a function of climatic, topographic variables and distance to the coast, showing that the climatic variables more relevant in the PPC

distribution are minimum temperature of coldest month, annual precipitation and precipitation of driest quarter. Short distance to the coast resulted also a relevant variable to explain presence of PPC in the Spanish Iberian Peninsula.

Figure 6. Risk percentage of PPC occurrence as defined by a species distribution model (From Serra-Varela et al., 2017).

Management of pitch canker disease is based on cultural, biochemical and biological control, as well as quarantine measures. In spite of the effort done in control measures, the pathogen has not been eradicated from nurseries and forest plantation and often results in significant economic and environmental losses. Therefore, selection of genetically resistances genotypes against *F. circinatum* seems to be the most optimistic approach for disease management (Gordon, Swett and Wingfield, 2015).

1.2.2. *Fusarium circinatum* as an endophyte in pines

Several studies have described the existence of a latent stage of *F. circinatum*, within the host tissue of pine seedlings without causing visible damage (Elvira-Recuenco, Iturrutxa and Raposo, 2015; Martín-Rodrigues *et al.*, 2015; Swett, Kirkpatrick and Gordon, 2016). *F. circinatum* has been reported to remain in this symptomless stage in *P. radiata* seedlings for up to 2 years (Swett and Gordon, 2017). Similarly, Elvira-Recuenco *et al.* (2015) detected the fungus in asymptomatic *P. radiata* seedlings up to 475 days post-inoculation. The pathogen was always detected in the roots of seedlings, but its population was lower in the roots of symptomless seedlings than in those showing symptoms. Further, Swett *et al.* (2016) established that shoot symptoms only develop when fungal colonization reaches the root collar, and plants remained symptomless if the fungus stayed only in the roots.

Many fungi that establish symptomless associations with their hosts are obligate biotrophs or hemibiotrophs (Stergiopoulos and Gordon, 2014). In the case of *F.*

circinatum this association with *P. radiata* have been described as hemibiotrophic, maintaining an initial biotrophic relationship with living host cells followed by a necrotrophic one (Swett, Kirkpatrick and Gordon, 2016). Martín-Rodrigues *et al.*, (2015) described a possible biotrophic phase of *F. circinatum* during root penetration of *P. radiata* seedlings, based on the formation of bulbous and narrow hyphae inside epidermal cell during root colonization, which resemble specialized hyphae associated with biotrophic fungal invasion.

The role of *F. circinatum* as an endophyte in pines is not clear. However, there are some studies that evidence the possibility of a role in enhancing plant fitness. Swett and Gordon, (2017) reported that *P. radiata* seedlings grown in infected sand have larger root and shoot biomass as well as an increase in root-associated mycorrhizas. Indeed, plants previously exposed to *F. circinatum* expressed elevated resistance to posterior infections (Bonello, Gordon and Storer, 2001; Swett and Gordon, 2017).

In addition, cryptically infected seedlings can constitute a vehicle for dissemination of the disease, especially from nurseries to plantations. These infected but symptomless seedlings may develop disease symptoms after planted in a new plantation. Indeed, the stress produced during transplanting of seedlings can make the plant more susceptible for the fungus and then produce disease (Mitchell *et al.*, 2012; N. B. Jones *et al.*, 2014), which may have important implications in the epidemiology and disease management. Furthermore, persistence of *F. circinatum* in the apparently healthy tissues of host trees makes particularly difficult to formulate or impose measures to control movement of the fungus to new environments.

1.2.3. *Fusarium circinatum* as an endophyte in alternative hosts

F. circinatum has been always considered a pathogen of conifers; however, the fungus has been recently detected colonizing grasses (family Poaceae) as an endophyte, without inducing symptoms. These grasses were growing beneath the canopy of a pine plantation with symptoms of pitch canker disease in South Africa (Swett *et al.*, 2014) and California (Swett and Gordon, 2012). Swett *et al.*, (2014) reported the presence of the fungus in grass species including *Briza maxima*, *Ehrharta erecta* var. *erecta*, *Pentameris pallida* and an unidentified species. *F. circinatum* is also able to colonize endophytically corn under laboratory conditions by growing in the intercellular space in the cortical and epidermal tissue and without inducing damage. The fungus colonizes roots, stem and developing ears of *Zea mays*, when seeds were inoculated (Swett and Gordon, 2015). Several species of the genera *Fusarium* have the capacity to colonize asymptotically grasses. The close and interfertile species with *F. circinatum*, *F. subglutinans*, is also able to colonize corn, as well as other phylogenetically related species of the same genera such as *F. verticillioides*. Therefore, the presence of the fungus in grasses is not surprising. However, it is possible that the host range of the fungus can be even larger than thought and may include other species which differ from conifers and grasses. Swett and Gordon, (2015) hypothesized that *F. circinatum* may be a commensal associated of grass hosts and that

pathogenesis on susceptible pines may be incidental. However, there is not solid evidence for this hypothesis.

Whether *F. circinatum* is an endophyte in grasses with a role in improving fitness of the host or whether conferring resistance to pathogens, is unknown. What is known is that grass-associated isolates were all pathogenic to pines after stem inoculation, with similar lesion length to the one of a known virulent isolate (Swett *et al.*, 2014). Thus, grasses may facilitate dissemination of the fungus in nurseries and may constitute a source of inoculum for new plantations. However, the risk will depend on the capacity of *F. circinatum* to sporulate in grass hosts that can facilitate the dispersion from infected to uninfected pines in nurseries and plantations. If infected grasses constitute a new source of inoculum and the implication of this alternative hosts to pines in the epidemiology of the disease needs to be determined. Insight into these questions will improve the knowledge of the endophytic stage of *F. circinatum*.

1.3. *Pinus* spp.

Coniferous forests dominate large ecosystems in the Northern Hemisphere and are the main source for timber and paper production worldwide, being of great economic and environmental importance. In Spain, conifers occupy 6,403,653 ha, which means the 34.5 % of the total forestry surface. Regarding *Pinus* genus, the most common species is *P. halepensis*, which occupies 21 % of the total area populated by conifers, followed by *P. sylvestris* (10.5 %), *P. pinaster* (7.5 %), *P. sylvestris* (6.5 %), *P. nigra* (3.4 %), *P. pinea* (2.1 %), *P. uncinata* (0.5 %) and *P. canariensis* (0.4 %), as well as forests where diverse *Pinus* species are mixed (source: MAGRAMA, 3º I.F.N., 2009). *P. radiata* is the only exotic species, introduced in northern Spain in the nineteenth century, and currently occupies 287,771 ha. The most productive plantations in Spain are found in the north, along the Atlantic coastal zone, and are composed mostly of pines (*P. pinaster* and *P. radiata*) and eucalypts (*Eucalyptus globulus* and *E. nitens*). Most of the Spanish plantations of *P. radiata* are grown in this zone, whereas many of the native forests of *P. pinaster*, although present in this zone, are located in north-western Spain (Serrada, Montero and Reque, 2008). The forestry industry in north-western Spain, based on the production of solid timber and pulp products, has a positive effect on the economy of the region.

Resistance or susceptibility to *F. circinatum* varies among the different *Pinus* species. As mentioned above, the exotic commercial and broadly planted *P. radiata* represents one of the most susceptible species, whereas the native *P. pinaster* has shown a moderate resistance to *F. circinatum* (Iturrutxa *et al.*, 2011, 2012). However, natural infections have been also reported in northern Spain where disease pressure is high (Iturrutxa *et al.*, 2013). *P. pinaster* is of highly economic importance due to its use for timber production, pulp, paper making industry and resin for turpentine and rosin production, besides its ecological importance as a native species in Europe. Therefore,

the presence of the fungus in the area represents a serious threat for the species, especially in the Atlantic coast of Spain, Portugal and France.

Pinus pinaster Ait. is commonly known in Europe as maritime pine. It is original from the western Mediterranean Basin and is distributed in the Iberian Peninsula, South France, West Italy, Western Mediterranean isles, North Morocco, Algeria and Tunisia (Figure 7). *P. pinaster* is a fast-growing, medium-sized (20 – 35 m) coniferous tree, with an orange-red bark, thick and fissured at the base of the trunk. Needles are in pairs, thick, large and blue-green to yellow-green colours. Cones are conics and 10-20 mm large. Maritime pine is an ecologically versatile species and is present in a broad range of elevations, climates and soils, showing a high genetic variation as a result. Elvira-Recueno *et al.* (2014) reported that this species shows a moderate to high genetic variation in response to *F. circinatum* in different *P. pinaster* populations. The moderate resistance that *P. pinaster* shows against the pathogen in comparison to other pine species such as *P. radiata* and *P. patula* makes this species an excellent model for determining which mechanisms and metabolic pathways are responsible for resistance of *Pinus* to *F. circinatum*.

Figure 7. Map of *Pinus pinaster* distribution in Europe. Source: Distribution map of Maritime pine (*Pinus pinaster*) EUFORGEN 2009, www.euforgen.org.

1.4. Molecular processes involved in pathogen-host interaction

Plants are able to protect themselves against pathogens in such a way that only a small fraction of fungi have the capacity to cause disease (Heath, 1997). In this sense, plants are generally resistant to most pathogens. Since plants are sessile, they rely on their immune system to detect a pathogen and to respond to it. One of the most important challenges in phytopathology is to determine how pathogens are recognized by their hosts and how is established a resistant or susceptible interaction between them. At molecular level, this interaction involves the activation of multiple biochemical

pathways, phytohormone regulation, transcription factors activation, which ultimately leads to the reprogramming of gene expression.

Nowadays, the understanding of this interactions has raised to the development of a model, known as zig-zag model, of the function and evolution of the immune system (Jones and Dangl, 2006). Typically, plants recognize the fungus by detection of pathogen-associated molecular patterns (PAMPs), which depends on proteins known as pattern recognition receptors (PRRs) localized on the plant cell membrane. This first response based on the interaction between PAMP-PRR is called PAMP-triggered immunity (PTI), and in some cases, this response is enough to prevent pathogen colonization. Other group of pathogens (successful pathogens) secrete effectors, which contribute to virulence by suppressing basal defence and giving name to the effector-triggered susceptibility (ETS). Nevertheless, plants have developed a more specialized mechanism to prevent infection and recognize this effectors by resistance proteins, either indirect or through direct binding to the NB-LRR (nucleotide binding - leucine rich repeat) domain, resulting in effector-triggered immunity (ETI) (Jones and Dangl, 2006; Veluthakkal and Dasgupta, 2010), which often ends with hypersensitivity response (HR) in the local site of infection (Greenberg and Yao, 2004). However, natural selection pressure drives pathogens to modify its effector or even acquire new one to suppress ETI. On the contrary, plants develop new resistance specificities so that ETI can be triggered again (Jones and Dangl, 2006).

1.4.1. Plant defence response

The first step in any infection is recognition of the pathogen by the host aimed at the inhibition of pathogen entrance. Host cell walls constitute a physical barrier for pathogen penetration and is generally based on an extensive network of cellulose micro-fibrils cross-linked with hemicellulose (Hématy, Cherk and Somerville, 2009; Scheller and Ulvskov, 2010; Pauly *et al.*, 2013; Malinovsky, Fangel and Willats, 2014). In the primary walls this network is embedded in a matrix of pectic polysaccharides, whereas in the secondary wall of mature tissue pectin is less abundant and walls are lignified (Miedes *et al.*, 2014). In response to a pathogen attack, plants induce modification in cell wall structure in order to difficult pathogen penetration and further infection (Malinovsky, Fangel and Willats, 2014). The accumulation of callose at infection site prevents the penetration of the fungus and makes the cell wall more resistant to enzyme degradation (Hückelhoven, 2007). Suberin is secreted by cells of the vascular parenchyma to prevent colonization of the vascular system, while cutin makes the cuticle stronger. Production of waxes on the surface difficults spore germination. Lignin is other of the main components of the cell wall, providing structural support and impermeability (Albersheim *et al.*, 2011; Sattler and Funnell-Harris, 2013).

Once the pathogen is detected, the early response of the plant consists of rapid ion fluxes across the plasma membrane, activation of mitogen-activated protein kinases (MAPKs), calcium-dependent protein kinases (CDPKs), production of reactive oxygen species (ROS) and induction of defence-related genes (Lecourieux *et al.*, 2002; Thatcher, Anderson and Singh, 2005). The accumulation of ROS in the infection site,

known as oxidative burst, contributes to regulation of HR, a form of fungal cell death, in order to detain nutrient availability and fungal infection (Thatcher, Anderson and Singh, 2005; Tanaka *et al.*, 2006; Heller and Tudzynski, 2011). ROS also acts as signalling molecules mediating defence gene expression either by redox control of transcription factors or by interacting with other signalling components such as phosphorylation cascades. Plants also have diverse enzymes responsible for controlling the levels of ROS, such as catalase (CAT), superoxide dismutase (SOD), peroxidases, ascorbate peroxidases (APX) and glutathione peroxidases (GPX), in order to reduce the high amount of ROS accumulated during the early oxidative burst and protect cells from the toxic effects (Heller and Tudzynski, 2011).

Pathogenesis related (PR) proteins are strongly induced by plants in response to pathogens (Van Loon and Van Strien, 1999). Chitinases and β -1,3-glucanases are well known PR proteins, belonging to the PR3 and PR2 families, respectively (Van Loon and Van Strien, 1999; Ebrahim, Usha and Singh, 2011; Balasubramanian *et al.*, 2012). They can act alone or in combination contributing to plant defence by degrading the cell wall of the fungus. Chitinases hydrolyzes chitin of the fungal cell wall, being chitin one of the most common PAMPs for fungal recognition in plants (Nürnberg *et al.*, 2004). Oligosaccharides derived from chitinase and β -1,3-glucanase activities act as elicitors of defence responses (Kurosaki, Tashiro and Nishi, 1988; Klarzynski *et al.*, 2002). Thaumatin-like proteins (TLPs), which belong to the PR5 family proteins, have antifungal activity, also related to the enzymatic capacity to degrade the cell wall of the fungus (Liu, Sturrock and Ekramoddoullah, 2010; Wang *et al.*, 2010). As an example, TLP of *Castanopsis chinensis* has shown antifungal activity against *Botrytis cinerea*, *F. oxysporum*, *Mycosphaerella arachidicola* and *Physalospora piricola* (Chu and Ng, 2003).

Plant hormones and their coordination and interaction play a crucial role in plant defence. Hormones initially described in this process were salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), as primary signals for induction of defence response (Pieterse *et al.*, 2012); but later studies have also indicated that abscisic acid (ABA), gibberellic acid (GA), auxins, cytokinins (CK), brassinosteroids (BR) and peptide hormones are also implicated in plant defence (Bari and Jones, 2009). SA is commonly involved in the activation of defence response against biotrophic or hemibiotrophic fungi and in the establishment of systemic acquired resistance (SAR), a long-lasting and broad-spectrum resistance in distal parts of the infection site (Ton *et al.*, 2002; Glazebrook, 2005; Truman *et al.*, 2007; Vlot, Dempsey and Klessig, 2009). SA is also involved in the expression of PR proteins. PR1 expression is SA responsive and it is commonly used as a molecular marker for SAR response (Loake and Grant, 2007). By contrast, ET and JA act usually synergistically in response to the infection of necrotrophic fungi (Glazebrook, 2005). JA and ET are also implicated in long distance signalling, named as induced systemic resistance (ISR) (Thatcher, Anderson and Singh, 2005; Truman *et al.*, 2007; Pieterse *et al.*, 2012).

This division of JA/ET or SA mediated resistance based on the biotrophic or necrotrophic lifestyle of the pathogen is only a very simplistic view of classification and crosstalk among phytohormones are much more complex and context dependent. Indeed, in many cases pathogen lifestyle classification into pure biotroph or necrotroph cannot be assigned. Although an antagonistic relation between JA and SA has been reported (Loake and Grant, 2007; Norton *et al.*, 2007; Thaler, Humphrey and Whiteman, 2012), this antagonism seems to be absent in the defence response of *Arabidopsis* to *Plectosphaerella cucumerina* (Berrocal-Lobo and Molina, 2004), *Pseudomonas syringae* and *Peronospora parasitica* (Clarke *et al.*, 2000). A cooperation between the two phytohormones has also been described in *Picea abies* (Arnerup, 2011) and *Zea mays* (Engelberth, Viswanathan and Engelberth, 2011). How JA, SA and ET coordinate to regulate the expression of defence genes is still not clear.

Some studies have suggested a role of auxins in plant-pathogen interaction resulting in positive or negative effect in resistance (Bari and Jones, 2009; Pieterse *et al.*, 2012). Suppression of auxin responses resulted in enhanced resistance to *P. syringae* and *Hyaloperonospora arabidopsidis*, suggesting that repression of auxin signalling is involved in defences against pathogens (Navarro *et al.*, 2006; Robert-Seilanianantz *et al.*, 2011). In contrast, Llorente *et al.*, (2008) reported that repression of auxin compromises resistance of *Arabidopsis* plants to necrotrophic fungi such as *Plectosphaerella cucumerina* and *Botrytis cinerea*. In addition, a role of auxins in modulating signalling pathways of other hormones have been suggested (Pieterse *et al.*, 2012); however, the complex network of cross-talk between the different phytohormones is still poorly understood.

A role of ABA as a negative regulator of defence responses have been reported in various pathosystems, such as in tomato plants against *B. cinerea* (Audenaert, De Meyer and Höfte, 2002), *Oidium neolycopersici* (Achuo and Prinsen, 2006) and *Erwinia chrysantemi* (Asselbergh *et al.*, 2008), as well as in *Arabidopsis* plants against *F. oxysporum* (Anderson *et al.*, 2004). However, the role of ABA as a positive regulator of defence has also been reported (Mauch-Mani and Mauch, 2005). There are evidences that GA, CK and BR are also involved in the regulation of plant defence responses against some pathogens, although its role in plant defence is poorly understood (Bari and Jones, 2009).

Plant secondary metabolites are also commonly induced against pathogens, some of them with antifungal activities. An important defence response of conifers is the production of phenolic compounds, such as flavonoids, lignans and tannins. For example, Norway spruce accumulates phenolics upon pathogenic infection in specialized phloem polyphenolic parenchyma cells (Franceschi *et al.*, 2005). Furthermore, the oxidation of phenolic compounds contributes to ROS production and subsequent oxidative burst (Barbehenn *et al.*, 2009). Production of oleoresin is known to be a common defence response in conifers composed mainly by monoterpenes and diterpenes with smaller amounts of sesquiterpenes (Keeling and Bohlmann, 2006), which provide an obstacle for invasion.

1.4.2. Pathogen response

Fungal cell wall is the first part of the fungus to be in contact with the host and its integrity needs to get adapted to the new environment in order to survive. It consists of a highly dynamic structure primarily formed by polymers including chitin, glucans and mannan/galactomannan (Bowman and Free, 2006). The cell wall integrity is regulated by a complex signalling cascade partially conserved in different fungal species and integrate cell surface sensor receptors which activate the downstream signalling by the stimulation of Rho small GTPases, protein kinase (PK) C and MAPKs, which ultimately regulate transcription factors that modulate changes in cell wall integrity (Turrà, Segorbe and Di Pietro, 2014). Martínez-Rocha *et al.*, (2008) reported that mutants for the orthologue Rho1 in *F. oxysporum* not only showed alterations in cell wall structure and in expression of cell wall biogenesis genes, but also a reduction in fungal virulence in tomato plants.

When the fungus reaches the surface of a host, it must face the first line of defence, the host cell wall. The fungus secretes extra-cellular cell wall degrading enzymes (CWDEs) to overcome this barrier and enhance its penetration and infection (Kikot, Hours and Alconada, 2009; Kubicek, Starr and Glass, 2014). Pectinolytic enzymes are the most important groups of CWDE (Have *et al.*, 1998; Valette-Collet *et al.*, 2007) and are considered important virulence factors in some pathogenic *Fusarium* spp. (García-Maceira *et al.*, 2001). These enzymes consist of four classes of enzymes: pectine lyase, polygalacturonase, pectin methylesterase and rhamnogalacturonase. They are usually the first enzymes to be secreted by the fungus and the degradation of pectins makes the cell wall of the host more accessible (Martínez *et al.*, 2006; Niture, Kumar and Pant, 2006). A polygalacturonase mutant in *B. cinerea* resulted in reduce virulence in tomato plants (Have *et al.*, 1998). Similarly, the expression of a pectate lyase from *Colletotrichum gloeosporioides* in *C. magna* resulted in an increase of virulence when infecting watermelon plants (Yakoby *et al.*, 2001). Other type of CWDEs, besides pectinolytic enzymes, are cellulases and xylanases, which degrade cellulose and xylan (a main component of the hemicellulose), respectively. *Fusarium graminearum* and *F. culmorum* produce cellulases, xylanases and pectinases when infecting wheat spikes during the early stages of infection (Kang and Buchenauer, 2000; Wanjiru, Zhensheng and Buchenauer, 2002). Xylanases and cellulases are also secreted by other in *Fusarium* spp. when infecting wheat leaves (Wanjiru, Zhensheng and Buchenauer, 2002).

Sterols are essential structural components in eukaryotic cellular membranes with vital roles in regulating membrane fluidity and permeability. In fungal membranes ergosterol constitutes the major component (Osswald, Höll and Elstner, 1986; Mille-Lindblom, Von Wachenfeldt and Tranvik, 2004). Ergosterol has been shown to have a role in virulence in *F. graminearum* (Liu *et al.*, 2013). Treatment with azole fungicide blocks ergosterol biosynthesis by inhibiting the enzyme that catalyses the conversion of lanosterol into ergosterol (Price *et al.*, 2015). The inhibition of ergosterol

biosynthesis through host-induced silencing of this enzyme results in protection against *F. graminearum* (Koch *et al.*, 2013).

Besides the cell wall barrier, the pathogen has to overcome the plant defensive oxidative burst by trying to reduce ROS levels by ROS scavenging enzymes, such as CAT, GPX and SOD. However, ROS production is not exclusive of plants, since some fungi are able to produce superoxide during the infection process by NoxR gene, a NADPH oxidase (Egan *et al.*, 2007). Similarly, *Aspergillus* mycotoxin ochratoxin A induces necrosis in leaves through oxidative burst induction resulting in increased ROS level and down-regulating the activity of plant antioxidant enzymes (Peng *et al.*, 2010). However, the reason why some fungi promote ROS production is still poorly understood.

Pathogens have developed the ability to manipulate plant defence regulatory network by producing the same hormones as the plant, which results in host hormone imbalance and in inappropriate defence response (Chanclud and Morel, 2016). Some species in the *Fusarium fujikuroi* species complex are known to produce GA, contributing to plant disease (Bömke and Tudzynski, 2009). Other studies have also reported a role of ET and auxins produced by fungi in perturbing plant phytohormone homeostasis (Van Bockhaven *et al.*, 2015; Chanclud and Morel, 2016). Fungal hormone production, with focus on *F. circinatum*, will be discussed in chapter 6.

1.5. Molecular processes involved in endophyte-host interaction

Endophytes have to avoid or overcome host defence to successfully penetrate and colonize plant tissue. However, the metabolic and molecular basis of host-endophyte interaction is poorly understood and studies are still scarce. Common pathways involved in plant defence against pathogens are also induced in the presence of an endophyte, including calcium ion fluxes, receptor-like kinases (RLKs), oxidative burst and phytohormone signalling (Kogel, Franken and Hüchelhoven, 2006). Based on a comparative analysis of sequenced genomes from mutualistic or parasitic bacteria associated to plants, Karpinets *et al.* (2014) determined that the biosynthetic functions are enriched and more diverse in plant mutualists whereas processes and functions involved in degradation and host invasion are enriched and more diverse in pathogens. Endophytes have a complex nutrient transport system and genes involved in the uptake of capsular polysaccharides, organic ions, peptides, amino acids and carbohydrates are commonly over-expressed in comparison with pathogens (Hardoim *et al.*, 2015).

As in plant-pathogen interactions, the plant commonly produces ROS under an endophyte invasion. The expression of enzymes with detoxification capacities, such as GPX and CAT, are commonly synthesized by endophytes in order to counteract the oxidative burst and protect plant cells against the oxidative damage (Hardoim *et al.*, 2015). As mentioned above, the fungal Nox genes constitute a NADPH oxidase

complex which participates in the production of ROS. Disruption of genes NOX1 and NOX2 in *Magnaporthe grisea* (Egan *et al.*, 2007) and bcnoxA and bcnoxB in *Botrytis cinerea* (Segmüller *et al.*, 2008) resulted in reduction of pathogenicity, suggesting a role of Nox in virulence. However, noxA genes are essential for maintaining a mutualistic association between the *Epychloë festucae* with ray grass, since mutants lacking noxA gene switch from mutualistic to pathogenic interaction (Eaton, Cox and Scott, 2011). Therefore, Nox genes may have a role in determining mutualistic/pathogenic interaction.

Endophytic fungi are also able to produce hormones, such as auxins and gibberellins, with a role in growth promotion in the plant (Long, Schmidt and Baldwin, 2008; Shi, Lou and Li, 2009). GA production by endophytic fungi isolated in various crop plants have been reported (Khan *et al.*, 2015). It has been also proposed that the auxin indol-3-acetic acid (IAA) facilitates endophyte colonization (Suzuki, He and Oyaizu, 2003) possibly via interference with the host defence system (Navarro *et al.*, 2006). CK biosynthesis is commonly observed in endophytes and deletion of genes related to CK biosynthesis in *Piriformospora indica* result in disruption of plant growth promotion (Vadassery *et al.*, 2008). The role of ET in plant-endophyte interaction has been studied (Long, Schmidt and Baldwin, 2008; Camehl *et al.*, 2010; Kusari, Pandey and Spiteller, 2013) and seems to have a role in determining the beneficial and non-beneficial balance of the interaction (Camehl *et al.*, 2010).

1.6. *Pinus-Fusarium circinatum* interaction

Despite the economic and ecological importance of conifers, genomic studies are limited by the large size of their genomes, which range from 20 to 40 Gb, approximately 200 times the size of the Arabidopsis genome and 7 times the size of human genome. Genomes are also complex with highly repetitive sequences, retrotransposons, accumulation of non-coding regions and gene duplication (Mackay *et al.*, 2012). However, in the last years, the development of high-throughput sequencing- techniques (Next Generation Sequencing, NGS) has allow a big progress in the field, and the genomes of Norway spruce (Nystedt *et al.*, 2013), white spruce (Birol *et al.*, 2013) and loblolly pine (Wegrzyn *et al.*, 2013) have been sequenced. In Europe, special effort has been done in the study of *P. pinaster* and a database, EuroPineDB, integrating genome information has been generated (Fernández-Pozo *et al.*, 2011; Canales *et al.*, 2014).

RNA sequencing (RNA-seq) approach allows the characterization of the transcriptome even in species with no reference or incomplete genome available, in which RNA-seq reads can be *de novo* assembled into a transcriptome (Robertson *et al.*, 2010; Canales *et al.*, 2014; Visser *et al.*, 2015). RNA-seq has been used to obtain transcriptomic data of plant tissues, allowing determining differential expression of transcripts between samples. A variant of this technology is the dual RNA-seq, which offers the possibility of analysing the transcriptome of the plant and pathogen simultaneously, allowing

determination of gene expression in the two organisms (Westermann, Gorski and Vogel, 2012; Hayden *et al.*, 2014; Meyer *et al.*, 2016; Naidoo *et al.*, 2018).

Concerning *F. circinatum*-*Pinus* pathosystem, the transcriptome of *P. radiata* inoculated seedlings was recently published (Carrasco *et al.*, 2017) showing induction of genes related to phytohormone signalling, PR proteins, phosphorylase family protein and physical and chemical barriers to restrict pathogen invasion. Similarly, Donoso *et al.*, (2015) detected an increase of transcription levels of TLPs (PR5 family) in *P. radiata* resistant genotypes by a reverse transcriptase-quantitative PCR (RT-qPCR) assay, as well as peroxidase, phenylalanine ammonia lyase (PAL), pinosylvin synthase and phenylcoumarin benzylic ether reductase. Davis *et al.*, (2002) reported SA and JA induction of chitinases of the PR3 family in *P. eliotii* seedlings inoculated with *F. circinatum*, and chitinases induction was earlier in resistant genotypes than in susceptible ones, suggesting the potential role of PR3 chitinases in pine defence response.

Bonello *et al.*, (2006) suggest that systemic-induced resistance (SIR) represents an important mechanism in coniferous trees involved in the balanced allocation of resources between growth and defence. Bonello, Gordon and Storer, (2001) reported SIR in *P. radiata* seedlings inoculated with *F. circinatum*, since repeated mechanical inoculations in the same tree resulted in a progressive reduction of lesion under field and greenhouse conditions.

In *F. circinatum*-*Pinus* interaction, most of the studies have focused their attention on host defence (Donoso *et al.*, 2015; Carrasco *et al.*, 2017), while little is known about *F. circinatum* genes involved in pathogenicity. Muñoz-Adalia *et al.*, (2018) suggested 5 candidate genes (*Fcfga1*, *Fcfgb1*, *Fcac*, *Fcrho1* and *FcpacC*) that could be involved in *F. circinatum* virulence based on the high similarity at nucleotide and protein levels with genes previously described in other *Fusarium* species. Although this is probable, the annotation could not be directly assigned (Punta and Ofran, 2008). Recently, by a dual RNA-seq approach, Visser (2019) reported that *F. circinatum* ergosterol biosynthetic genes are involved in virulence when infecting *P. tecunumanii* and *P. patula* seedlings and highlighted the role of phytohormone signalling in pine defence response.

Although oleoresin production is a common mechanism defence of conifers against pathogens, *F. circinatum* is able to grow in oleoresin and invade resin ducts (Martin-Rodrigues *et al.*, 2013). In addition, infection of the fungus results in induction of traumatic resin ducts in the xylem of *P. radiata* seedlings. Therefore, resin production in pines as a defence strategy is not solid, at least against *F. circinatum*.

Despite the importance of *P. pinaster* in the Mediterranean area, little is known about *F. circinatum*-*P. pinaster* interaction at molecular level. Application of dual RNA-seq during the infection of *P. pinaster* by the fungus will allow elucidating the mechanisms involved in the interaction. This will be the focus of chapters 5 and 6 of the present dissertation.

Chapter 2: OBJECTIVES

The objective of this thesis is to study *Fusarium circinatum* endophytic and pathogenic association with pines and alternative hosts in order to explain the ecological and molecular aspects of the interaction. The main objective can be split into the following specific objectives:

1. Describe and characterize the endophytic association of *F. circinatum* with herbaceous plants growing in a *P. radiata* plantation with PPC in northern Spain.
2. Determine the presence of *F. circinatum* in roots of non-symptomatic mature trees in *P. radiata* and *P. pinaster* plantations where the pathogen is known to be causing canker symptoms.
3. Determine the role of non-symptomatic herbaceous hosts as reservoir of inoculum.
4. Study genetic diversity of *F. circinatum* subpopulations, obtained from (a) cankers of symptomatic pine trees, (b) non-symptomatic herbaceous plants and (c) roots of both symptomatic and non-symptomatic trees.
5. Generate a high quality *de novo* *P. pinaster* transcriptome assembly to create a reference for downstream gene expression analysis.
6. Elucidate the molecular processes involved in the moderate resistance of *P. pinaster* to *F. circinatum*, with emphasis in phytohormone signalling, as well as determine the key steps where the pathogen could be manipulating host defence to its own benefit, leading to host susceptibility.

Chapter 3: MATERIALS AND METHODS

3.1. Ecological aspects of *Fusarium circinatum* endophytic lifestyle

3.1.1. Plant material sampling

3.1.1.1. Herbaceous plants sampling

Non-symptomatic host plants were collected beneath the canopy of a *P. radiata* plantation (100 x 55 m of surface) showing symptoms of PPC in Laukiniz (Basque Country, NE Spain) (Figure 9, point 1). Sampling was initially planned to be carried out in three one-meter square plots randomly marked in the plantation, but this method was discarded when we saw that these plots were only covered with the two most abundant species (*Agrostis capillaris* and *Pseudarrhenatherum longifolium*). These species were collected at four different points of the plantation chosen arbitrarily. For less abundant species, they were collected where found. Plants were sampled in three different months of 2016: April, July and August (Table 1). The entire plant was collected and morphologically identified. A total of 165 plants were collected (Table 1).

In order to determine if herbaceous plants are infected out of the plantation and in case of being infected, till how far, another sampling was carried out in June 2018. The aerial part of the plants was collected inside the plantation and at a distance of 3, 6 and 13 m from one of the borders (Figure 8). The same species identified in the other sampling dates were collected when present, as well as four new species, *Daboecia cantabrica*, *Holcus lanatus*, *Potentilla* sp. and an unidentified grass (Table 2). A total of 168 plants were collected.

Figure 8. Direction and distance of sampling from the border of a *Pinus radiata* plantation.

3.1.1.2. Pine cankers sampling

Cankers from 39 symptomatic pine trees in the same plantation were sampled to collect an isolate of *F. circinatum* from each tree.

3.1.1.3. Pine roots sampling

In four *Pinus* plantations in Basque Country (NE Spain) showing symptoms of pitch canker disease, roots from ten non-symptomatic and ten symptomatic trees were sampled. Trees were considered as “non-symptomatic” when resinous cankers were not present, neither in the main stem nor in the principal branches. On the contrary, trees with at least one resinous canker were considered as “symptomatic”. Three of the plantations were *P. radiata*, located at Bizkaia (100 x 55 m of surface, same plantation above) (1), Gipuzkoa (130 X 80) (2) and Arava (160 X 70) (3). The fourth one was a *P. pinaster* plantation located at Gipuzkoa (90 X 65) (4) (Figure 9). Roots were sampled at a maximum distance of 1 m from the collar region of the tree and within the first 20 cm of depth. Approximately 100 g of roots were collected for each sample with a root diameter of 0.2 to 5 mm. All roots sampled were apparently healthy and no differences were observed between roots collected from non-symptomatic or symptomatic pine trees. Climatological conditions for each plantation are provided in Supplementary Figure 1.

Figure 9. Location of the sampling plantations. Plantations 1, 2 and 3 were *Pinus radiata*, located at Bizkaia (1), Gipuzkoa (2) and Arava (3). The fourth one was a *Pinus pinaster* plantation located at Gipuzkoa (4).

3.1.2. *Fusarium circinatum* isolation

For each herbaceous plant sampled, the stem and leaves were separated from the root system and from seeds when they were present. Fungal endophytes were isolated following the disinfestation method described by Márquez *et al.*, (2010). Leaves and stems were surface disinfested by immersion in a 20 % commercial bleach with Tween 20 (1 drop / 100 ml) solution for 10 minutes and soaked twice in sterile distilled water. Roots were carefully washed under tap water to remove any adhering soil particles, dipped into 70 % ETOH for 1 minute and then followed the same procedure as leaves and stems. When seeds were present (seeds from six plants of *Pseudarrhenatherum longifolium*, two from *Sonchus oleraceus*, two from *Centaurea debeauxii* and one from *Hypochaeris radicata*), they were briefly rinsed in 70 % ETOH and immersed in a 20 %

commercial bleach with Tween 20 solution for 3 minutes, followed by two sterile distilled water washes. Plant material was aseptically transferred to sterilized filter paper and when dried, cut into 5-mm segments and placed in petri dishes with FSM (Aegerter and Gordon, 2006) and incubated at 25°C. In the case of seeds, all collected seeds for each plant were mixed, crushed using a sterile stick and placed on FSM. For further characterization, one isolate from each plant was chosen.

Cankers from symptomatic pine trees of the same plantation were sampled and surface disinfected by immersion in a 30 % commercial bleach solution with Tween 20 for 1 minute and washed twice in sterile distilled water (Pérez-Sierra *et al.*, 2007). Canker wood was cut into 1-cm pieces, placed on FSM and incubated at 25°C. One isolate per tree were used for the rest of the analyses.

Roots from symptomatic or non-symptomatic pine trees were carefully washed under tap water to remove any soil particle adhered. For surface disinfestation, roots were dipped into 70 % ETOH for 1 minute, submerged in a 30 % commercial bleach with Tween 20 (1 drop / 100 ml) solution during 15 minutes and soaked twice in sterile distilled water. The thinnest roots (less than 1 mm diameter) were immersed in the same commercial bleach solution but for 10 minutes instead of 15. Surface disinfested roots were aseptically transferred to sterilized filter paper and when dried, transversally cut into 5 mm segments and placed in petri dishes with FSM. The thickest roots were first longitudinally divided in two and then cut into 5mm pieces. Three to 6 petri dishes per sampled root were cultured depending on the root thickness. One isolate was selected per sampled tree for the rest of the analyses. Plates were incubated in darkness at 25°C and evaluated every three days.

3.1.3. Morphological and molecular identification of *Fusarium circinatum*

For morphological fungal identification, putative colonies of *Fusarium* growing on FSM were transferred to a SNA medium (Nirenberg, 1981) to confirm the species. Plates were incubated for 7-10 days at 25°C and then microscopically inspected for the formation of coiled sterile hyphae characteristic of *F. circinatum* (Nirenberg and O'Donnell, 1998). Monosporic isolates were obtained and conserved on paper (Whatman nº 1) at -20 °C until use.

Molecular confirmation of *F. circinatum* was done by PCR using specific primers CIRC1A (CTTGGCTCGAGAAGGG) and CIRC4A (ACCTACCCTACACCTCTCACT), as described by (Schweigkofler *et al.*, 2004) in a final volume per reaction of 25 µl with 1 µl DNA template. DNA was extracted from mycelia growing on PDA using an E.Z.N.A. Plant DNA Kit (Omega Biotek), following the manufacturer's instructions and stored at -20°C. PCR amplification was performed in a Veriti 96 well Thermal Cycler (Applied Biosystems), and its product was visualized in a 1 % agarose gel (Agarose MS-12, Pronadisa) stained with RedSafe (RedSafe Nucleic Staining Acid Solution, Intron Biotechnology) and visualized under UV light. A 100 bp ladder was used as a molecular weight marker (Biotools).

The identification was confirmed by sequence analysis. PCR-amplified fragments were purified with Montage Genomic kit (Millipore Corporation, Bedford, USA) following the manufacturer's instructions and sequenced using the CIRC1A primer. The resulting

sequences were compared with the ones of *Fusarium* species present in the GenBank database (NCBI) by a BLAST nucleotide search analysis.

3.1.4. Isolate characterization: genetic variability and pathogenicity

3.1.4.1. Mating type determination

Idiomorphs MAT-1 or MAT-2 were determined for each isolate. A multiplex PCR assay was performed using primers MAT1p1 (AGAACTGACTGATACATCAAGGGG) – MAT1p3 (TCATAAGAAGTGTGAAGGAATCACAG) and GcHMG1 (CTTTACCGTAAGGAGCGTCACCAT) - GcHMG2 (TGATCCGCCATCTGCTTG TAGAGT) for alleles MAT-1 and MAT-2, respectively, as described by Wallace and Covert (2000) and Schweigkofler et al. (2004). A final volume of 25 µl with 1 µl of DNA template per reaction was used following the amplification conditions described by Berbegal et al. (2013). PCR products were visualized in a 1.5 % agarose gel under UV light. A 100 bp ladder was used as a molecular weight marker. MAT-1 isolates amplified a 380 bp fragment, while MAT-2 produced a 190 bp amplicon.

3.1.4.2. Genetic diversity

The whole number of isolates (74 isolates) were used for genotype analyses, 29 obtained from non-symptomatic herbaceous hosts (Table 1), 39 from *P. radiata* cankers and 6 from symptomatic and non-symptomatic pine roots. A reference isolate representing the single haplotype identified in Basque Country (isolate CECT20759) (Iturrutxa et al., 2011; Berbegal et al., 2013) and a negative control with no DNA were also included. Primers for eight SSR loci (FCM-2, FCM-4, FCM-6, FCM-7, FCM-19, FCM-24, FCM-25 and FCM-26) previously characterized and selected for reproducible polymorphism were used (Berbegal, Armengol and Grünwald, 2013). Fluorescent labelling for each SSR locus was performed in one reaction using three primers (Schuelke, 2000): an SSR forward primer with a M13 tail (TGTAACGACGGCCAGT) at the 5' end, an SSR reverse primer, and the universal fluorochrome 6-carboxy-fluorescein (FAM) labelled M13 primer. The PCR reaction was performed as described by Berbegal et al. (2013) in a final volume of 10 µl. Amplifications were conducted in 96 well plates and PCR products were purified by adding ExoSAP-IT PCR Product Cleanup (Affimetrix) following the manufacturer's instructions. PCR products were sized by capillary electrophoresis (Macrogen Europe Lab). Allele calls were performed using the Geneious R10 10.0.9 Software (Biomatters ApS, Europe).

3.1.4.3. Pathogenicity tests

A pathogenicity test was conducted with all isolates obtained from non-symptomatic herbaceous hosts and one known virulent strain of *F. circinatum* representative of the fungal population analysed in the Basque Country (Isolate CECT20759) (Iturrutxa et al., 2011) as a positive control. Two-year-old *P. radiata* commercial seedlings were acclimatized in a greenhouse for 1 week at 20 – 22°C and a photoperiod of 12 h light / 12 h darkness. Each isolate was cultured on PDA for 7-10 days in darkness. The mycelium of each isolate was scraped off the agar plate with a sterile needle and inoculated by wound in the pine stem parallel to the stem axis (Elvira-Recuenco et al.,

2014). The wound was made approximately at 10 cm above the soil level. A sterile needle without inoculum was used to wound plants as a negative control. Plants were covered with a plastic bag for 24 h in order to maintain high humidity and favour fungal infection. Five seedlings were randomly inoculated for each isolate. Lesion length was measured at the 5th week post-inoculation. Re-isolation of the pathogen from 2 plants of each isolate on FSM and SNA were conducted to confirm that inoculated isolates were responsible for the lesions observed. A one-way ANOVA with Tukey's comparison test was performed using Statgraphics Centurion (Statpoint Technologies, Inc, United States) to compare lesion lengths.

3.1.5. Horizontal transmission from herbaceous plants to pines under controlled conditions

A qualitative assay was performed to evaluate whether pine seedling colonization was viable by inoculum coming from non-symptomatic herbaceous hosts but infected by *F. circinatum*. To do this, the remaining aerial plant segments of those herbaceous plants from which the fungus was previously isolated were surface disinfested following the same procedure as before and cut into 1-cm length fragments. These fragments were scattered over a sterile peat substrate (autoclaved for 1 hour, three days in a row). A total of 60 *P. radiata* seeds were sown in two boxes of 13 x 10 cm containing the substrate described above, and the other 30 pine seeds were sown in a box with no inoculum to serve as a control. The boxes were incubated at 25°C with a photoperiod of 12 h light/12 h darkness and irrigated periodically. Seedlings were observed for one month and sampled when they showed symptoms of damping-off or at the end of the experiment. Pine seedlings were carefully washed with tap water to eliminate any adhered substrate particles and then immersed in 70 % ETOH solution for 30 s, a 20 % commercial bleach with Tween 20 (1 drop / 100 µl) solution for 2 minutes, followed by two washes in sterile distilled water (Swett et al., 2016). Dry and disinfected pine material was cut into 5-mm segments and cultured on FSM. *F. circinatum* cultures grown in this medium were transferred to SNA for morphological confirmation.

3.1.6. *Fusarium circinatum* isolation from field-collected soil

Soil from Bizkaia plantation (*P. radiata*) was tested for the presence or absence of *F. circinatum*. Ten soil samples were collected in 10 points of the plantation. Approximately 500 g within 15 cm of the soil surface were collected and any debris in the soil was removed. Each soil sample was air dried and passed through a 2 mm sieve. Two soil suspensions were prepared for each soil and a part of the subsample was kept at 100°C for 48 hours to calculate the dry weight later. Soil suspension was prepared by adding 10 g of soil sample to 100 ml sterile distilled water and shaken at 150 rpm for 30 minutes. Three serial 10 fold dilutions were prepared and 400 µl of each suspension was added to 4 petri dishes containing FSM with some modification (3 gL⁻¹ streptomycin sulphate, 50 mgL⁻¹ Rose Bengal, 1 gL⁻¹ PCNB, 1 gL⁻¹ neomycin). Plates were incubated in darkness at 25°C and checked during the next 3 weeks. Putative colonies of *F. circinatum* were transferred to SNA or PDA medium respectively, in order to identify them. The experiment was repeated twice.

3.2. Molecular aspects of *Fusarium circinatum* pathogenic lifestyle

3.2.1. Plant and fungal material

Seven-month-old *P. pinaster* seedlings were used for the experiment. Seedlings were maintained in a greenhouse at 20 - 22°C and a photoperiod of 12 h light / 12 h darkness and inoculated after two weeks of acclimation.

For inoculations, a virulent *F. circinatum* isolate from Basque Country (Northern Spain) (Isolate CECT20759; isolated from *P. radiata* tree) (Iturrity et al., 2011) was used. A spore suspension in sterile distilled water was prepared after one week of culture of the fungus in PDA by scraping the plate surface and passing through two layers of glass wool. Spore concentration was measured with a hemocytometer and adjusted to 5×10^5 spores/ml.

3.2.2. Inoculation and microscopic observation

A total of 90 six-month-old *P. pinaster* seedlings were inoculated with a GFP-tagged strain of *F. circinatum*. The first 2 cm of the shoot tip were excised and a 2 µl drop of the spore suspension (1000 conidia) was deposited in the wound with a micropipette. A set of 54 seedlings were mock-inoculated with sterile distilled water. Plants were covered with a plastic bag for 24 h in order to maintain high humidity and favour fungal infection. Plant tissue of 6 inoculated and 2 mock-inoculated plants were daily visualized under an epifluorescence microscope (Nikon EFD-3) during the first 8 days after inoculation and at 10, 14 and 17 days post-inoculation (dpi). Cross-sections were placed on glass slides, covered with cover glasses and visualized under the epifluorescence microscope. The progression of the pathogen within host tissue served to determine the times of sampling for RNA-seq analyses and 3, 5 and 10 dpi were selected for sampling.

3.2.3. Inoculation and tissue sampling for RNA extraction

A total of 288 *P. pinaster* seedlings were used for the experiment. Half of them were inoculated with the pathogen as explained above while the remaining half was mock-inoculated with sterile distilled water. For sampling, the top 1.5 cm of shoot tissue was harvested for each seedling at three different times: 3, 5, and 10 dpi, for both inoculated and mock-inoculated seedlings. We used 4 biological replicates (BR) per group (mock and inoculated) with a pool of 8 individuals each. Plant material was immediately frozen in liquid nitrogen and stored at -80°C until use.

A total of 16 inoculated and 16 non-inoculated seedlings were maintained for visualizing disease progress. Lesion length was measured at the end of the experiment (33 dpi). By that time, 3 cm of the tip of 6 randomly collected plants of each group (inoculated and mock-inoculated) were surface sterilized by immersion in 70 % EtOH

for 1 minute. Plant tissue were transversally cut and cultured on FSM to verify the efficacy of the inoculation.

3.2.4. RNA extraction and sequencing

Total RNA was extracted using a Plant/Fungi Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Ontario) following the manufacturer's instructions and stored at -80 °C. The protocol included a DNase treatment step for a maximum removal of residual DNA (Norgen's RNase-free DNase I Kit). The integrity and concentration of the extracted RNA was assessed using a 2100 Bioanalyzer (Agilent Technologies). RNA was considered good quality when RNA Integrity Number (RIN) > 7. Two biological replicates for inoculated samples at 10 dpi did not pass the RIN threshold and were excluded for the rest of the analysis.

Approximately 40 µl of total RNA for each sample was submitted to Macrogen (Macrogen Korea). 24 TruSeq mRNA stranded libraries with polyA selection and an insert size of 300 bp was prepared. 101 bp paired-end reads were generated with Illumina HiSeq 4000, with a sequencing depth of 90 million reads per sample. Samples were multiplexed in 4 lanes, with 6 samples per lane. In order to avoid technical errors due to sample position in the sequencer, each BR of each sample was included in a different lane.

3.2.5. *De novo* *Pinus pinaster* transcriptome assembly

3.2.5.1. Raw data quality control and filtering

Quality control of Illumina raw reads was performed by FastQC analyses v0.11.7 (Andrews, 2012). For trimming and filtering of low-quality raw reads and remove adapters, Trimmomatic 0.36 software was used (Bolger, Lohse and Usadel, 2014). Bases with a Phred Score below 30 and reads shorter than 40 bp were removed. The first 15 bases of all reads were trimmed to remove sequencing biases. The quality of trimmed and filtered reads was checked again by FastQC.

3.2.5.2. Preliminary assemblies

Diverse studies have shown a high variability in the assembly of reads when using different assemblers (Zhao *et al.*, 2011; Bradnam *et al.*, 2013). Therefore, the *de novo* transcriptome assembly of *P. pinaster* was created using two different assemblers: Trinity v.2.4.0 (Grabherr *et al.*, 2011) and transABySS v.1.5.4 (Robertson *et al.*, 2010), programs specially developed for *de novo* transcriptome assembly of RNA-seq short-read data. A multi kmer strategy was adopted; therefore, De Bruijn graphs were built over different kmer values and the resulting assemblies were then merged to improve sensitivity and for an accurate gene set reconstruction (Surget-Groba and Montoya-Burgos, 2010; Zhao *et al.*, 2011; Durai and Schulz, 2016).

Seven assemblies were created with Trinity with kmer values of 19, 21, 23, 25, 27, 29 and 31 and the next set of parameters: minimum contig length of 350 bp and maximum read coverage of 50 for in silico normalization. Other 5 assemblies were generated with kmer sizes of 19, 21, 23, 25 and 27 but without normalization of the reads. For TransABYSS, we run 9 different *de novo* assemblies with a kmer value ranging from 21 to 77, with a step size of 8 as well as a kmer value of 25. The minimum output sequence length was set to 350 bp for all TransABYSS assemblies. Quality of each preliminary assembly was checked by Transrate v.1.0.3 (Smith-Unna *et al.*, 2016).

3.2.5.3. Merging assemblies

Best quality preliminary assemblies were merged into one dataset using the EvidentialGene tr2aacds pipeline version 2017.12.21 (Gilbert 2013), which builds the most optimal assembly from a pool of different assemblies. It uses fastanrdb of exonerate package v 2.2.0 (Slater and Birney, 2005) for pairwise sequence comparison based on protein qualities for predicting the best coding DNA sequences (CDS) among identical sequences and reduce redundancy. Then, cd-hit-est v.4.7 (Li and Godzik, 2006) and BLASTn (blast v.2.7.0) are used to cluster nucleotide sequences with a 98 % of similarity into loci. EvidentialGene tr2aacds returns the subset of most accurate coding genes, classified into alternate or main (primary) CDS. Additionally, an “okay” or “drop” value is assigned using scores of alignment and protein quality to separate “useful” and “not useful” transcripts, respectively. The “drop” class contains redundant and uninformative mRNA transcripts and they were discarded for the rest of the analyses. The quality of the transcript sequences derived from the merged assembly were checked by Transrate v.1.0.3.

3.2.5.4. Annotation

Both reference transcriptomes were annotated using EnTap (Eukaryotic Non-Model Transcriptome Annotation Pipeline) version 0.8.2 (Hart *et al.*, 2018). Coding regions of the transcripts were selected by GenemarkS-T v5.1 March 2014 (Tang, Lomsadze and Borodovsky, 2015). The annotation process integrates similarity search across different databases with a minimum query and target coverage of 80 and 60 %, respectively. NCBI non-redundant protein, RefSeq, SwissProt and Arabidopsis proteome databases were used for BLASTp alignment using Diamond 0.9.9 (Buchfink, Xie and Huson, 2014). Non-pine origin sequences were removed from the assembly by significant alignment to fungal, bacterial, viral, insect, archaea, opisthokonta and amoebozoa sequences. For orthologous group and gene ontology (GO) assignment InterProScan v5.28-67.0 (P. Jones *et al.*, 2014) and EggNOG v0.12.7 (Huerta-Cepas *et al.*, 2016) were used. Finally, all contaminants and non-frame selected and unannotated sequences were manually filtered. Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) annotation was also assigned by using GhostKOALA (Kanehisa, Sato and Morishima, 2016).

Mercator tool (Lohse *et al.*, 2014), with default parameters and including all the databases available, were used to assign predicted proteins into MapMan v.3.5.1R2

(Thimm *et al.*, 2004), a tool that allows the visualization of metabolic pathways and processes of a large set of data.

3.2.5.5. Assembly validation

Completeness and contiguity of the assembly was checked using BUSCO v3.0 (Benchmarking of Single-Copy Orthologs) (Simão *et al.*, 2015). We used the eukaryote_odb9 and embryophyta_odb9 lineages to identify putative core eukaryotic genes (CEGs) and universal single copy orthologs (USCOs) in the assembly.

Figure 10. Workflow followed for the generation of *de novo Pinus pinaster* transcriptome assembly. First box: check of quality and filtering of raw reads. Second box: assembly. Third box: annotation.

3.2.6. *Fusarium circinatum* reference transcriptome

Fusarium circinatum reference transcriptome was obtained from Visser (2019). In this study, they extracted the longest transcript sequence for all predicted genes (15,049) from the *F. circinatum* (strain FSP34) genome sequence (Wingfield *et al.*, 2012) and they annotated transcript sequences with EnTAP.

3.2.7. Mapping, differential expression (DE) and gene ontology (GO) enrichment analysis

F. circinatum transcriptome and *P. pinaster* *de novo* transcriptome assembly were combined in a single dataset. This dataset was used to map reads with the software Kallisto v.0.44.0 (Bray *et al.*, 2016) with sequence bias correction and bootstrap samples set to 100. Kallisto abundance output file (transcript abundance estimates) for each read was import to R 3.5.1 with tximport package v.1.6.0 (Soneson, Love and Robinson, 2016). DESeq2 package v.1.18.1 (Love, Huber and Anders, 2014) was used for DE analysis, with a p-value < 0.05 and $|\log_2(\text{Fold change})| > 0.5$. Transcripts with less than 20 reads in at least 3 samples were filtered for the rest of the analysis. For host DE analysis *F. circinatum* data was removed and expression levels at different time points (3, 5 and 10 dpi) were compared between inoculated and mock-inoculated samples. For *F. circinatum* DE analyses, *P. pinaster* data was filtered and gene expression levels between time points were compared between the inoculated samples (3 versus 5 dpi, 3 versus 10 dpi and 5 versus 10 dpi). To excluded potential endophyte contamination and confirm the confidence of *F. circinatum* expressed genes, a high confidence DE analysis (inoculated relative to mock-inoculated) with all the data set (host and pathogen) was performed and then compared to the one with only inoculated samples. Genes considered not high confident at all time points were discarded. GOSeq v.1.34.0 package (Young, Wakefield and Smyth, 2010) was used to assess GO terms significantly overrepresented (FDR p value < 0.10) in the DE data. RamiGO v.1.28.0 package (Schröder *et al.*, 2013) was used for visualization of GO trees. GO enrichment was based on the annotated transcriptomes for both species.

A principle component analysis (PCA) was performed for *P. pinaster* and *F. circinatum* normalize counts (regularized-logarithm transformation or rlog - DESeq2 rlog function) for all the samples, to visualize the overall effect of experimental covariates and batch effects.

Significant DE genes for each species were clustered based on the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) or $\log_2$ (Fold Change) values by using Hmisc v.4.1-1 R package, with a correlation of 0.5 and using Pearson correlation method. Gene ontology for genes of each cluster was assigned with GOSeq. *F. circinatum* DE genes were also searched (PHI-BLAST tool, default parameters) in the pathogen-host interactions (PHI) database 4.2 (Urban *et al.*, 2017) to identify potential pathogenicity and virulence factors.

The most up-regulated gene in *P. pinaster* dataset coded for a disease resistance (R) protein. BLASTP search against the non-redundant protein NCBI database was conducted. BLASTP search by including only *Arabidopsis* species was also done in order to assign any putative molecular function.

Figure 11. Workflow followed for mapping, differential expression (DE) and gene ontology (GO) enrichment analysis.

Chapter 4: ECOLOGICAL ASPECTS OF
FUSARIUM CIRCINATUM ENDOPHYTIC
LIFESTYLE

4.1. Introduction

Fusarium circinatum is a fungus with a duality in pathogen-endophyte lifestyle. As a pathogen, it is the causal agent of PPC. As an endophyte, *F. circinatum* colonizes grasses growing under *P. radiata* trees with symptoms of PPC (Swett and Gordon, 2012; Swett *et al.*, 2014). Like other fungi, *F. circinatum* cannot be distinguished morphologically when isolated from symptomatic or non-symptomatic plants, that is, pines or grasses (Swett *et al.*, 2014). The fact that *F. circinatum* colonizes grasses in plantations with symptoms of PPC raises a relevant question about the potential extension of non-symptomatic hosts serving as reservoirs of inoculum. Thus far, pathogen survival was studied in pines debris, not taking into account that it could colonize species other than *Pinus* spp. Survival in infected needles and wood fragments of *P. radiata* was measured on the soil surface over time, showing that the pathogen can hardly be recovered from debris after two years (Serrano *et al.*, 2016). However, the colonization of non-symptomatic grasses by *F. circinatum* highlights a new inoculum source that needs to be studied.

Endophytes live at least part of their life cycle within plant tissues (Zabalgogezcoa, 2008; Hardoim *et al.*, 2015). They infect new plants by either horizontal or vertical transmission. When a fungus is horizontally transmitted, plant to plant, it emerges to sporulate in plant or senescent tissue (Stone, Polishook and White Jr, 2004). If the fungus is vertically transmitted, which is less frequent than horizontal transmission, it is via host seeds (Hardoim *et al.*, 2015). There is no evidence of how transmission of *F. circinatum* may occur in hosts other than pines, and whether it occurs not only among herbaceous plants but also from herbaceous plants to pines. The only information that suggests co-infection is that isolates of *F. circinatum* obtained from vegetative tissues of non-symptomatic grasses were pathogenic to artificially inoculated pines, with virulence similar to isolates obtained from symptomatic trees (Swett *et al.*, 2014).

The genetic structure of the population of *F. circinatum* was previously studied in Basque Country (NE Spain) with isolates coming from symptomatic trees. Isolates made up a homogeneous population, with only two VCG (vegetative compatibility groups), all isolates of mating type 2, and three genotypes distinguished by AFLP markers (Iturrutxa *et al.*, 2011). A recent study (Bergebég, Armengol and Grünwald, 2013) using eight microsatellites or simple sequence repeats (SSR) markers described by Santana *et al.*, (2009) to analyse a broader sample revealed that the Spanish population of *F. circinatum* is structured into two distinct groups, each of which included a dominant haplotype. This study also found only one haplotype in Basque Country exclusively of mating type 2. Therefore, given the clonality of *F. circinatum* in this region and the presence of only one genotype in the area identified by SSR markers, this condition offers a unique opportunity to genetically compare both populations coming from symptomatic and non-symptomatic hosts.

Presence of *F. circinatum* in pine roots has been underestimated in the lifecycle of the pathogen and existing research on the role that infected roots play in symptom expression and disease development is limited. *Fusarium circinatum* has been detected in roots of pine seedlings (Viljoen, Wingfield and Marasas, 1994; Storer, Gordon and Clark, 1998) causing disease symptoms. Likewise, the fungus has the ability to colonize

roots of pine seedlings as an endophyte, that is, without causing apparent symptoms (Elvira-Recuenco, Iturrutxa and Raposo, 2015; Martín-Rodrigues *et al.*, 2015; Swett, Kirkpatrick and Gordon, 2016), but it has never been reported in roots from asymptomatic mature trees. Seedling roots can be colonized from infected soil (Martín-Rodrigues *et al.*, 2015; Swett, Kirkpatrick and Gordon, 2016) or by transmission from infected emerged seeds (Elvira-Recuenco, Iturrutxa and Raposo, 2015), and aerial symptoms are not visible until the fungus reaches the collar region (Swett, Kirkpatrick and Gordon, 2016). In addition, results indicate that this occurs when the fungus population in the root is above a certain level (Elvira-Recuenco, Iturrutxa and Raposo, 2015).

The final objective developed in this chapter is (1) to describe and characterize the endophytic association of *F. circinatum* with herbaceous plants growing in a *P. radiata* plantation with PPC in northern Spain; and (2) to determine the presence of *F. circinatum* in roots of non-symptomatic mature trees (no cankers) in *P. radiata* and *P. pinaster* plantations where the pathogen is known to be causing canker symptoms. Related to the first objective, we explored the non-symptomatic host range of *F. circinatum*, studying if they are isolated from aerial or root tissues, and its transmission. We assayed whether non-symptomatic herbaceous plants may act as reservoir of inoculum infecting pine seedlings through horizontal transmission. In addition, we studied whether all subpopulations obtained from (a) cankers of symptomatic pine trees, (b) non-symptomatic herbaceous plants isolates and (c) roots of both symptomatic (cankers were present) and non-symptomatic (cankers not present) trees population, are genetically differentiated by a microsatellite analysis approach. Furthermore, field-collected soil in one of the plantations will be tested for the presence of the fungi. These results will provide information to elucidate the role of endophytic colonization in the lifecycle of the pathogen.

4.2. Results

4.2.1. *Fusarium circinatum* endophytic isolation

4.2.1.1. Isolation in herbaceous plants

Based on morphological and molecular analyses, 28 isolates of *F. circinatum* were recovered from 165 non-symptomatic plants sampled from a *P. radiata* plantation (Table 1). These plants were identified as being of 16 different species (Table 1; Figure 12), from which *F. circinatum* was isolated in 7 species, including in several plant families: *Agrostis capillaris* (Poaceae), *Pseudarrhenatherum longifolium* (Poaceae), *Centaurea debeauxii* (Asteraceae), *Teucrium scorodonia* (Lamiaceae), *Sonchus oleraceus* (Asteraceae), *Rubus ulmifolius* (Rosaceae) and *Hypochaeris radicata* (Asteraceae). The fungus was recovered from the leaves and stems and was never obtained from the root system. Seeds were processed when present, and the fungus was only isolated from *H. radicata*; thus, vertical transmission can be possible, at least in this species (Table 1).

Figure 12. *Pseudarrhenatherum longifolium* (Poaceae) at the left, *Sonchus oleraceus* (Asteraceae) in the middle and *Teucrium scorodonia* (Lamiaceae) at the right.

Table 1. Non-symptomatic herbaceous species sampled in a *Pinus radiata* plantation with symptoms of pitch canker disease and frequency of infected plants from which *Fusarium circinatum* was recovered.

Family	Specie	No. of infected plants (total sampled)				Infected plant part ^a
		April	July	Augt	Total	
Poaceae	<i>Agrostis capillaris</i>	0 (10)	6 (12)	0 (8)	6 (30)	L, S
	<i>Pseudarrhenatherum longifolium</i>	1 (23)	3 (15)	7 (22)	11 (60)	L, S
	<i>Anthoxanthum odoratum</i>	0 (10)	- ^b	-	0 (10)	-
	<i>Holcus mollis</i>	0 (1)	-	-	0 (1)	-
	<i>Brachypodium rupestre</i>	0 (2)	-	-	0 (2)	-
	<i>Dactylis glomerata</i>	0 (4)	-	-	0 (4)	-
	<i>Cortaderia selloana</i>	0 (4)	-	-	0 (4)	-
Asteraceae	<i>Centaurea debeauxii</i>	1 (4)	2 (7)	-	3 (11)	S
	<i>Taraxacum officinalis</i>	0 (3)	-	-	0 (3)	-
	<i>Sonchus oleraceus</i>	2 (4)	2 (2)	-	4 (6)	S
	<i>Hypochaeris radicata</i>	-	1 (5)	0 (6)	1 (11)	S, Se

Iridaceae	<i>Crocasmia crocosmiiflora</i>	0 (3)	-	-	0 (3)	-
Lamiaceae	<i>Teucrium scorodonia</i>	2 (4)	0 (1)	-	2 (5)	S
Juncaceae	<i>Luzula multiflora</i>	0 (3)	-	-	0 (3)	-
Polygonaceae	<i>Rumex acetosa</i>	0 (3)	-	-	0 (3)	-
Rosaceae	<i>Rubus ulmifolius</i>	-	1 (9)	-	1 (9)	S
	Total sampled	6 (78)	15 (51)	7 (36)	28 (165)	

^a infected plant part: L: leaves; S: stem; Se: seeds

^b not sampled

Two species were infected in high proportion, although they were sampled at a low number. They were *S. oleraceus*, which was the species most infected (67 %), with 4 infected plants out of 6, followed by *T. scorodonia* (40 %) with 2 infected plants out of 5 (Figure 13). *H. radicata* plants had the lowest frequency of infection (9 %). The most abundant species in the plantation were grasses (Poaceae), with a total of 60 plants of *P. longifolium* and 30 plants of *A. capillaris*. Despite being the most abundant during the three months of sampling, the number of infected plants from which *F. circinatum* was recovered was lower. Only 20 % and 18 % of plants were infected for *A. capillaris* and *P. longifolium* (Figure 13), respectively. *F. circinatum* was not recovered from *Crocasmia crocosmiiflora*, *Anthoxanthum odoratum*, *Taraxacum officinalis*, *Luzula multiflora*, *Holcus mollis*, *Brachypodium rupestre*, *Dactylis glomerata*, *Rumex acetosa* and *Cortaderia selloana*. *F. circinatum* isolated from cankers of symptomatic pine trees was also confirmed by morphological and molecular methods.

Figure 13. Number of infected plants (green) over the total sampled (green and blue) for each species in which *Fusarium circinatum* was detected.

4.2.1.1.1. Dissemination from the border of the plantation

A total of 168 plants were analysed, 56 inside the plantation, 39 at 3 m from the plantation, 40 at 6 m and 43 at 13 m (Table 2). A total of 7 plants (15.22 %) were infected inside the plantation; these plants include species *H. radicata*, *H. lanatus* and *Potentilla* sp. At a distance of 3 m from the border of the plantation 3 plants resulted infected (7.69 %), including species *H. lanatus* and *C. debeauxii*. *F. circinatum* was not detected in any of the analysed plants at 6 and 13 m from the plantation.

Table 2. Number of infected plants for each species sampled inside the plantation and at a distance of 3, 6 and 13 meters from the border of the plantation. Samp.: number of sampled plants; Inf.: number of infected plants; NI grass: non-identified grass. – Not sampled.

Species sampled	Inside		3 m		6 m		13 m	
	Samp.	Inf.	Samp.	Inf.	Samp.	Inf.	Samp.	Inf.
<i>Hypochaeris radicata</i>	7	4	10	0	5	0	3	0
<i>Teucrium scorodonia</i>	4	0	-	-	4	0	-	-
<i>Rubus ulmifolius</i>	15	0	-	-	-	-	4	0
<i>Agrostis capillaris</i>	8	0	10	0	10	0	10	0
<i>Holcus lanatus</i>	4	2	8	2	5	0	10	0
<i>Potentilla</i> sp.	8	1	-	-	-	-	-	-
<i>Centaurea debeauxii</i>	-	-	6	1	11	0	6	0
<i>Daboecia cantabrica</i>	-	-	5	0	5	0	-	-
NI grass	-	-	-	-	-	-	10	0
Total	46	7 (15%)	39	3 (8%)	40	0	43	0

4.2.1.2. Isolation in pine roots

F. circinatum was only isolated from roots at Bizkaia plantation (6 isolates from 6 trees) where half of sampled roots of non-symptomatic trees (5 roots out of 10) were colonized by the pathogen. In roots of symptomatic trees only one sampled root was colonized by the fungus (Figure 14). *F. circinatum* was not detected in any of the other *P. radiata* locations for either symptomatic or non-symptomatic trees. Neither was isolated from roots of either non-symptomatic or symptomatic trees at *P. pinaster* plantation (Gipuzkoa-P).

Figure 14. Percentage of roots of symptomatic non-symptomatic trees from which *Fusarium circinatum* was isolated in Bizkaia plantation (the only plantation where the fungus was detected in roots).

4.2.2. Isolate characterization: genetic variability and pathogenicity

4.2.2.1. Mating type

Regarding determination of *F. circinatum* mating types, results showed that all isolates were exclusively MAT-2 type.

4.2.2.2. Genetic diversity

Genetic diversity analysis of *F. circinatum* isolates by SSR markers revealed that all isolates were identical, and they did not show any polymorphism for any SSR loci. Haplotypes were not differentiated on the basis of their origin, roots of non-symptomatic or symptomatic trees, tree cankers or asymptomatic herbaceous plants. All isolates presented only peak sizes of 178, 153, 251, 190, 173, 124, 204 and 244 bp resulted for the SSR loci FCM-2, FCM-4, FCM-6, FCM-7, FCM-19, FCM-24, FCM-25 and FCM-26, respectively. No peak was detected in the negative controls. The exception to this genetic uniformity was due to one isolate coming from a canker, which showed polymorphism for the FCM25 SSR locus. The SSR peak had a size of 199 bp instead of 204 bp. A sequence analysis of this amplicon showed that it had one repetition less (AGACA) than the two other isolates used for comparison.

4.2.2.3. Pathogenicity test

All *F. circinatum* isolates from roots of non-symptomatic and symptomatic pine trees and non-symptomatic herbaceous plants were pathogenic to *P. radiata*. Inoculation was done in separate trials for isolates obtained from non-symptomatic herbaceous plants or from symptomatic and non-symptomatic pine roots. The lesion size measured at the 5th week after inoculation for herbaceous plants isolates ranged from 2.4 ± 0.75 to 4.5 ± 0.45 cm, and the mean lesion caused by the pathogenic reference isolate was 3.5 ± 1.02 cm. For isolates obtained from pine roots, the lesion size ranged from 4.05 ± 0.73 to 4.76 ± 1.11 cm, and the mean lesion caused by the pathogenic reference isolate was 4.98 ± 1.65 cm. No statistical significant differences were found between isolates.

No lesions were developed in the non-inoculated plants. The fungus was successfully recovered from the sampled inoculated pines and its identity was morphologically confirmed.

4.2.3. Horizontal transmission from non-symptomatic plants to pines under controlled conditions

Results show that non-symptomatic herbaceous host segments that had fallen on the soil but were infected by *F. circinatum* may act as inoculum reservoir. Pine seedlings were infected with *F. circinatum* when growing in peat soil with senescent infected plant tissue on it. In the first and second boxes, 80 % and 87 % of emerged plants developed disease symptoms, respectively, while those emerged seedlings grown in sterile peat did not show any disease symptoms. When plating symptomatic pine seedlings on FSM, all of them were infected by the fungus, and no pathogen was detected in seedlings growing in sterile soil. *F. circinatum* was detected generally in the collar region of pine seedlings. Regarding emergence, in the first box only the 63 % of seedlings referred to the control emerged (24 seedlings out of 30), while in the second box, 96 % of seedlings emerged.

4.2.4. Detection in field-collected soil

F. circinatum was not isolated from any of the ten soil samples at Bizkaia plantation where symptomatic *P. radiata* trees were present.

4.3. Discussion

We confirmed the presence of *F. circinatum* in alternative non-symptomatic hosts to pines in Spain, as reported before in South Africa and California (Swett and Gordon, 2012; Swett *et al.*, 2014). Furthermore, we report for the first time the presence of *F. circinatum* in plants of families different from the Poaceae (grasses) where the fungus was initially found. In addition to grasses, the families reported here are the dicotyledoneae Asteraceae, Rosaceae and Lamiaceae, and this finding implies that the non-symptomatic host range is broader than initially thought. Endophytic association of *F. circinatum* with grasses was explained, at least in part, by the fact that this fungus is phylogenetically close to other *Fusarium* species that are able to colonize grasses, such as *F. subglutinans*, which is also interfertile with *F. circinatum* (Desjardins, Plattner and Gordon, 2000). However, the fact that the fungus is found in other plant families not close to grasses suggest that this endophytic association is related to the capacity of *F. circinatum* to adopt different lifestyles other than parasitism when colonizing their hosts.

There are other reports that include some examples of fungi with different lifestyles according to hosts: *Colletotrichum magna*, which causes anthracnose in cucurbit plants and colonizes non-cucurbit species in an endophytic lifestyle (Kogel, Franken and Hückelhoven, 2006); *Verticillium dahliae*, pathogenic in dicots while endophytic in

monocots (Malcolm *et al.*, 2013); *F. oxysporum*, a pathogen that can colonize root systems and move through the cortical tissue of susceptible hosts, causing wilt disease in many economically important crops (Gordon and Martyn, 1997) and at the same time is a fungi that commonly appears as an endophyte in other plants (Kuldau and Yates, 2000). Indeed, some fungi known as endophytes can behave as pathogens under altered environmental conditions (Delaye, García-Guzmán and Heil, 2013). For example, the same strain of *Leptosphaeria maculans* was found in completely asymptomatic *Arabidopsis thaliana* plants under natural conditions, but the fungus become necrotrophic in a more stressful situation (Junker, Draeger and Schulz, 2012). Similarly, the *Diplodia mutila*, endophyte living within tropical palm can cause necrosis under high light intensity (Álvarez-Loayza *et al.*, 2011).

Regarding our method to isolate endophytic fungi, we used longer times for surface sterilization of plants than those used in previous studies. Swett *et al.*, (2014) immersed plant material for 10 s in 70% EtOH followed by 30 s in 0.1% NaOCl; Photita *et al.*, (2004) surface sterilized in 75% EtOH for 1 min followed by 1% sodium hypochlorite for 3 min and 95% EtOH for 30 s. Those times and concentrations are less than we used here. It is possible that by using shorter times for the surface sterilization of plants before plating, such as those used in previous studies, the frequency of colonization by *F. circinatum* could be higher than we found here or new species could even be reported.

The outcome of a plant-microbe interaction depends on many factors that include plant and microbe genotypes and environmental conditions (Redman, Dunigan and Rodriguez, 2001; Hardoim *et al.*, 2015; Hily *et al.*, 2016). We have detected a unique haplotype (with only one exception) in the fungus population collected either from symptomatic pines or non-symptomatic herbaceous plants, suggesting that the same haplotype of *F. circinatum* can have a lifestyle as an endophyte or as a pathogen. Molecular markers do not represent the total variation in the genome that there might be if those populations have mutations allowing adaptation to specific hosts that they colonize. However, detection of only one haplotype together with the fact that all isolates were pathogenic to pines regardless of from where they were isolated, suggest all isolates may occur in pines and in herbaceous plants. This situation, where the host plays no role in the haplotype distribution of the fungus population, has also been reported for *Lasiodiplodia theobromae*, where the same haplotypes were found in all host species (Mohali, Burgess and Wingfield, 2005). Also for *Botryosphaeria dothidea*, where presence of some haplotypes occurred on many different hosts and across broad geographic range (Marsberg *et al.*, 2017; Mehl *et al.*, 2017).

Based on our results, *F. circinatum* is a foliar endophyte in herbaceous plants that colonizes the aerial part of the plant. The fungus was only isolated from leaves and/or stems, but never from roots. Indeed, *F. circinatum* was not isolated from soil samples. This agrees with a previous study where *F. circinatum* was not found in the soil sampled from different plantations with symptoms of PPC (Serrano *et al.*, 2016). The composition of endophyte populations is partially shaped by the environment in which it originates (Hardoim *et al.*, 2015); that is, the air for the foliar endophytes and the soil for root endophytes. Non-symptomatic hosts may be colonized by spores from the air as airborne inoculum of *F. circinatum* is detected with seasonal differences throughout the year (Schweigkofler, O'Donnell and Garbelotto, 2004). The availability of aerial

inoculum is associated with trees displaying different levels of disease symptoms (Garbelotto, Smith and Schweigkofler, 2008) and it is not detected in areas where disease does not occur (Wingfield *et al.*, 2008); thus it is likely that conidia coming from tree cankers are the source of inoculum for herbaceous plants in the understory of the affected Monterey pine plantation. Our results show that herbaceous plants at a distance of more than 3 meters from the border of the plantation are not infected, supporting this hypothesis. If this is the case, the incidence of colonized plants might decrease or even disappear once diseased trees are removed.

Fungal endophytes in grasses spread horizontally from plant to plant, frequently by emergence and sporulation in senescing plants (Márquez *et al.*, 2012). Endophytes are also predominantly horizontally transmitted in conifers, woody and herbaceous angiosperms (Rodríguez *et al.*, 2009). Certain grass endophytes, such as *Neotyphodium coenophialum*, are primarily transmitted vertically via seed infections (Saikkonen, Ion and Gyllenberg, 2002). They never leave their hosts, being regarded as true mutualists because they confer toxicity to mammals and herbivores. However, recent reports suggest that vertical transmission is more frequent in dicot species than initially thought (Hodgson *et al.*, 2014). We isolated *F. circinatum* from seeds of one plant of *H. radicata*, likely colonizing the inner seed and not its coat (seeds were surface sterilized before plating). Although we did not demonstrate that the fungus was recovered from seedlings grown from those infected seeds (seed sampling destructive), our study reveals that this possibility may occur. Furthermore, a previous study (Storer, Gordon and Clark, 1998) hypothesized an endophytic stage of *F. circinatum* in pine seeds, suggesting that the fungus remains quiescent within the seeds.

F. circinatum is transmitted in pines by spores either through the air or via insects (Wingfield *et al.*, 2008). Our assay has shown that pine seedlings can be infected from senescent tissue of non-symptomatic plants colonized by the fungus. Some herbaceous plants sampled here are annual or biannual, so when plants die, senescent tissue falls on the soil. Other plants are perennial and when a new leaf has been established, the older and lowermost leaves often senesce (Moore and Moser, 1995). The sporulation of endophytes frequently occurs this way when infected host tissue dies (Sánchez Márquez, Bills and Zabalgogezcoa, 2007; Zabalgogezcoa, 2008). Our results thus suggest that non-symptomatic plants may constitute an inoculum source in a plantation or even in new plantations. Currently in Spain, there is a mandatory minimum period of two years following felling of infected pines before a new plantation can be established. The importance that non-symptomatic plants may have as inoculum reservoirs needs to be quantified, since the frequency of infected plants and inoculum density seems to decrease in the absence of symptomatic hosts.

In the present study, we also report for the first time the presence of *F. circinatum* in roots of asymptomatic *P. radiata* mature trees. In previous studies the pathogen has been detected within roots of seedlings that appear healthy under controlled conditions and in nurseries (Storer, Gordon and Clark, 1998; Elvira-Recuenco, Iturrutxa and Raposo, 2015; Martín-Rodrigues *et al.*, 2015; Swett, Kirkpatrick and Gordon, 2016). The results presented here are obtained from pine roots naturally colonized by *F. circinatum* under field conditions. A previous work (Elvira-Recuenco, Iturrutxa and Raposo, 2015) determined that pine seedlings can remain symptomless 475 days after

inoculation under controlled conditions. In another study (Swett, Kirkpatrick and Gordon, 2016), colonized pine seedlings remained symptomless for up to 52 weeks from initial exposure to the pathogen. Seedlings are moved from nurseries to forest plantations approximately when seedlings are 1 year old, but seedlings can remain symptomless for a longer period of time. In the present study, the fungus was detected in a higher proportion from roots of non-symptomatic trees (with no cankers). This observation raises the question how these roots became infected and the implications of root colonization in the disease cycle for the fungi.

Since *F. circinatum* was only isolated at the Bizkaia plantation, with almost 30% of trees with cankers, the highest rate of the four plantations tested, it seems that the presence of the fungus in roots of both symptomatic and asymptomatic trees is related to disease incidence, with no detections for plantations lower than 13%. According to Garbelotto, Smith and Schweigkofler (2008), different inoculum loads were associated with trees displaying different levels of disease symptoms. This could be one of the reasons why *F. circinatum* was only isolated at the Bizkaia plantation. Proximity to the sea and mild temperatures are known to be optimal for disease development caused by *F. circinatum* (Serra-Varela *et al.*, 2017). Distance to the coast is similar for all plantations, except the Araba plantation. However, all plantations had similar climatic conditions, and therefore, no assumptions can be made about influence of these factors in the presence of *F. circinatum* in the plantations tested.

F. circinatum was not isolated from the soils sampled in a *P. radiata* plantation with symptoms of pitch canker disease. Although results have to be interpreted with caution due to the relatively low number of samples, they suggest that the ability of the fungus to survive in soil after a disease outbreak under field conditions is limited, thus, we propose that roots are not infected from soilborne inoculum. The lack of chlamydospores or other survival structures could explain why this fungus does not persist in soil. In a previous study (Serrano *et al.*, 2016), soils of eight *P. radiata* plantations with symptoms of pitch canker disease in Basque Country, were tested for the presence of *F. circinatum*, but the fungus was never detected in any of the soils sampled. In the same study, survival of fungal conidia in infected soil was measured under controlled temperature, but no conidia were recovered after 8 months at temperatures of 20 and 30°C. One possible explanation for our isolation of *F. circinatum* from roots of symptomless mature trees and not from the soil could be that some symptomless but infected seedlings may be moved from nurseries to plantations and once there, the pathogen can persist in a latent stage for a long period in the root system. This latent stage within root tissue could be related to fungal population in roots. Elvira-Recuenco, Iturritxa and Raposo, (2015) found that *F. circinatum* population present in roots of non-symptomatic pine seedlings was lower than the population obtained in the symptomatic ones. Furthermore, following the stress induced when moving asymptomatic but infected pine seedlings from nurseries to forest plantations, *F. circinatum* colonization could progress and disease may develop,

symptoms become visible and the pathogen can spread horizontally by airborne spores or via insects from pine to pine.

Other possible way of root infection is that infested debris or herbaceous plants on soil act as a source of inoculum. *F. circinatum* was recovered after 507 days in up to 27% of the wood pieces from naturally colonized branches and needles placed on soil at two locations (Serrano *et al.*, 2016). We report that senescent segments of herbaceous plants fallen in the soil but infected by the fungus can cause disease in pine seedlings growing next to them. Therefore, one possible explanation for the root colonization is that colonized debris on/in soil may infect roots by spore washing or by walking, cattle, or machinery moving.

Further research on the role that roots of felled trees may play in the dispersal of the disease is needed. Several measures were undertaken when the pathogen was first detected in Europe (Landeras *et al.*, 2005), like an eradication and control program in Spain (RD 637/2006), which included cutting down and elimination of the aerial part of symptomatic trees and demarcation of buffering areas. However, root system of these trees remained in the plantation and therefore it should be investigated if root colonization of felled trees might be an important factor in disease dispersal for the pathogen.

Interestingly, the pathogen was not isolated from *P. pinaster* roots of non-symptomatic and symptomatic trees and this could be due to differences in host susceptibility. Based on lesion length measurement after stem inoculation with a known virulent isolate, several studies have reported *P. radiata* as highly susceptible to the pathogen while *P. pinaster* has shown moderate resistance (Iturrutxa *et al.*, 2013). Besides, no colonization in root systems of *P. pinaster* for both symptomatic and non-symptomatic trees would indicate that maybe there is also a defence strategy for *P. pinaster* not to be colonized as an endophyte without causing any symptoms in the tree.

No genetic variability was found in the whole population obtained from the same plantation, independently from the origin of the isolate: herbaceous plants, pine cankers or pine asymptomatic roots. All isolates shared a common haplotype, the same that is more abundant in this northern region (Berbegal, Armengol and Grünwald, 2013). Likewise, all isolates were only of one of the two mating types (MAT-2), the one that is present in this region (Iturrutxa *et al.*, 2011; Berbegal, Armengol and Grünwald, 2013). The genetic homogeneity of *F. circinatum* isolates sampled in this study can be explained by the relatively short history of the presence of the fungus in the area (Iturrutxa *et al.*, 2011).

Several studies attribute ecological functions to endophytes in plants they are colonizing. These studies refer especially to an increase in resistance or tolerance to plant stress, either of biotic or abiotic conditions (Redman *et al.*, 2011; Hardoim *et al.*,

2015). Increases in tolerance to water stress (Ravel *et al.*, 1997; Bae *et al.*, 2009), in resistance to diseases and herbivory are reported (Siegel *et al.*, 1990; Herre *et al.*, 2007), but there are other studies that do not find any ecological function (at least so far) for the endophytes (commensalism). The physiological and ecological role the fungus plays as endophyte in herbaceous plants and pine roots is unknown. When causing no symptoms in roots of pines seedlings, the growth rate increases and the mycorrhizal community associated become more extensive (Swett and Gordon, 2017). Furthermore, when roots were previously exposed to the pathogen, resistance to posterior stem infection increases (Swett, Kirkpatrick and Gordon, 2016). Bonello, Gordon and Storer (2001) pointed out that the lesion length of trees previously inoculated with the pathogen was shorter than lesions of trees with no prior exposure to the fungus. Therefore, it is possible that the presence of *F. circinatum* in roots confers to mature trees less vulnerability to future infections. This could explain why the fungus was isolated in high proportion from roots of mature non-symptomatic trees in the present study.

These findings suggest that an endophytic stage of *F. circinatum* in pines is an important phase of its lifecycle which precedes a pathogenic lifestyle. Our findings also show the potential risk of spread of *F. circinatum* across non-symptomatic species that serve as reservoirs of inoculum in plantations and may contribute to long distance spread through seedlings from nurseries. Further studies that try to determine the role of *F. circinatum* as an endophyte are needed in order to create a more comprehensive understanding of the evolution, dynamics and ecology of plant-fungi interactions.

Chapter 5: *DE NOVO PINUS PINASTER* TRANSCRIPTOME ASSEMBLY

5.1. Introduction

In the last decade, transcriptomic information has been commonly used in a wide range of biological studies providing fundamental insights into biological processes. In the area of phytopathology, the study of gene expression profiles for both host and pathogen during the infection process is especially useful (Conesa *et al.*, 2016; Carrasco *et al.*, 2017). In RNA-seq experiments, an accurate quantification of gene expression requires a high quality reference for read mapping. In these cases, reads are directly mapped to a reference and abundance of transcripts between treatments and samples can be estimated (Wang, Gerstein and Snyder, 2009). However, in species in which a reference genome is not available or is incomplete, the assembly of reads into a reference has become a challenge (Robertson *et al.*, 2010; Grabherr *et al.*, 2011). This is the case that occupies conifers, in which genomic resources are limited due to the large size and complexity of their genomes (Mackay *et al.*, 2012).

De novo transcriptome assembly for some *Pinus* species, such as *P. patula* (Visser *et al.*, 2015), *P. tecunumanii* (Visser *et al.*, 2018), *P. radiata* (Carrasco *et al.*, 2017), *P. monticola*, (Liu, Sturrock and Benton, 2013) and *P. pinaster* (Canales *et al.*, 2014) have been generated. *De novo* *P. pinaster* draft transcriptome was generated by deep sequencing of different organ and tissues, containing 26,020 unique transcripts. Although the genetic resources available for *P. pinaster* have improved in the last years (SustainPineDB) (Fernández-Pozo *et al.*, 2011; Canales *et al.*, 2014), the fragmented state of the assembly can limit comprehensive transcriptome analysis (Salzberg and Yorke, 2005).

Although alternative splicing events and genes with overlapping regions can difficult the assembly and introduce biases (Conesa *et al.*, 2016), the development in the last decade of new improved algorithms and bioinformatic tools successfully allow the generation of high quality *de novo* assemblies, even for the assembly of short reads (Haas *et al.*, 2013; HersHKovitz *et al.*, 2013; Visser *et al.*, 2018). There is not a consensus about the most appropriate assembly program since diverse studies have shown a high variability in the quality of the assembly when using different tools (Zhao *et al.*, 2011; Bradnam *et al.*, 2013). Several assembly programs such as Trinity (Grabherr *et al.*, 2011), TransABYSS (Robertson *et al.*, 2010), Oases (Schulz *et al.*, 2012) and SOAPdenovoTrans (Xie *et al.*, 2014), has been widely used in *de novo* assembly. Trinity is a program especially developed for the assembly of RNA-seq short reads and is known to perform well and be more sensitive than other assembly programs. Trinity generate *de Bruijn graph* from the large amount of short reads, use an algorithm to determine the different possible branches and paths and finally select the most reasonable transcripts or isoforms. TransABYSS has also been successfully used in the assembly of short reads.

The goal of this chapter is to generate a high quality *de novo* *P. pinaster* transcriptome assembly to build a reference for downstream gene expression analysis in *P. pinaster*-*F. circinatum* interaction. For this purpose we generate 21 preliminary assemblies by

using two different programs (Trinity v.2.4.0 and transABYSS v.1.5.4) to finally merge the best preliminary assemblies into a unique dataset. In addition, these results will contribute with the current *P. pinaster* genetic resources available and could provide novel transcripts involved in defence mechanism in pines.

5.2. Results

5.2.1. Pathogen colonization and symptom development

F. circinatum was observed growing in the resin drop during the first three days. At 4 dpi the pathogen entered within the host tissue in only one plant out of six analysed while at 5 dpi it had penetrated in all of them. During the following days the fungus continued growing within the host and progressed from the tip along the stem. At 8 dpi some of the plants showed visible damage at the inoculation site. At 11 dpi inoculated seedlings had a lesion length of approximately 5 mm in the tip. The sampling times chosen for the RNA-seq assay were: 3 dpi (the fungus has not penetrated within the host and is growing in the resin drop), 5 dpi (the fungus penetrated within host tissue) and 10 dpi (lesion is visible in the shoot tip of all seedlings) (Figure 15 and 16).

Figure 15. A-C: symptoms at the shoot tip of inoculated *Pinus pinaster* seedlings at each sampling time point. A: 3 dpi, no visible symptoms. B: 5 dpi, no visible symptoms. C: 10 dpi, symptoms at the inoculation site. E, G, I: transverse sections of *P. pinaster* inoculated shoot tip visualized under epifluorescent microscope at each sampling time after inoculation. D, F, H: growth of the fungus in the resin drop at each time point. D-E: 3dpi. F-G: 5 dpi. H-I: 10 dpi. White arrows indicate colonization of the fungus within host tissue. Bar 100 µm.

Figure 16. Progression (mm) of a green fluorescent protein (GFP)-tagged *Fusarium circinatum* strain within *Pinus pinaster* shoot tissue at different days after inoculation. The progression of the pathogen within the host tissue served to determine the times of sampling for RNA-seq analyses (3, 5 and 10 dpi).

By the end of the experiment, all inoculated seedlings showed symptoms of disease including discoloured brown stem, necrosis and needle desiccation at the tip (Supplementary Figure 2). Inoculated seedlings showed a lesion length at the tip of $1.5 \text{ cm} \pm 0.59$ (SD) and *F. circinatum* was re-isolated from all 6 tips cultured on FSM. Mock-inoculated seedlings did not show symptoms of disease and the fungus was not recovered from any of them.

5.2.2. RNA isolation and quality analysis

All RNA samples at 3 and 5 dpi passed the quality control parameters established, with a RIN value higher than 8.4. At 10 dpi, the 4 biological replicates for mock-inoculated samples passed control analyses but not 2 of the 4 biological replicates for inoculated ones, with a RIN of 5.4 and 5.8 and a low concentration. Neither passed de library quality control. Therefore, these two samples were discarded for the analysis. A total of 2,576 million reads of 101 bp PE (142 Gb) of raw data were generated for the 22 libraries. After filtering and trimming raw data, 942 million of 87 bp PE reads remain for the downstream analysis, which means that a 9.01 % of not quality reads was removed (Supplementary Table 1).

5.2.3. Preliminary assemblies

A total of 21 different Trinity and TransABySS preliminary assemblies were built, showing differences in quality based on the parameters used (Table 3). None of the assemblies produce transcripts with unknown bases and the GC content were similar between them. In silico normalization of Trinity assemblies produced highest number of transcripts, mean length and better N50 values. However, in non-normalized

assemblies more fragments were mapped and the percentage of good mapped contigs was higher (Supplementary Table 2). Therefore, all Trinity assemblies (normalized and non-normalized) were used for building the final *de novo* transcriptome assembly. When comparing Trinity and TransABYSS assemblies at the same kmer value, Trinity showed better quality statistics, therefore TransABYSS assemblies with the same kmer value as Trinity were discarded. As Trinity does not allow the use of kmer values higher than 32, all TransABYSS assemblies with higher kmer value were conserved.

Table 3. Statistics for each TransABYSS and Trinity assembly. N seq: number of transcripts; N bases: number of bases; Mean length: mean length of the transcripts; N50: N50 value; Ns: number of unknown bases; % GC: guanine and cytosine content; trinity-N: in silico normalized trinity assembly; trinity-nN: non-normalized trinity assemblies. * Best quality preliminary assemblies selected to generate the final assembly.

Software	kmer	N seq	N bases	Mean length	N50	Ns	% GC
transABYSS	21	155,002	130,062,963	839	987	0	0.42
transABYSS	25	177,632	156,537,679	881	1073	0	0.42
transABYSS	29	194,496	171,614,178	882	1077	0	0.42
transABYSS *	37	216,505	191,809,279	885	1082	0	0.42
transABYSS *	45	175,922	157,983,806	898	1149	0	0.42
transABYSS *	53	227,127	200,483,638	882	1067	0	0.42
transABYSS *	61	216,262	192,122,235	888	1065	0	0.42
transABYSS *	69	185,869	168,685,723	907	1094	0	0.43
transABYSS *	77	123,114	116,609,159	947	1164	0	0.43
trinity-N *	19	220,217	218,692,491	993	1248	0	0.42
trinity-N *	21	221,484	306,982,861	1386	2013	0	0.42
trinity-N *	23	222,996	324,494,700	1455	2117	0	0.42
trinity-N *	25	254,226	338,343,159	1330	1939	0	0.42
trinity-N *	27	251,125	339,266,456	1350	1964	0	0.42
trinity-N *	29	245,476	338,191,077	1377	1997	0	0.42
trinity-N *	31	240,841	337,312,739	1400	2030	0	0.42
trinity-nN *	19	192,124	187,315,834	974	1246	0	0.42
trinity-nN *	21	193,012	227,145,680	1176	1681	0	0.42
trinity-nN *	23	194,285	234,918,497	1209	1754	0	0.42
trinity-nN *	25	214,734	247,127,783	1150	1647	0	0.42
trinity-nN *	27	213,152	249,048,800	1168	1686	0	0.42
Merged assembly	19-77	49,624	68,941,872	1389	1861	0	0.45

5.2.4. Merged assembly

A total of 18 high quality preliminary assemblies were used as input for the EviGene pipeline to build the *P. pinaster de novo* transcriptome (Table 3). When merging best quality assemblies, EviGene pipeline classified the 90.7 % of the sequences as

redundant and uninformative, which means that 49,624 sequences (9.3 %) were non redundant coding genes and were used to generate the *P. pinaster* transcriptome.

BUSCO analysis against the embryophyta_odb9 lineage database identified 1,261 (87.57 %) complete BUSCOs in a total of 1,440 BUSCO groups searched, from which 1,109 (77.01 %) were single copy, 152 (10.56 %) duplicated, 145 (10.07 %) missing and 34 (2.36 %) fragmented. Regarding the eukaryote_odb9 database, 294 completed (97.03 %) BUSCOs were identified out of 303 groups searched, and 213 (70.30 %) were single copy, 81 (26.73 %) were duplicated, 7 (2.31 %) were missing and only 2 (0.66 %) fragmented (Supplementary Figure 3).

5.2.5. Annotation

Coding regions were predicted for 46,576 sequences with GeneMarkS-T. Of the aligned sequences, 29.15 % (8,584) were classified as non-pine origin contigs, mainly belonging to the *Fusarium* genera (*F. mangiferae*, *F. fujikuroi*, *F. proliferatum*, *F. nygamai*, *F. oxysporum*). After filtering contaminants, non-frame selected and unannotated sequences, the final *P. pinaster de novo* transcriptome assembly were represented by 24,375 sequences, from which 17,593 (72.18 %) were full length genes. Best hit selection of BLAST alignment against the 4 databases generated a total of 20,864 (85.60 %) unique sequences, from which 9,957 (47.72 %) were informative. EggNOG annotation predicted 23,674 sequences with family assignment, 5,425 with at least one pathway (KEGG) assignment and 22,863 predicted protein domains. EggNOG associated 7614, 11096 and 5050 GO terms to biological process (BP), cellular compartment (CC) and molecular function (MF) category, respectively. InterProScan predicted 20,188 protein domains and associated 6293, 1929 and 9346 GO terms to BP, CC and MF category, respectively (Table 4). Among the top MF terms were catalytic activity, binding, hydrolase activity, organic-cyclic compound binding, transferase activity, ion binding and protein binding. The most common BP terms were cellular metabolic process, organic substance metabolic process, response to stimulus and biological regulation. CC terms were cell, cell part, intracellular, organelle, membrane-bounded organelle and cytoplasm (Figure 17).

Table 4. *De novo Pinus pinaster* transcriptome annotation after filtering contaminants, non-frame selected and unannotated sequences.

<i>De novo P. pinaster</i> transcriptome annotation	
Frame selected	24,375
Complete	17,593
Sequence unique alignment	20,864
Informative (known description)	9,957
EggNOG functional annotation	
Description	23,674
KEEG	5,425
Protein domain (EggNOG)	22,863
GO BP (total/unique)	7,614
GO CC (total/unique)	11,096
GO MF (total/unique)	5,050
InterProScan functional annotation	
Protein domain (EggNOG)	20,188
Pathways	4,133
GO BP (total/unique)	6,293
GO CC (total/unique)	1,929
GO MF (total/unique)	9,346

GhostKOALA assigned 12,741 K numbers (of 35,090) mainly classified according to the KEGG Orthology System in the functional categories genetic information processing, environmental information processing and cellular processes. Mercator assigned functional annotation to 15,347 *P. pinaster* transcripts to MapMan.

Figure 17. Distribution of molecular function (MF), biological process (BP) and cellular compartment (CC) Gene Ontology (GO) terms in the *Pinus pinaster* assembled transcriptome.

5.3. Discussion

In the forest field and particularly for conifers, sequencing of genomes is still costly and challenging, likely due to their huge genome size. We used a transcriptome assembly approach to generate a high quality *de novo* *P. pinaster* reference. This reference was constituted by 24,375 sequences, from which 17,523 were full length genes. The completeness and contiguity of the assembly is affected by several parameters such as the assembly programs and parameters set (Zhao *et al.*, 2011). Comparison of BUSCO results of the generated *P. pinaster* transcriptome with the last *Pinus* transcriptomes published reflects the effectiveness of the assembly pipeline used. We identified 87.57 % of complete BUSCOs, of which the 10.56 % were duplicated, similar to the results obtained for the recently published *P. patula* and *P. tecunumanii de novo* assemblies (Visser *et al.*, 2018). Therefore, this *P. pinaster* quality transcriptome represents a valuable genomic source for further research of maritime pine trees.

In order to maximise diversity of assembled transcripts, we generated a total of 21 different preliminary assemblies, 12 and 9 assemblies for Trinity and TransABYSS programs, respectively. As previously reported in other studies (Zhao *et al.*, 2011; Bradnam *et al.*, 2013), differences between assembly programs are evident. We found Trinity generally performs better than TransABYSS, and differences were found within Trinity assemblies when in silico normalization or non-normalization was applied. In silico normalization is known to reduce the quantity of the input reads to be assembled while maintaining transcriptome complexity and capability for full length transcript reconstruction, improving assembly efficiency and computational requirements (Brown *et al.*, 2012; Haas *et al.*, 2013). Grabherr *et al.*, (2013) found that normalization of RNA-seq reads from fission yeast and mouse resulted in full length reconstruction similar to the entire read dataset. In the present study, in silico normalization performed better for the number of transcripts, mean length and N50 values, while more fragments were mapped and the percentage of good mapped contigs was higher in non-normalized assemblies.

The selection of a single or multi kmer strategy significantly influences in the quality of the assembly (Zhao *et al.*, 2011; Durai and Schulz, 2016). Short kmer lengths will use most of the reads and increase the chance for all kmers to overlap, which results in more complete assemblies, but the risk of missassemblies is higher due to path ambiguity. The long kmer assemblies use fewer reads, but resolve repeats and tend to have fewer mistakes than short kmer assemblies. Therefore, the sensitivity of the assembly increase by using a multi kmer approach and then merging the resulting assemblies for an accurate gene set reconstruction (Surget-Groba and Montoya-Burgos, 2010; Zhao *et al.*, 2011; Durai and Schulz, 2016). Because Trinity does not allow kmer values higher than 32, we maintained TransABYSS preliminary assemblies with kmer values of ranging from 37 to 77. These results indicate that the use of different assembly programs, with different parameters (such as kmer values and normalization or non-normalization of reads), is a preliminary approach that warranties the selection of the best assemblies. This strongly enhance the quality of the final assembly.

The selected 18 preliminary assemblies used as input for EviGene pipeline classified the 9.3 % of the reads as non-redundant coding genes, which means 49,624 sequences. This result was similar to the one obtained by Visser *et al.*, (2015) in which the 7 % of *P. patula* reads were maintained as non-redundant when using a similar pipeline. After filtering non-pine sequences, contaminants, non-frame selected and unannotated sequences, the 49 % of the transcripts were successfully annotated, represented a wide variety of GO terms in the MF, BP and CC categories. The most represented GO terms are indicative of the quick and extensive metabolic activity in the analysed tissue, as reported in the interaction of *Pinus* with *F. circinatum* in other studies (Carrasco *et al.*, 2017; Visser *et al.*, 2018). The remaining 51 % of the transcripts were predicted as potential coding genes by EviGene pipeline but they were not annotated when searched by similarity against the four databases used, or if annotated they were discarded as contaminants. Likewise, Visser *et al.*, (2015) annotated the 60 % of the transcripts of assembled *P. patula* transcripts.

Sequencing of shoot tissue of inoculated and mock-inoculated seedlings at 3, 5 and 10 dpi generated 1,288 million reads and quality filtering of data resulted in the removal of a 9 % of the reads, similar to results obtained in other studies (Visser *et al.*, 2015; Carrasco *et al.*, 2017), indicating the high quality of the original reads. Indeed, we followed a PE strand specific sequencing approach and included 4 BR, which are factors known to influence the efficacy of the assembly because are more informative (Ozsolak and Milos, 2011; Conesa *et al.*, 2016).

The high quality of *de novo* *P. pinaster* transcriptome assembly generated means a valuable genomic source for future studies and can be integrated in the *P. pinaster* database available (SustainPineDB). This transcriptome will be used as a reference for the study of gene expression during the interaction of *P. pinaster* with *F. circinatum*, which is the focus of the next chapter.

Chapter 6: EXPRESSION PROFILE IN *FUSARIUM*
CIRCINATUM AND *PINUS PINASTER*
INTERACTION

6.1. Introduction

Progress in NGS technology and bioinformatics has allowed more in-depth investigation of transcriptomic responses in non-model species such as pines. Dual RNA-seq allows the characterization of host and pathogen gene expression profiles simultaneously, highlighting molecular processes involved in the defence and virulence mechanism, respectively (Westermann, Gorski and Vogel, 2012; Hayden *et al.*, 2014; Meyer *et al.*, 2016; Naidoo *et al.*, 2018). The transcriptome profile of *P. radiata* seedlings inoculated by *F. circinatum* was recently published (Carrasco *et al.*, 2017). They identified genes involved in mechanisms related to physical and mechanical barriers as the main differential expressed gene group to restrict pathogen invasion, including genes related to phenylpropanoid metabolism, cell wall biosynthesis and remodelling and proteolysis, as well as chitinase and thaumatococin. The second most important category of genes detected were those involved in phytohormone signalling including ABA, SA, JA and auxins. Similarly, Visser (2019) highlighted the role of phytohormones in resistance to the pathogen by comparing transcriptional profiles of *P. patula* and *P. tecunumanii* inoculated seedlings, species with disparate disease phenotypes.

Much effort has been dedicated to understand phytohormone signalling in plant defence, mainly dependent on SA, JA and ET, which interactions can range from synergic to antagonistic, as explained before. By contrast, knowledge in fungal hormone production and how this affects plant hormone homeostasis is limited. A remarkable aspect of *Fusarium* species in the *Fusarium fujikuroi* species complex (FFC), is the ability to synthesize phytohormones, including gibberellins (Bömke and Tudzynski, 2009; Troncoso *et al.*, 2010) and auxins (Tsavkelova *et al.*, 2012), that contribute to plant disease. However, the underlying molecular mechanism as well as their role in plant interactions remains unclear. Two mechanisms have been suggested: perturbing plant processes to favour invasion and nutrient uptake, and/or acting as signals for the fungus to engage appropriate physiological processes to get adapted to the new environment (Chanclud and Morel, 2016). GA production has been well described in the rice-infecting fungus *F. fujikuroi* (Bömke and Tudzynski, 2009) and a correlation between GA levels and virulence has been reported (Desjardins *et al.*, 2000). GA biosynthetic genes are organized in a gene cluster, and while most of the species of the FFC have the entire GA biosynthetic gene cluster, *F. circinatum* was reported to have only one gene (Malonek *et al.*, 2005; Bömke and Tudzynski, 2009). IAA, the most common form of auxins, can be synthesized from tryptophan by the indol-3-acetamide (IAM) pathway, and IAM-related genes have been reported in four *Fusarium* pathogenic fungi: *F. verticillioides*, *F. oxysporum*, *F. fujikuroi* and *F. proliferatum* (Tsavkelova *et al.*, 2012). In the same study, the deletion of an IAM-related gene resulted in drastic reduction of IAA production in *F. proliferatum*. Similarly, *F. oxysporum* transgenic lines containing two IAM genes significantly produce more IAA than the wild type when infecting *Orobancha* (Cohen *et al.*, 2002). ET producer fungi ranged from necrotrophic, like *B. cinerea*, to biotrophic, such as *F.*

oxysporum f. sp. *pini*, and a role in perturbing the plant phytohormone homeostasis has been suggested (Chanclud and Morel, 2016). Silicon treatment has been shown to induce brown spot resistance in rice against *Cochliobolus miyabeanus* by disarming fungal ET (Van Bockhaven *et al.*, 2015). In the case of *Colletotrichum* sp. pathogens, ET is required for the formation of appressoria (Flaishman, 1994). To our knowledge, expression of *F. circinatum* genes related to hormone biosynthesis or signalling, besides GA, has not been studied.

The aim of this chapter is to elucidate the molecular processes involved in the moderate resistance of *P. pinaster* to *F. circinatum*, as well as determine the key steps where the pathogen could be manipulating host defence to its own benefit, leading to host susceptibility. For this purpose, we determine by a dual RNA-seq approach the expression profiling of both organisms during host-pathogen interaction at different times after inoculation (3, 5 and 10 dpi).

6.2. Results

6.2.1. Mapping to the host and pathogen reference transcriptomes

Kallisto mapped a total of 964 million reads to the *F. circinatum* and *P. pinaster* combined dataset, which means the 74.93 % of the reads were mapped. In total, 72.97 % of the reads were mapped to the *P. pinaster de novo* transcriptome reference, with similar mapping percentage between time points and between inoculated and mock-inoculated samples. No reads of the mock-inoculated samples mapped to the *F. circinatum* reference transcriptome. For inoculated samples, 1.95 % of the reads mapped to *F. circinatum* and an increase in the number of mapped reads was observed when increasing the sampling time due to the growth of the pathogen (Table 5).

Table 5. Percentage of total mapped reads for each species. Number of differential expressed (DE) genes for *Pinus pinaster* and high confident (HC) differential expressed genes for *Fusarium circinatum* at each time point in inoculated samples (p-value < 0.05; $|\log_2(\text{Fold Change})| \geq 0.5$). Ppin: *P. pinaster*; Fcir: *F. circinatum*.

	Total mapped reads (%)	Ppin mapped reads (%)	Fcir mapped reads (%)	DE genes Ppin (up-down regulated)	HC genes Fcir
Mock inoc-3dpi	75.86	75.86	0	1,438-679	4,07
Inoc-3dpi	75.7	75.55	0.14		
Mock inoc-5dpi	75.78	75.78	0	3,783-2447	4,366
Inoc-5dpi	75.95	75.31	0.64		
Mock inoc-10dpi	73.71	73.7	0	6,577-6102	4,372
Inoc-10dpi	72.58	61.65	10.92		
Total	74.93	72.98	1.95	13,323	4,374

PCA for *P. pinaster* and *F. circinatum* rlog data classified separately inoculated and mock-inoculated samples. The replicate samples show a high similarity with respect to the first two principal components for each time point. A small within group variance and a good separation of groups reflects the good quality of the analysis (Supplementary Figure 4).

6.2.2. Host and pathogen DE genes

A total of 13,323 DE genes were identified among inoculated *P. pinaster* samples compared to mock-inoculated ones. A notable increase of DE genes from 3 to 10 days post-inoculation was observed (Table 5).

For *F. circinatum*, at 3, 5 and 10 dpi 93.17 % (4,070 genes), 99.8 % (4,366 genes) and 99.9 % (4,372 genes) of the DE genes were considered HC expressed genes, respectively (Table 5). When comparing DE genes at 3 versus 5 dpi in inoculated seedlings, 3,427 genes were down-regulated and only 11 up-regulated. A similar pattern was observed between 3 versus 10 dpi with 4,307 up-regulated and 21 down-regulated genes and between 5 and 10 dpi where 1,599 genes were down-regulated and 24 up-regulated.

6.2.3. *Pinus pinaster* transcriptome profile

6.2.3.1. Over-represented GO terms in *Pinus pinaster* DE gene clusters

Significant *P. pinaster* DE genes (13,323 genes) were classified in 53 clusters by using Hmisc R package. Due to the complexity, we set the $|\log_2(\text{Fold Change})|$ cutoff value to 1 (8,802 DE genes) in order to reduce the number of clusters. DE genes were classified into 30 clusters, from which 12 had enriched GO terms and represent the 92.7 % of the DE genes (Figure 18; Supplementary Table 3).

Cluster 1 was up-regulated at 5 and 10 dpi and slightly at 3 dpi. Terms included in the BP category were related to response to stimulus, response to chitin, regulation of ROS, response to oxidative stress, positive regulation of cell death and phosphorylation transduction, all common responses related to biotic stress. Phytohormone signalling was also evident, since terms related to ET, JA and SA were detected in cluster 1, as well as SAR mediated by SA terms. In the MF category, terms were related to glycosyltransferase activity, catalytic activity, lipase activity and lyase activity. In the CC category, only two terms were enriched, plasma membrane and cell periphery.

Genes in cluster 2 were highly up-regulated at all time points with an increase from 3 to 10 dpi, and highly up-regulated at 3 dpi in comparison with the other clusters. In the BP category we found terms related to cellular metabolic processes, cellular localization, cellular component organization or biogenesis, cytosolic transport, protein-containing complex subunit organization, vesicle-mediated transport and proteasome-mediated ubiquitin-dependent protein catabolic process. In the MF, terms

were mostly related to binding activities, protein binding, heterocyclic compound binding, nucleotide binding and phosphorylase and hydrolase activities. CC terms were related to protein containing complex, vacuole membrane, membrane coat, vacuole coat and site of polarized growth.

Terms in cluster 3 in the BP category were related to flavonoid and anthocyanin metabolic processes, hormone transport, response to oxidative stress, response to jasmonic acid and auxin polar transport. No terms were enriched in the MF category. NADH dehydrogenase complex, vacuole and mitochondria were found in the CC category. Genes of this cluster were mostly up-regulated at 10 dpi.

In cluster 5 only three terms in the BP were enriched, related to transport. In cluster 11, a response to ethanol term was enriched in the BP.

Genes classified in clusters 18, 19, 27 and 29 were down-regulated mostly at 10 dpi and slightly at 3 and 5 dpi (Figure 18). Terms in these clusters were related to growth, development, reproduction, morphogenesis and photosynthesis. Terms related to isoprenoid, terpenoid and carotenoid metabolic processes were found.

Figure 18. Heatmap representing clusters with gene ontology (GO) enrichment for the significant *Pinus pinaster* differential expressed (DE) genes (p -value < 0.05 ; $|\log_2(\text{Fold Change})| = 1$) at each time point (3, 5 and 10 days post-inoculation).

6.2.3.2. *Pinus pinaster* phytohormone signalling

Four phytohormones seem to have a major role in *P. pinaster* defence response; they are JA, ET, SA and auxins. ABA, CK and GA seem to be suppressed. Log₂(Fold Change) values for genes related to phytohormone signalling is provided as supplementary material (Supplementary Table 5).

6.2.3.2.1. Jasmonic acid

JA is synthesized from linolenic acid by the activity of diverse enzymes that catalyses essential steps in JA synthesis pathway. These enzymes include lipoxygenases (LOX), allene oxidase synthase (AOS), allene oxidase cyclase (ACO) and 12-oxo-PDA-reductase (OPR). During *F. circinatum* infection LOX and OPR were up-regulated at all time points in inoculated seedlings. AOC was only up-regulated at 10 dpi, while AOS was up-regulated at 5 and 10 dpi but also down-regulated at 10 dpi. JA is synthesized and conjugated with isoleucine to produce jasmonoyl-isoleucine (JA-Ile), the active form, and is then perceived by the receptor Coronatine Insensitive 1 (COI1). In the presences of JA-Ile, COI1 degrade JAZ (jasmonate ZIM domain) proteins, which are repressor of JA signalling. JAZ proteins bind to MYC2 transcription factor family, which regulates expression of JA responsive genes. Interestingly, although JA biosynthesis seems to take place since 3 dpi, a COI1 gene was down-regulated at 5 and 10 dpi. A JAZ gene was highly up-regulated at all time points, while MYC transcription factor was down-regulated at 5 and 10 dpi. TOPLESS (TPL) and NOVEL INTERACTOR of JAZ (NINJA) corepressors were down- and up-regulated at 10 dpi, respectively. Jasmonate methyl transferase (JMT) genes were up-regulated at all time points, indicating JA conversion to methyl jasmonate (MeJA) for systemic signalling. JA induce genes encoding PR proteins such as chitinases (PR3) and β -1,3-glucanases (PR2) (Davis *et al.*, 2002). A total of 15 *chitinase* genes were up-regulated at all time points, mainly belonging to chitinase class VII, and a PR2 gene was also up-regulated. Some PR9 can be induced by methyl jasmonate (Curtis *et al.*, 1997), and we detected up-regulation of 49 PR9 genes at all times, especially at 10 dpi. However, 14 genes were also down-regulated at 10 dpi (Figure 19; Supplementary Table 6).

6.2.3.2.2. Ethylene

ET is synthesized from the amino acid methionine and converted to S-adenosyl-L-methionine (SAM) by the action of the enzyme SAM synthase. Two enzymes catalyse the final conversion of SAM to ET, first the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and finally the ACC-oxidase (ACO). In inoculated *P. pinaster* seedlings, ACS was up-regulated at all time points while ACO was up-regulated at 5 and 10 dpi. ET is perceived by endoplasmic reticulum-associated receptors (ETR), upstream of several positive regulators of ET signalling. ETR2 was up-regulated at 5 and 10 dpi; however, ETR1 was down-regulated at 10 dpi. The downstream ETHYLENE INSENSITIVE 2 (EIN2), essential for positive regulation of ET signalling, was down-regulated at 5 and 10 dpi. EIN3 and EIL1 (EIN3-LIKE1) are downstream of EIN2, and were up-regulated at 5 and 10 dpi. ERF/AP2 ethylene-responsive transcription factors were up-regulated at all

time points, especially at 5 and 10 dpi. *EBF1* (EIN3-binding F-box protein 1) degrade EIN3/EIL1 in the absence of ET and was down-regulated at 5 dpi.

6.2.3.2.3. Salicylic acid

Isochorismate synthase (ICS) is the key enzyme involved in SA biosynthesis from the chorismate pathway and a *ICS* gene was down-regulated at 5 and 10 dpi. The non-expressor of PR1 (*NPR1*) protein directly binding SA and interacts with the bZYP transcription factor in the TGA family responsible of SA regulation. No differential expression of *NPR1* was found, however, *TGA* was up-regulated at 10 dpi, suggesting SA signalling. Two *NPR1* homologs, *NPR3* and *NPR4*, were shown to bind SA and target *NPR1* for ubiquitin-mediated protein degradation (Fu et al. 2012), but none of them were differentially expressed. SA can be also synthesized from the PAL pathway, and *PAL* was up-regulated at all time points. However, some *PAL* genes were also down-regulated. SA accumulation require two proteins, *EDS1* (enhanced disease susceptibility 1) and *PAD4* (lipase) (Pieterse *et al.*, 2012). Both *EDS1* and *PAD4* were up-regulated at 5 and 10 dpi. SA can be glycosylated by UDP-glycosyltransferase (UGT) to the inactive form 2-O- β -D-glucoside (SAG). Terms related to glycosyltransferase activity were enriched in cluster 1 genes. PR1 is a PR protein commonly induced in defence response and its expression is SA responsive. Three *PR1* genes were up-regulated at all time points (Figure 19). PR5 is SA, JA and ABA-responsive (Wang *et al.*, 2010) and 10 *PR5* genes were up-regulated at all time points, although two genes were also down-regulated (Figure 19, Supplementary Table 6).

6.2.3.2.4. Auxins

Auxins are mostly synthesized from tryptophan by the action of diverse enzymes like indole-3-pyruvate monooxygenase. We found two *indole-3-pyruvate monooxygenase* genes down-regulated at all time points, although one gene was also up-regulated at 10 dpi. TIR1 (Transport Inhibitor response) is receptor of auxins (Kepinski and Leyser, 2005) and a *TIR1* gene was up-regulated at 10 dpi. The family of transcriptional repressors auxin/indole-3-acetic acid (Aux/IAA) suppress the activity of transcriptional activators of the auxin response factor (ARF) family (Tiwari, 2004). Auxins promote the interaction of Aux/IAA proteins with the ubiquitin protein ligase SCF^{TIR1} for Aux/IAA degradation. Two *Aux/IAA* family genes were down-regulated at 10 dpi and 9 *ARF* genes were also down-regulated at 5 and 10 dpi. Small auxin up RNAs (*SAURs*), the largest family of auxin response genes, were up- and down-regulated at 10 dpi, and several auxin-responsive genes were up- and down-regulated at all time points. Gretchen Hagen 3 acyl acid amido synthetase family proteins (GH3) inactivate IAA by the transformation of IAA-amido conjugates (Woodward and Bartel, 2005) and *GH3* genes were both up- and down-regulated at 5 and 10 dpi. The enzyme IAA carboxyl methyltransferase (IAMT) catalyses the conversion of IAA into the inactive form methyl IAA (MeIAA) (Qin *et al.*, 2005) and four *IAMT1* genes were up-regulated at all dpi. The auxin efflux carriers PINFORMED (*PIN*) and P-glycoproteins (*PGP*) genes were both down-regulated at 5 and 10 dpi, while a *AUX3* gene, an auxin influx transporter related gene, was up-regulated at all time points.

6.2.3.2.5. Absciscic acid

Some *9-cis-epoxycarotenoid dioxygenase* (*NCED*) genes, which participate in ABA biosynthesis, were up-regulated at all dpi, although one *NCED* gene was also down-regulated at 10 dpi. Zeaxanthin epoxidase (*ZEP*) also participates in ABA biosynthesis and was down-regulated at 10 dpi. Furthermore, ABA irreversible degradation was observed since an *ABA 8-hydroxylase* gene was up-regulated at 5 and 10 dpi, which indicates ABA is converted to phaseic acid, with low activity. However, one *ABA 8-hydroxylase* gene was also down-regulated at 10 dpi. ABA signalling transduction is inhibited by type 2C protein phosphatases (PP2C), and *PPC2* was up-regulated at 5 and 10 dpi, although one gene was also down-regulated. Thus, ABA does not seem to have a major role in *P. pinaster* response to *F. circinatum*.

6.2.3.2.6. Gibberellic acid

Two key genes involved in GA biosynthesis, *ent-kaurene synthase* (*KS*) and *ent-kaurene oxidase* (*KO*), were down-regulated at 5 and 10 dpi. GA 20-oxidases (*GA20ox*) gene, involved in conversion of GA₁₂ to the active forms of GAs, was also down-regulated. GA 2-oxidases (*GA2ox*) convert bioactive gibberellins to their inactive form, and a *GA2ox* gene was highly up-regulated at all time points. These results suggest GA suppression.

6.2.3.2.7. Cytokinins

CKs bind to a HK receptors and this initiates a signalling phosphorylation cascade which ultimately activates a family of transcription factors, ARR. We founded down-regulation of *HK* genes at 5 and 10 dpi. No changes in *ARR* genes were detected. A *CK-O-glucosyltransferase* gene was up-regulated at 5 and 10 dpi, which participates in CK degradation. Therefore, CK signalling seems to be suppressed in inoculated seedlings at 5 and 10 dpi, with no CK signalling activity at 3 dpi.

6.2.3.2.8. Brassinosteroids

Some genes involved in BR biosynthesis were up-regulated at 5 and 10 dpi (*DET2*, *BR6OX*) while others were down-regulated (*DWF4* and *DWF1*). The receptor-like kinases BRI1 can physically bind BRs and can interact with BAK1, a co-receptor BRI1-associated receptor kinase (Li *et al.*, 2002). We found down-regulation of a *BRI1* gene at 5 and 10 dpi. The downstream transcription factors BZR1 and BES1 regulate BR signalling by activating or repressing BR biosynthetic genes via a feedback loop. *BES1/BZR1* was up-regulated at 5 and 10 dpi. Brassinosteroid-responsive RING-H2 (*BRH1*) was also up-regulated at all time points. However, BRH1 is not only involved in response to BR stimulus, but also in response to chitin, and we found up-regulation of *chitinase* at all dpi.

Figure 19. Expression pattern of differential expressed (DE) genes related to pathogenesis related (PR) proteins in *Pinus pinaster* inoculated seedlings. For each PR protein, the average of the $\log_2$ (Fold Change) value is represented at 3, 5 and days post-inoculation. Error bars represent the standard error of the mean.

6.2.3.3. *Pinus pinaster* disease resistance protein

The most highly up-regulated gene at all dpi was annotated as an unknown disease resistance gene, with $\log_2$ (Fold Change) values of 20.1, 31.8 and 23.5 at 3, 5 and 10 dpi respectively, which means around 3-times fold the second most up-regulated gene. BLASTP alignment against the non-redundant NCBI database resulted in best hits corresponding to disease R proteins of *Picea sitchensis* (unknown), *Pinus lambertiana* (CC-NBS-LRR like), *P. monticola* (CC-NBS-LRR) and *P. taeda* (NBS-LRR), with identities ranging from 46 to 64 %. The *P. sitchensis* unknown disease R protein, matched with a query coverage of 98 %, identity of 56.23 % and an E-value of 0.0. This protein had a LRR, NB-ARC (nucleotide-binding adapter shared by APAF-1, R proteins and CED-4) and RPW8 (Resistance To Powdery Mildew 8) family domains. BLASTP against the model species *Arabidopsis* result in best hits to an ADR1-like 1 gene, that codes for NB-LRR ADR1 proteins, with a query coverage of 96-71 %, identity of 35-37 % and significant e-values.

6.2.3.4. *Pinus pinaster* cell wall related genes

Genes related to cell wall modifications were differentially expressed. Genes coding for xyloglucan galactosyltransferase, an enzyme that participates in galactose binding within the xyloglucan structure, were up-regulated at 5 and 10 dpi. Several *xyloglucan endotransglucosylase hydrolase* genes were both up- and down-regulated. Expansins

related genes were also induced, mostly up-regulated at 5 and 10 dpi, although some genes were also down-regulated. A *cinnamyl alcohol dehydrogenase* gene was also up-regulated at all time points. In *A. thaliana* this enzyme is involved in the synthesis of lignin (Sibout, 2005). Several *laccase* genes were both up- and down-regulated, involved in lignin degradation. We found down-regulation of cellulose synthase genes, especially at 10 dpi, as well as *CESA1*, a catalytic subunit of cellulose synthase that is responsible for the production of cellulose in the primary wall (Endler and Persson, 2011). *Pectinesterase*, *polygalacturonase* and *pectate lyase* genes were also up- and down-regulated (Supplementary Table 7).

6.2.3.5. *Pinus pinaster* terpenoid related genes

Some terpenoid genes related to oleoresin biosynthesis were up-regulated from the early stage at 3 dpi, such as *α-bisabolene synthase* and *monofunctional isopimaradiene synthase*. One *Alpha-pinene synthase* gene was up-regulated at 5 and 10 dpi, however five genes were down-regulated at 10 dpi. Other genes related to oleoresin biosynthesis were down-regulated at 10 dpi such as *carene synthase*, *δ-selinene synthase* and *longifolene synthase* (Supplementary Table 8).

6.2.4. *Fusarium circinatum* transcriptome profile

6.2.4.1. Over-represented GO terms in *Fusarium circinatum* DE gene clusters

Significant DE genes for *F. circinatum* (p-value < 0.05; $|\log_2(\text{FoldChange})| > 0.5$) were classified into 7 clusters (Figure 20) and 2 of them had enriched GO terms. These two clusters (cluster 3 and 4) represent 98 % of the DE gene dataset and almost all of them (92.07 %) were classified into cluster 3 (Supplementary Table 4). The pattern of expression of both clusters was similar, increasing from 3 to 10 dpi (Figure 20).

In cluster 3, terms were related to generation of precursor metabolites and energy, such as mitochondrial ATP synthesis coupled electron transport, establishment of protein localization, intracellular transport, oxidative phosphorylation and oxidation-reduction process. In the CC category terms related to mitochondrion, ribosome, cytoplasm, organelle membrane and endoplasmic reticulum. Phospholipid binding, phosphatidylinositol binding, lipid binding and structural constituent of ribosome were enriched in the MF.

In cluster 4, terms were related to perception and metabolic processes. In the BP category, terms were related to regulation of cellular and metabolic processes, regulation of cyclin-dependent protein serine/threonine kinase activity, positive regulation of biological process and signal transduction. Site of polarized growth was the only term enriched in the CC. Osmosensor activity and cyclin-dependent protein serine/threonine kinase regulator activity terms were found in the MF category.

Figure 20. Heatmap representing clusters with gene ontology (GO) enrichment for *Fusarium circinatum* FPKM values of the differentially expressed (DE) genes at each time point (3, 5 and 10 days post-inoculation).

6.2.4.2. *Fusarium circinatum* hormone production related genes

In the present study, two genes related to GA biosynthesis (*gibberellin cluster-C13-oxidase*, *gibberellin cluster-GA14-synthase*) were induced by *F. circinatum* between all time points. Genes that participate in geranyl geranyl diphosphate (GGDP) and entkaurene synthesis (*gibberellin cluster-kaurensynthase* and *gibberellin cluster-GGPP-synthase*) were also induced, with an increase from 3 to 10 dpi (Figure 21, Supplementary Table 9). Gibberellins in fungi are synthesized from acetyl-CoA. In the fungus *G. fujikuroi*, GA12-aldehyde is first 3-beta-hydroxylated (catalysed by P450 monooxygenase) to GA14-aldehyde and then oxidized to GA14. Then, GA14 is converted to GA4 and finally desaturated to produce GA7 and the active gibberellin GA3.

ET produced by fungi is synthesized from 2-keto-4-methylthiobutyric acid, derived from methionine, or from 2-oxoglutarate (Van Bockhaven *et al.*, 2015). Two genes (*2-oxoglutarate-dependent ethylene succinate-forming enzyme* and *2-keto-4-methylthiobutyrate-dependent ethylene-forming enzyme*) coding enzymes involved in ET biosynthesis were up-regulated by *F. circinatum* at all time points, especially at 10 dpi (Figure 21). An auxin efflux gene was also up-regulated at all time points (Figure 21, Supplementary Table 9).

Isochorismatase hydrolase (ICSH) family proteins catalyse the hydrolysis of isochorismatase, a key metabolite for SA biosynthesis from the chorismate pathway. Two *ICSH* genes were up-regulated by *F. circinatum* and showed increased expression over time (Figure 21, Supplementary Table 9).

Figure 21. Expression profile of differentially expressed (DE) genes related to virulence by *Fusarium circinatum*. For each gene, FPKM values at each time point (3, 5 and 10 days post-inoculation) is indicated.

From the DE *F. circinatum* genes mentioned above, 4 of them showed hits to the PHI database with E-values $< 1e^{-4}$ (Supplementary Table 12). Knockout of these genes in other pathogens showed reduced virulence in their hosts. Interestingly, one of this genes (*2-keto-4-methylthiobutyrate-dependent ethylene-forming enzyme*) presented a 90 % of similarity with a *F. oxysporum* gene, which knockout resulted in reduced virulence in tomato plants (Niño-Sánchez *et al.*, 2016).

6.2.4.3. *Fusarium circinatum* cell wall degrading enzymes related genes

Genes related to CWDEs were DE between time points in *F. circinatum* including endoglucanases and β -glucosidases for degradation of cellulose, xylanolytic enzymes, pectinolytic enzyme for pectin degradation, as pectate lyase, polygalacturonases, and cutinases which hydrolyses cutin, facilitating fungus penetration through the cuticle (Supplementary Table 13).

6.2.4.4. *In silico* characterization of possible *Fusarium circinatum* virulence factors

Some top DE genes in *F. circinatum* dataset showed hits against the PHI database (Supplementary Table 13). Sequence similarity (ranging from 20-30 %) to genes related to ergosterol biosynthesis in *F. graminearum*, *Alternaria alternata*, *Candida albicans* and *Aspergillus fumigatus* were found. The knockout of these genes resulted in reduced virulence or loss of pathogenicity in their hosts. In addition, we also identified 16 genes related to ergosterol biosynthesis differentially expressed in *F. circinatum* dataset (Supplementary Table 11), suggesting ergosterol as a potential *F. circinatum* virulence factor. Some *F. circinatum* genes showed sequence similarity (46-54 % similarity) to *chitin synthase* genes of *Ustilago maydis* and *F. oxysporum*, which knockout resulted in reduced virulence in corn and tomato plants (Supplementary Table 13). Indeed, 19 *chitin synthase* genes were differentially expressed *F. circinatum* dataset (Supplementary Table 11). Other top *F. circinatum* DE genes showed hits to indole-3-acetic acid biosynthesis and pectate lyase related genes in the PHI database (Supplementary Table 13).

6.3. Discussion

This chapter provides a comprehensive characterization of the underlying molecular mechanism for defence and pathogenicity response in the *P. pinaster* – *F. circinatum* pathosystem. The moderate resistance the maritime pine has shown to the pathogen (Iturrirxa *et al.*, 2013) can be explained at least in part by the early induction of defence-related genes and complex phytohormone signalling including SA, JA and ET. We also hypothesised the key steps where the pathogen could be manipulating host defences to its own benefit by altering host hormone homeostasis.

The early activation of *P. pinaster* defence response can be deduced from genes classified in cluster 2, up-regulated at 3 dpi, before the fungus has penetrated within the host tissue. At 5 and 10 dpi, when the fungus has penetrated and invaded the host tissue, the expression of genes in cluster 1 highly increased and GO terms related to phytohormone signalling, regulation of ROS, oxidative stress, positive regulation of cell death and signal transduction were found. This indicates activation of the classical PTI in response to the pathogen. Terms related to response to chitin were enriched in this cluster and chitinases are commonly induced by pathogen attack in several trees such as *Picea abies*, *Pinus elliottii*, *Pinus sylvestris* and *Fagus sylvatica* (Sharma P. *et al.*, 1993; Davis *et al.*, 2002; Hietala *et al.*, 2004; Nagy *et al.*, 2004; Schlink, 2009). Rice and *Arabidopsis* perceive fungal chitin through the lysine motif (LysM) RLK CERK1 which induces CERK1 dimerization, essential for the activation of downstream signalling (Miya *et al.*, 2007; Wan *et al.*, 2008). We found up-regulation of genes encoding LysM motif RLK and chitinase in inoculated seedlings since 3 dpi, suggesting fungal recognition at the early stage. Furthermore, oligosaccharides release from chitin degradation can serve as PAMPs for activation of PTI signalling in the host (Ren and West, 1992; Montesano, Brader and Palva, 2003).

A common plant defence response is the reinforcement of cell wall in order to difficult pathogen penetration and further infection (Hématy, Cherk and Somerville, 2009; Malinovsky, Fangel and Willats, 2014; Miedes *et al.*, 2014). We founded induction of genes involved in xyloglucan and lignin biosynthesis, as well as suberin and cutin related genes, suggesting reinforcement of the cell wall in *P. pinaster*. The up- and down-regulation of *laccase*, *pectinesterase* and *polygalacturonase* genes also reflect the active remodelling of cell wall structure in the presence of the pathogen. On the contrary, we identified up-regulation of several *F. circinatum* genes related to CWDE that contributes to host cell wall degradation and favours its penetration. Several species of the genera *Fusarium*, such as *F. graminearum*, contribute to pathogenesis by secretion of CWDE (Hématy, Cherk and Somerville, 2009; Kikot, Hours and Alconada, 2009). By search in the PHI database we found a gene with sequence similarity to a *pectate lyase* gene of *Phytophthora capsici* and the knockout of this genes resulted in reduced virulence in pepper plants (Fu *et al.*, 2015). In addition, some *expansin* genes were up-regulated and expansins are known to induce extensibility and relaxation of the cell wall, needed for the insertion of some components of the cell wall (Catalá, Rose and Bennett, 2000). However, this relaxation can also make the plant cell wall more vulnerable to fungal penetration (Li *et al.*, 2008; Fu and Wang, 2011).

Several studies have demonstrated the crucial role of phytohormones in host defence response (Pieterse *et al.*, 2012). In *P. pinaster* - *F. circinatum* pathosystem terms related to ET, JA and SA were over-represented in cluster 1. By contrast, GA, CK and ABA signalling seems to be suppressed. Genes involved in JA and ET biosynthesis were up-regulated since the early stage of 3 dpi and both phytohormones have been shown to cooperate under biotic stress conditions (Berrocal-Lobo, Molina and Solano, 2002; Lorenzo *et al.*, 2003, 2004). Despite the active biosynthesis of JA, the down-regulation of *COI1* and *MYC2* at 5 and 10 dpi, together with the up-regulation of *JAZ* genes reflect the block of JA signalling. *COI1* protein has an F-box domain and is involved in the formation of Ubiquitin ligase E3 SCF complex, for protein degradation (Devoto *et al.*, 2002). In the presences of JA-Ile, *COI1* degrade *JAZ* proteins, which are direct targets of the SCF *COI1* E3 ubiquitin-ligase (Chini *et al.*, 2007; Thines *et al.*, 2007). In the absence of *COI1*, *JAZ* proteins repress *MYC* transcription factor suppressing the expression of JA responsive genes. Based on the fact that *COI1* is a key element for regulation of JA signalling pathway (Devoto *et al.*, 2002) and the importance this phytohormone has shown in defence response against several necrotrophic pathogens (Glazebrook, 2005), we hypothesise that *F. circinatum* could be blocking JA signalling by *COI1* suppression. Similarly, (Thatcher, Manners, and Kazan 2009) suggested that *F. oxysporum* hijacks *COI1*-mediated JA signalling to promote disease in *Arabidopsis* plants.

The induction of genes related to ET biosynthesis, together with the up-regulation of ethylene-responsive regulator genes *ERF*, *ETR* and *EIN3/EIL1* suggests an active role of this phytohormone in *P. pinaster* defence response. However, the down-regulation of *EIN2* at 5 and 10 dpi, essential for positive regulation of ET, could interfere with ET signalling. Interestingly, we found up-regulation of 2 genes related to ET biosynthesis

in *F. circinatum* and we suggest this could have a role in perturbing ET homeostasis in the host. Similarly, De Vleeschauwer *et al.*, (2010) reported that ET produced by the rice brown spot pathogen *Cochliobolus miyabeanus* is a virulence factor and suggest that fungal ET interferes with the rice ET signalling route to suppress effective defence pathways. Indeed, one of these two genes related to ET production in *F. circinatum* showed hits to the PHI database and the knockout of this gene in *F. oxysporum* resulted in a reduced virulence phenotype in common bean plants (Niño-Sánchez *et al.*, 2016).

ET and JA participate in induction of expression of certain PR proteins (Curtis *et al.*, 1997; Belhadj *et al.*, 2008; Wang *et al.*, 2010). In the present study, we detected up-regulation of *PR1*, *PR2*, *PR3*, *PR5* and *PR9* genes. Rakwal *et al.*, (2004) reported a coordinated increase in ET and some PR3 in rice plants. Similarly, Carrasco *et al.* (2017) found synchronized increase between the induction of *PR5* and ET in *P. radiata* seedlings inoculated with *F. circinatum*. *PR2*, *PR3* and *PR5* participate in degradation of glucans of the cell wall of the pathogen, making the fungus more susceptible to cell lysis and attack by other plant defence molecules (Hématy, Cherk and Somerville, 2009). Similarly, during *F. culmorum* infection, two basic isoforms of *PR2* and three basic isoforms of *PR3* were induced in germinating wheat seeds upon infection (Caruso *et al.*, 1999). An increase of a class III *PR9* responsible for lignin biosynthesis and cell wall thickening was detected in *P. sylvestris* roots infected with *Heterobasidion annosum* (Adomas *et al.*, 2007). Indeed, induction of *PR5* in *P. radiata*, highly susceptible to the pathogen, infected by *F. circinatum*, occurs after 6 days post-inoculation, but no differences were found at 3 dpi between inoculated and mock-inoculated samples (Carrasco *et al.*, 2017). Here we detected up-regulation of PR proteins since the early time of 3 dpi, before the pathogen has penetrated, suggesting the quick activation of defence responses in *P. pinaster* against *F. circinatum*.

SA biosynthesis seems to occur from the PAL pathway and not from the chorismate pathway, since *ICS* was down-regulated at 5 and 10 dpi. Similarly, Ding *et al.*, (2011) found induction of *PAL* in a wheat variety after *F. graminearum* infection, while *ICS* was down-regulated, suggesting SA accumulation via the phenylpropanoid pathway. Interestingly, we found up-regulation of two *ICSH* genes by *F. circinatum*, which catalyses the hydrolysis of *ICS*. Moreover, *ICSH1* has been shown to accumulate in a highly aggressive *V. dahliae* isolate but not in a weakly aggressive isolate (El-Bebany, Rampitsch and Daayf, 2010). Indeed, cotton plants inoculated with a *V. dahliae* mutant lacking the *ICSH1* gene resulted in attenuated aggressiveness while higher levels of SA were detected (Liu *et al.*, 2014). Zhu *et al.*, (2017) propose that *ICSH1* is a virulence factor in *V. dahliae* and that the high level of *ICSH* in highly aggressive isolates lead to hijacking the SA pathway in potato plants by hydrolysing *ICS*. Similarly, we suggest that *F. circinatum* prevents SA biosynthesis from the chorismate pathway, although whether *ICSH* is a virulence factor in *F. circinatum* requires further investigation. In addition, *TGA*, *EDS1* and *PAD4* were up-regulated in inoculated *P. pinaster* seedlings, suggesting SA accumulation has a role in the host defence response. Furthermore, *PAD4* is also required for phytoalexin camalexin and *PR1* synthesis. *PR1* gene

expression is SA responsive and three *PR1* genes were up-regulated at all dpi. Carrasco *et al.* (2017) also found *PR1* genes highly up-regulated in *P. radiata* resistant genotypes.

Van der Does *et al.*, (2013) propose that suppression of the JA pathway by SA functions downstream of the E3 ubiquitin-ligase Skip-Cullin-F-box complex SCF^{COI1}. However, a cooperation between JA and SA has been also reported (Engelberth, Viswanathan and Engelberth, 2011; Thaler, Humphrey and Whiteman, 2012). Due to the up-regulation of genes related to SA and JA biosynthesis in inoculated *P. pinaster* seedlings and based on the fundamental role JA has shown against necrotrophic pathogens (Glazebrook, 2005), we suggest a SA and JA cooperation rather than an antagonism in *P. pinaster*-*F. circinatum* interaction.

Genes encoding enzymes that catalyses the methylation JA (MeJA) were up-regulated at all time points suggesting a role of systemic signalling in *P. pinaster* defence response. Furthermore, SAR mediated by SA term was enriched in cluster 1. Moreover, Truman *et al.*, (2007) suggest JA has an earlier role in SAR establishment before SA accumulation in *Arabidopsis*. Similarly, we found up-regulation of genes related to JA biosynthesis (*LOX*, *OPR*) since 3 dpi, before induction of SA related genes, mostly up-regulated at 5 and 10 dpi (*NPR1*, *TAG*, *PAD4*, *EDS1*), suggesting again cooperation between the two phytohormones. Foliar application of MeJA in *P. radiata* conferred increased resistance to the necrotroph *Diplodia pinea* (Gould *et al.* 2008) and against other pathogenic fungi in *P. abies* (Kozłowski, Buchala, and Métraux 1999; Franceschi, Krekling, and Christiansen 2002). However, MeJA pre-treatment was ineffective in protection against *F. circinatum* in *P. patula* (Fitza *et al.*, 2013) and *P. pinaster* seedlings (Vivas *et al.*, 2012). Sasaki *et al.*, (2001) described MeJA as a key component in the jasmonate signalling pathway by controlling its own expression *via* a feedback mechanism by inducing the expression of *LOX* and *AOS*, both enzymes that catalyse key steps in JA biosynthesis. This can explain the up-regulation of JA biosynthesis genes in spite of the suppression of JA signalling. Furthermore, studies have reported the role of MeJA in chalcone synthase (*CHS*) induction in soybean and parsley (Creelman, Tierney and Mullet, 1992) and *P. glauca* (Richard *et al.*, 2000). *CHS* is a key enzyme in the flavonoid biosynthesis pathway and flavonoids play an important role in plant defence against pathogens. We found up-regulation of *CHS* at all dpi in *P. pinaster* inoculated seedlings, with an increase from 3 to 10 dpi.

Although SA, JA and ET are the key players in *P. pinaster* response to the pathogen, auxins can also have a role since some *SAUR* genes and a *TIR1* gene were up-regulated. However, we also detected suppression in the biosynthesis and signalling (down-regulation of *indole-3-pyruvate monooxygenase* and *ARF*), as well as accumulation and conjugation to inactive forms inside the cell (up-regulation of *GH3* and a *AUX* gene encoding for influx proteins, and down-regulation of *PIN* and *PGP* genes encoding efflux proteins). *IAMT1* genes were up-regulated suggesting conversion of IAA into the inactive MeIAA form. Crosstalk between auxins and other phytohormones has been documented and an inhibitory effect of auxins in JA signalling in *Arabidopsis* seedlings

was reported (Liu and Wang, 2006). Moreover, auxins are inducers of expansins, involved in cell wall extensibility (Catalá, Rose and Bennett, 2000) and this could enhance pathogen penetration. Indeed, one of the principle roles of auxins are related to growth and development which are functions sacrificed by the plant to favour mechanisms involved in defence response. This trade-off between growth and defence is reflected by over-represented GO terms related to growth, morphogenesis and photosynthesis of clusters with down-regulated DE genes (clusters 18, 19, 27 and 29; Figure 18). Nonetheless, the role of auxins in *P. pinaster* defence response to *F. circinatum* is not clear and needs to be elucidated.

Furthermore, a gene related to auxin efflux was up-regulated by *F. circinatum* at all time points, suggesting auxin accumulation could have a role in virulence. Similarly, an over-accumulation of IAA in *F. oxysporum* was related to a hypervirulent phenotype on Orobanche (Cohen *et al.*, 2002). In addition, search of *F. circinatum* DE genes in the PHI database showed a hit with sequence similarity to an indole-3-acetic acid biosynthesis gene of *P. syringae*. The knockout of this gene in the bacteria resulted in reduced virulence in the model species *A. thaliana* (McClerklin *et al.*, 2018). GA production is common in species of the FFC. Although Malonek *et al.*, (2005) reported that only one gene in the GA biosynthetic cluster is present in *F. circinatum*, we found two potential genes related to GA biosynthesis up-regulated. This two genes presented hits to the PHI database, and the knockout of this genes in other pathogens resulted in reduced virulent and loss of pathogenicity in the host. However, these results must be considered with caution, since percentages of identity ranger from 22-29 %.

Ergosterol biosynthesis may have a role in *F. circinatum* virulence, since some top DE genes showed sequence similarity with ergortetol related genes when searching in the PHI database. The knockout of these genes in other fungi resulted in reduced virulence or loss of pathogenicity in their hosts. In addition, we identified 16 genes related to ergosterol biosynthesis in *F. circinatum* dataset. Similarly, Visser, (2019) found ergosterol biosynthesis related genes expressed in *P. patula* and *P. tecunumanii* seedlings inoculated by *F. circinatum*, with a lower relative expression of these genes in the resistant host, suggesting the existence of an ergosterol biosynthesis inhibition mechanism.

In conifers, oleoresin constitute one of the main chemical defences against pathogen invasion (Luchi *et al.*, 2005; Lombardero, Alonso-Rodríguez and Roca-Posada, 2012). In the case of *F. circinatum*, one of the typical symptoms of the disease is the abundant production of resin, given name to the disease it causes. Kim *et al.*, (2010) reported that *P. rigida* and *P. densiflora* seedlings inoculated with the pathogen produced two-fold higher levels of resin flow than in non-inoculated ones. However, *F. circinatum* is known to tolerate resin (Slinski, Zakharov and Gordon, 2014) and even is able to move within the resin ducts to colonize the plant (Martin-Rodrigues *et al.*, 2013). We also observed *F. circinatum* growing in the resin drop that stem from the cut done to inoculate seedlings at all sampling points. Conifer resin is composed by a mixture of terpenoids (Phillips and Croteau, 1999). We found terms related to terpenoid

metabolic processes overrepresented in a *P. pinaster* cluster which contained down-regulated genes, mostly at 10 dpi. We also found some specific oleoresin related genes up-regulated at the early stages, but down-regulated at 10 dpi. This suggests the possibility that *F. circinatum* is inducing a change in the composition of *P. pinaster* resin terpenoid.

An additional defence response seems to occur in *P. pinaster* seedlings at 10 dpi (cluster 3), since GO terms related to flavonoids and anthocyanins biosynthesis were over-represented. Flavonoids are secondary metabolites involved in defence response (Bennett and Wallsgrove, 1994; Treutter, 2006). Despite the effort done by the plant in synthesizing these secondary metabolites at the latter stage of infection, the host is not able to counteract fungal infection.

Plant R genes play a crucial role in detection of fungal effectors for activation of downstream defence signalling leading, in some cases, to host immunity (Jones and Dangl, 2006; Gururani *et al.*, 2012). In the present work, the most up-regulated gene was a disease R gene. BLASTP search indicates some structure of the protein, which contains a NB-LRR domain, present in most disease R proteins and known to have an important role in recognition specificity (Jones and Dangl, 2006). BLASTP against *Arabidopsis* showed hits to ADR1 family genes, which are known to be a family of NB-LRR receptors for pathogen effectors recognition and also regulates signal transduction processes leading to SA accumulation for downstream defence response (Bonardi *et al.*, 2011). We also found up-regulation of genes related to SA signalling in *P. pinaster* inoculated seedlings; therefore, this extremely expressed disease R gene may have similar function to ADR1 family genes and may participate in SA accumulation. However, this function cannot be directly assigned since BLASTP alignment resulted in low percentages of identity (35-37 %).

In summary, this work provides knowledge of the mechanism underlying the *Pinus* defence response against the pathogen *F. circinatum*, indicating a quick recognition and activation of the defence mechanism based on PR proteins and mainly regulated by a complex signalling pathway that involved crosstalk between SA, JA, ET and maybe auxins. Moreover, we hypothesise the key steps where the pathogen could be manipulating host defence on his favour, mainly by perturbing phytohormone signalling homeostasis in the host. Future work in measuring the content of SA, JA, ET and auxins *in planta*, as well as functional studies with *F. circinatum* mutants, will help to support this hypothesis.

Chapter 7: GENERAL DISCUSSION

Host-microbe interactions, and particularly fungi, have been traditionally studied from the perspective of pathogenesis and disease, supported by colonization of the host followed by appearance of disease symptoms. But nowadays it is generally accepted that fungi can exhibit diverse lifestyles with outcomes which varies from mutualistic to parasitic (Saikkonen *et al.*, 2004; Kogel, Franken and Hückelhoven, 2006; Hardoim *et al.*, 2015). Even when a fungus is colonizing the same host, the relation varies from beneficial to harmful or vice versa depending on the genotype of both partners, and on abiotic and biotic factors (Schulz and Boyle, 2005). This is the case of the fungus *F. circinatum*, traditionally known exclusively as pathogen of pines, which can also colonize herbaceous plants and roots of pine mature trees as an endophyte, without causing apparent damage. In the present study, we reported that the fungal host range of *F. circinatum* when behaving as an endophyte is larger than thought, and includes species from families that differ widely among them, in the dicots and monocots.

Endophytic fungi commonly spread horizontally by sporulating in host senescent tissue and infecting surrounding plants (Zabalgogezcoa, 2008; Rodriguez *et al.*, 2009; Márquez *et al.*, 2012). As we have reported, *F. circinatum* can sporulate in senescent tissue of herbaceous plants and infect pine seedlings growing close to them under controlled conditions. Although herbaceous plant colonization seems to be restricted to the pine infected plantation, our results suggest that these infected but symptomless herbaceous plants constitute a reservoir of inoculum. The time the fungus remains colonizing and/or spreading as an endophyte in a plantation across herbaceous species needs to be quantified. There is a mandatory lag period of two years before a new plantation can be planted, but this may not be enough time to prevent *Pinus* new infections. Therefore, endophytic colonization of herbaceous plants brings a new scenario that needs to be considered in disease management.

Besides *F. circinatum* endophytic colonization of herbaceous plants, a latent stage without inducing symptoms have been described in shoot and roots of artificially infected *Pinus* seedlings (Elvira-Recuenco, Iturritxa and Raposo, 2015; Martín-Rodrigues *et al.*, 2015; Swett, Kirkpatrick and Gordon, 2016). In the present work, we reported for the first time the presence of the fungus in pine roots naturally colonized under field conditions. The presence of the fungus in roots seems to be related to the disease intensity in the plantation, since it was only detected in roots of the *P. radiata* plantation with the higher disease incidence. This latent phase of the pathogen in roots of mature trees is of importance considering that isolation frequency was higher in non-symptomatic trees than in symptomatic ones. The role that this endophytic stage has in the disease lifecycle needs to be evaluated. When a plantation is cut down, tree roots remain in the soil, so their contribution in pathogen dispersal should be investigated.

Results of this study suggested that plants in the understory of a plantation are infected by airborne spores probably coming from sporulating cankers on infected trees. However, origin of symptomless colonization of roots was not determined. The lack of *F. circinatum* detection in soil samples suggests that roots are not infected from

soilborne inoculum. One possible explanation for root colonization is that symptomless but infected seedlings can be moved from nurseries to plantations and once there, stay as an endophyte in the root system. This theory is supported by two facts: first, that pine seedlings are moved from nurseries when they are one year old, and *F. circinatum* can remain symptomless in *P. radiata* seedlings for up to 475 days after inoculation; and second, that fungus colonizes the root but not the stem of a symptomless seedling (Gordon et al, 2017; Elvira-Recuenco, Iturrutxa and Raposo, 2015).

Several studies have reported that endophytic colonization can result in enhance fitness on the host and confer resistance to pathogens (Redman *et al.*, 2011; Hardoim *et al.*, 2015). Whether *F. circinatum* confers some benefit to the host is not clear and needs to be investigated, at least in herbaceous plants. The presence of *F. circinatum* in pine roots confers less vulnerability to future infections by the mechanism of induced resistance (Bonello, Gordon and Storer, 2001; Swett, Kirkpatrick and Gordon, 2016), and mycorrhiza community became more extensively in inoculated roots (Swett and Gordon, 2017).

We have reported that the endophytic stage is an import phase of the lifecycle of *F. circinatum*, by colonizing roots of mature trees as well as a wide range of herbaceous species that may constitute potential sources of inoculum. The lack of genetic variability in the whole population, independently from the host where the fungus was isolated (tree cankers, herbaceous plants, symptomless pine roots) indicates that the same haplotype of the fungus can behave as a pathogen or as an endophyte. This confirms that *F. circinatum* is not only a pathogen as originally was described, and it can adopt diverse lifestyles with different outcomes depending on the genotype of both partners, and on abiotic and biotic factors.

For a better understanding of how *F. circinatum* adopts a pathogenic or endophytic lifestyle it is important to elucidate the molecular mechanism and pathways responsible for the outcome of the interaction. The pipeline used for *de novo* assembly of raw sequencing reads has allowed the generation of a high quality *de novo* *P. pinaster* transcriptome assembly. Consequently, by a dual RNA sequencing approach, we deeply analyse the molecular processes involved in *P. pinaster* defence as well as *F. circinatum* virulence. The moderate resistance that *P. pinaster* shows against the pathogen in comparison to other pine species such as *P. radiata* and *P. patula* makes this species an excellent model for determining which mechanisms and metabolic pathways are responsible for resistance to *F. circinatum*.

The quick activation of plant defence response since the early stage of 3 dpi, mainly based on the induction of PR proteins, reinforcement of the cell wall, oxidative burst and phytohormone signalling, reflects that *P. pinaster* recognizes and activates the defence response before the fungus has penetrated. We reported that phytohormone signalling constitute an important *P. pinaster* defence mechanism to overcome the attack of the pathogen, mainly based on SA, JA and ET signalling. We also hypothesise

the key steps where *F. circinatum* may be manipulating phytohormone host balance by the secretion of metabolites that block either the biosynthesis or signalling of these hormones (blocking JA signalling by COI1 suppression, repressing SA synthesis from the chorismate pathway by ICSH1 expression, and altering ET host balance by inducing genes related to ET biosynthesis), leading to host susceptibility. The secretion of CWDE by *F. circinatum* also contributes to host susceptibility by favouring fungal penetration and further infection.

Resistance genes recognize pathogen effectors and this recognition can be sometimes enough to stop pathogen infection (Jones and Dangl, 2006). One of the most relevant results of this study is the identification of a disease resistance gene, the most up-regulated gene in *P. pinaster* dataset. Although BLAST against the non-redundant NCBI database showed a moderate percentage of similarity (46-64 %), we propose this is a disease resistance protein that may represent a good candidate to be included in a *P. pinaster* breeding program.

The continuum of lifestyles ranging from endophyte to necrotrophic pathogens makes it difficult to draw boundaries to separate different lifestyles (Schulz and Boyle, 2005). The molecular basis of pathogenic infections have been well studied in several pathosystems, pointing out several processes involved in host resistance defence as well as in pathogen virulence. On the contrary, only a few studies in the last decade have exposed results related to how microbes avoid host defence response for an endophytic colonization. Here, we have identified the molecular mechanisms and metabolic pathways involved in *P. pinaster* defence response to the pathogen *F. circinatum*, as well as genes involved in *F. circinatum* virulence. For an endophytic lifestyle, *F. circinatum* must suppress or avoid the plant defence mechanism; although how the fungus overcomes plant response is still unknown.

7.1. Future perspectives

In many cases, management activities have been unsuccessful in the eradication of some pathogens or in restoring forests. Recovery following spread of exotic pathogens is likely to be both slow and uncertain and may not be possible without intensive programmes involving selection and dissemination of genotypes resistant to the pathogen (Telford *et al.*, 2015). Classical breeding methods can produce genetically diverse and resistant populations; however, in the case of forest trees, programs for developing resistant populations must deal with relatively long generation times, as well as the long-lived nature of trees and the potential of the pathogen to evolve. The introduction of genes of interest in plants, by genetic engineering, is becoming a common strategy in plant breeding programs.

Knowledge of genes and molecular markers underlying a trait, such as resistance to a disease, enable the acceleration of breeding strategies (Mehraj and Ahmad, 2018). While many simple traits have been well characterized at the genome level, many other complex traits remain poorly understood. Quesada *et al.*, (2010) suggest that pine resistance to *F. circinatum* is polygenic, controlled by several genes with small

effects. We have identified a highly expressed disease resistance protein in *P. pinaster* dataset, besides other less expressed resistance genes that could have small effects in pine resistance to PPC. The identified gene may be a candidate to be used as a disease resistance gene with applications in breeding programs using marker assisted selection (MAS) technique.

We identified target genes involved in the susceptible/resistance mechanism in pines in response to *F. circinatum* infection, mainly based in phytohormone related genes, as well as possible virulence factor that contribute to *F. circinatum* virulence. By inoculation of pine species which differ in their degree of susceptibility to the pathogen, such as the susceptible species *P. radiata* and *P. patula*, and the resistance species *P. sylvestris* and *P. tecunumanii*, we can quantify the expression of the determined target genes by a RT-qPCR approach in order to compare the defence mechanisms between susceptible and resistant genotypes to *F. circinatum*. Furthermore, abiotic factors influencing the virulence of the interaction could be determined and become a useful tool for disease management.

The possibility to knock out or to over-express genes in plants or in plant pathogenic organisms is a very powerful tool. Targeted functional testing using *F. circinatum* mutants in future studies would be needed to support our suggestions. Future work in measuring the content of SA, JA and ET *in planta* will be needed to confirm our hypothesis. In addition, Di et al. (2016) suggested that a combination of effectors, enzymes and hormone-like secondary metabolites in *F. oxysporum*, determine the outcome of an interaction, endophytic with potential beneficial effects for the host or pathogenic with various levels of disease. Whether phytohormone balance play a role in determining the outcome of the interaction in *F. circinatum* needs further investigation. The combination of “omic” technologies will contribute to a better understanding of fungal-host interactions.

The role of *F. circinatum* as an endophyte is not clear and whether the fungus has any ecological role by enhancing plant fitness as other endophytes do remains also a challenge. However, the presence of the fungus in alternative hosts to pines that constitute an inoculum source represents a new scenario that needs to be considered for disease management. It is essential to account for all lifestyle stages of the fungus, for a better understanding of disease epidemiology and management. Understanding the molecular and ecological basis of *F. circinatum* endophytic and pathogenic interactions with pines and alternative hosts will contribute to understand the factors that contribute to this lifestyle duality.

Chapter 8: CONCLUSIONS

Conclusions

The main conclusions from this dissertation can be summarized in the following points:

1. We report the presence of *Fusarium circinatum* as an endophyte in alternative hosts to pines and grasses, including dicot species from the families Asteraceae, Rosaceae and Lamiaceae.
2. We describe *F. circinatum* as a foliar endophyte in herbaceous plants, since the fungus was isolated from the aerial part and never from roots. The fungus was also isolated in seeds of one species; therefore, vertical transmission should not be discarded.
3. Herbaceous plants are not infected at a distance larger than 3 meters from the border of the plantation, suggesting these species are colonized by airborne spores coming from tree cankers that do not disperse long distances.
4. Colonized but symptomless herbaceous plants can serve as reservoir of inoculum when the fungus sporulates in senescent tissue, from which surrounding pine seedlings may be infected.
5. We report the presence of *F. circinatum* in roots of *Pinus radiata* mature trees, with a higher frequency in roots of asymptomatic trees than symptomatic ones. We propose that nurseries are the potential origin of roots infection, since soil in the plantation does not constitute a source of inoculum.
6. No genetic diversity is found in *F. circinatum* isolates regardless their origin: tree cankers, herbaceous plants or asymptomatic pine roots. Therefore, the same fungal haplotype can adopt different lifestyles, as an endophyte or as a pathogen.
7. The moderate resistance that *Pinus pinaster* shows against the pathogen is explained by a quick activation of the defence response based mainly on pathogenesis related proteins and a complex phytohormone signalling that involves salicylic acid, jasmonic acid and ethylene.
8. *F. circinatum* virulence is based on secretion of cell wall degrading enzymes and on perturbing phytohormone homeostasis in *P. pinaster*, favouring its penetration and infection.
9. We identify a potential protein responsible for disease resistance in *P. pinaster* against the pathogen, *F. circinatum*.

Conclusiones

Las principales conclusiones de este trabajo pueden resumirse en los siguientes puntos:

1. *Fusarium circinatum* puede encontrarse como endófito en huéspedes alternativos a pinos y gramíneas, incluyendo plantas herbáceas dicotiledóneas pertenecientes a las familias Asteraceae, Rosaceae y Lamiaceae.
2. El hongo solo se aísla de la parte aérea de plantas herbáceas, nunca de raíz, por lo que describimos *F. circinatum* como un endófito foliar. También se ha aislado de semillas en una de las especie, por lo que la transmisión vertical no debe ser descartada.
3. Las plantas herbáceas no se infectan a una distancia mayor de 3 metros desde el borde de la parcela, lo que sugiere que estas especies son colonizadas a partir de inóculo aéreo procedente de chancros de árboles enfermos y que no se dispersa a largas distancias.
4. Las plantas herbáceas asintomáticas pero infectadas pueden constituir un reservorio de inóculo, de forma que cuando el hongo esporula en tejido senescente, las plántulas de pino creciendo alrededor se pueden resultar infectadas.
5. *F. circinatum* se detecta en raíces de árbol adulto de *Pinus radiata*, con una mayor frecuencia en raíces de árboles asintomáticos que sintomáticos. Proponemos que los viveros pueden ser el origen potencial de la infección de raíces, ya que el suelo de la plantación no constituye una fuente de inóculo.
6. No se encuentra diversidad genética en la población de *F. circinatum* independientemente del origen del asilado: chancros de árboles enfermos, plantas herbáceas o raíces de pinos asintomáticos. Por tanto, el mismo haplotipo del hongo puede adoptar diferentes estilos de vida, como endófito o como patógeno.
7. La moderada resistencia que *P. pinaster* presenta frente a al patógeno puede explicarse por la rápida activación de la respuesta de defensa basada principalmente en proteínas relacionadas con la patogenicidad (PR) y una compleja señalización hormonal que involucra al ácido salicílico, ácido jasmónico y etileno.
8. La virulencia de *F. circinatum* se basa en la secreción de enzimas degradadoras de la pared celular y en la perturbación de la homeostasis hormonal en el huésped, favoreciendo su penetración e infección de *P. pinaster*.

Conclusiones

9. Identificamos una proteína potencialmente responsable de la resistencia a la enfermedad en *P. pinaster* frente al patógeno *F. circinatum*.

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Supplementary material

Supplementary Figure 1: Climatological conditions at each plantation: temperature average, minimum and maximum temperatures and precipitation per month in the period 2008-2017. *P. radiata* plantations: P1 (Bizkaia), P2 (Gipuzkoa-R), P3 (Araba). *P. pinaster* plantations: P4 (Gipuzkoa-P). (Chapter 4).

Supplementary Figure 2: Symptoms at the shoot tip of inoculated (left side) and mock-inoculated (right side) *Pinus pinaster* seedlings by the end of the experiment (33 dpi). (Chapter 5).

Supplementary Figure 3: Contiguity and completeness assembly comparison by BUSCO analysis against the embryophyta lineage database, comparing the last *Pinus de novo* transcriptomes published. *P. patula* v1.0 (Visser et al. 2015); *P. patula* v2.0 and *P. tecunumanii* (Visser et al. 2018). (Chapter 5).

Supplementary Figure 4: Principal component analyses (PCA) for *Pinus pinaster* (above) and *Fusarium circinatum* (below) rlog data of the differential expression gene analysis (DESeq2). In red: mock-inoculated samples; in blue: inoculated samples at 3 dpi; in green: inoculated samples at 5 dpi; in yellow: inoculated samples at 10 dpi. (Chapter 6).

Supplementary Table 1: Read counts for each RNA sample at each time point, before and after filtering and trimming. Dpi: days post-inoculation; BR: biological replicate, RIN: RNA Integrity Number; Q 30: Phred quality score 30. (Chapter 5).

Group	Dpi	BR	RIN	Raw data			Trimmed and quality data					
				Total sequences	length	Q 30 (%)	Surviving sequences		Dropped sequences		length	Q 30 (%)
Mock-inoculated	3	1	9.1	47560680	101	95.432	43524073	92%	4036607	8%	87	100%
		2	9.3	49304321	101	93.399	42888235	87%	6416086	13%	87	100%
		3	9.4	55202436	101	95.335	50469767	91%	4732669	9%	87	100%
		4	9.3	57664110	101	93.799	50255464	87%	7408646	13%	87	100%
	5	1	9.4	60020039	101	95.462	54989003	92%	5031036	8%	87	100%
		2	9.5	48698072	101	94.791	43962376	90%	4735696	10%	87	100%
		3	9.4	67646789	101	95.166	61685025	91%	5961764	9%	87	100%
		4	9.5	49237615	101	94.069	43128434	88%	6109181	12%	87	100%
	10	1	8.9	76067369	101	95.59	71868450	94%	4201529	6%	87	100%
		2	9.1	86509928	101	96.1	82513169	95%	3997603	5%	87	100%
		3	9.5	90517427	101	95.9	86045866	95%	4470914	5%	87	100%
		4	9.3	40772260	101	93.827	35479626	87%	5292634	13%	87	100%
Inoculated	3	1	9	47685626	101	95.324	43557040	91%	4128586	9%	87	100%
		2	8.7	48814763	101	94.603	44193721	91%	4621042	9%	87	100%
		3	8.4	53563502	101	95.721	49227053	92%	4336449	8%	87	100%
		4	9.3	70028248	101	94.056	61288640	88%	8739608	12%	87	100%
	5	1	9	56674997	101	95.408	51758117	91%	4916880	9%	87	100%
		2	9.1	50285308	101	95.160	45541827	91%	4743481	9%	87	100%
		3	9.3	56625000	101	94.341	50588768	89%	6036232	11%	87	100%
		4	9.4	46585164	101	94.170	40838549	88%	5746615	12%	87	100%
	10	1	7.4	80561442	101	93.880	76283629	95%	4278447	5%	87	100%
		2	8.9	48417238	101	95.88	42289518	87%	6127720	13%	87	100%
		3	5.4	-	-	-	-	-	-	-	-	-
		4	5.8	-	-	-	-	-	-	-	-	-
Total				1288442334			1172376350	91%	116069425	9%		

Supplementary Table 2: Comparative statistics between normalized (Norm) and non-normalized (N-norm) Trinity preliminary assemblies. Kmer value; % of mapped fragments; % of good mapping; AS: assembly score; OP: optimal score; OC: optimal cutoff; Number of good contigs; % good contigs. (Chapter 5).

	kmer	% mapped fragm	% good map	AS	OP	OC	N. good contigs	% good contigs
Norm	19	89	71	0.204	0.276	0.105	183,664	83
N-norm	19	93	76	0.252	0.334	0.15	161,595	84
Norm	21	92	76	0.211	0.330	0.124	179,704	81
N-norm	21	93	77	0.257	0.360	0.185	158,473	81
Norm	23	92	78	0.219	0.345	0.127	181,600	81
N-norm	23	94	77	0.263	0.366	0.191	159,712	82
Norm	25	91	75	0.204	0.326	0.133	202,702	80
N-norm	25	93	76	0.24	0.365	0.239	164,395	77
Norm	27	91	76	0.20	0.334	0.159	196,564	78
N-norm	27	93	77	0.244	0.371	0.222	166,792	78

Supplementary Table S3: Clustering of *Pinus pinaster* differential expressed (DE) genes with gene ontology (GO) enriched terms. For each cluster, number of genes and percentage for genes are indicated. (Chapter 6).

Cluster ID	Number of genes	% of genes
1	1,144	13.0
2	2,755	31.3
3	449	5.1
5	20	0.2
11	52	0.6
13	97	1.1
15	211	2.4
18	1,083	12.3
19	1,233	14.0
24	549	6.2
27	151	1.7
29	415	4.7
Total	8,159	92.7

Supplementary Table 4: Clustering of *Fusarium circinatum* high confident (HC) differential expressed (DE) genes. For each cluster, number of genes and percentage for genes are indicated. (Chapter 6).

Cluster ID	Number of genes	% of genes
1	7	0.16
2	9	0.21
3	4,027	92.07
4	261	5.97
5	24	0.55
6	39	0.89
7	7	0.16
Total	4,374	100

Supplementary Table 5: Phytohormone related differentially expressed (DE) genes in *Pinus pinaster*. (Chapter 6).

Log ₂ (Fold Change) value					
Sequence ID	3 dpi	5 dpi	10 dpi	Predicted Gene	Description
JASMONIC ACID					
C07_R5228618	2,1289	4,6156	5,4350	LOX	Plant lipoxygenase
C09_R2662631	0,0000	2,9804	2,9143	LOX	Plant lipoxygenase
B01_TRINITY_DN31213_c3_g2_i3	0,0000	-0,9703	-1,7292	LOX	Plant lipoxygenase
B01_TRINITY_DN27721_c0_g1_i1	0,0000	8,6093	10,5604	LOX	Plant lipoxygenase
C11_S1054541	0,0000	4,4010	6,5443	LOX	Plant lipoxygenase
A09_TRINITY_DN44224_c0_g2_i2	0,0000	2,3277	3,1065	LOX	Plant lipoxygenase
A05_TRINITY_DN21748_c0_g1_i1	2,7950	7,9549	5,3743	LOX	Plant lipoxygenase
A01_TRINITY_DN20252_c7_g3_i6	0,0000	1,4584	0,0000	AOS	Allene oxide synthase
A10_TRINITY_DN44886_c0_g1_i1	0,0000	0,0000	-2,4605	AOS	Allene oxide synthase
C12_R645204	0,0000	1,1187	1,7896	AOS	Allene oxide synthase
C10_R3672771	0,0000	0,0000	0,7665	AOC	allene oxide cyclase
C09_R2656666	0,0000	3,1570	3,9213	OPR1	12-oxophytodienoate reductase
C08_R3942250	1,1778	5,8780	9,1756	OPR3	12-oxophytodienoate reductase
C06_R6819728	0,6958	3,7818	5,0803	OPR1	12-oxophytodienoate reductase
B01_TRINITY_DN36246_c0_g1_i1	0,0000	5,5781	8,1268	OPR3	12-oxophytodienoate reductase
C12_R638632	0,0000	2,2439	3,4702	OPR1	12-oxophytodienoate reductase
C07_R5216897	0,0000	4,1656	5,3044	OPR1	12-oxophytodienoate reductase
C11_R1236778	3,2734	6,4141	8,6580	OPR3	12-oxophytodienoate reductase
A06_TRINITY_DN49730_c1_g1_i3	0,0000	0,6951	2,4733	OPR3	12-oxophytodienoate reductase
A02_TRINITY_DN31697_c0_g1_i1	0,0000	7,0675	11,3062	OPR1	12-oxophytodienoate reductase
C12_R667215	0,0000	-1,0431	-2,0981	COI1	Coronatine-insensitive protein
A01_TRINITY_DN22480_c2_g3_i2	2,6693	6,5607	7,5275	JAZ10	JAZ protein
A11_TRINITY_DN57362_c1_g1_i2	0,0000	-2,7045	-5,8402	MYC	Transcription factor

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C06_R6819006	0,0000	0,0000	-0,8030	TOPLESS	Topless-related protein
C08_R3964188	0,0000	0,0000	-1,7904	TOPLESS	Topless-related protein
A08_TRINITY_DN27200_c0_g1_i28	0,0000	0,0000	-2,5099	TOPLESS	Topless-related protein
A07_TRINITY_DN38457_c0_g1_i1	0,0000	0,0000	0,8461	NINJA	ninja-family protein
C08_R3943631	3,0314	6,0113	7,3537	JMT	Jasmonate methyl transferase
A08_TRINITY_DN27739_c0_g1_i8	2,6561	4,1398	5,0179	JMT	Jasmonate methyl transferase
A08_TRINITY_DN27739_c0_g1_i12	1,1274	2,4928	3,8332	JMT	Jasmonate methyl transferase
C09_S471408	3,5151	8,5109	10,4313	CHS1	Chalcone synthase
C07_R5214662	6,5095	8,6520	9,8821	CHS1	Chalcone synthase
A11_TRINITY_DN57940_c0_g2_i7	1,6979	3,6139	5,1316	CHS1	Chalcone synthase
A08_TRINITY_DN28267_c5_g1_i3	1,7493	4,4144	5,0850	CHS1	Chalcone synthase
B04_TRINITY_DN63024_c0_g1_i1	0,0000	3,1230	5,0519	CHS1	Chalcone synthase
A10_TRINITY_DN47312_c2_g1_i14	1,1813	2,3694	4,3565	CHS1	Chalcone synthase
A06_TRINITY_DN49747_c1_g1_i16	1,4808	3,3834	4,3380	CHS1	Chalcone synthase
A03_TRINITY_DN31253_c1_g1_i1	1,0031	2,2939	4,0304	CHS1	Chalcone synthase
C12_R628193	1,1166	2,2981	3,9063	CHS1	Chalcone synthase
C12_R641260	1,1042	2,3553	3,6066	CHS1	Chalcone synthase
A10_TRINITY_DN47312_c2_g1_i4	0,0000	3,3300	3,4982	CHS1	Chalcone synthase
C12_R626363	0,9438	1,5154	3,1318	CHS1	Chalcone synthase
A10_TRINITY_DN43382_c0_g1_i6	0,8992	1,7367	3,0954	CHS1	Chalcone synthase
C09_R2654120	1,0875	1,9760	2,6922	CHS1	Chalcone synthase
A11_TRINITY_DN41422_c1_g1_i13	1,0207	2,1734	2,5656	CHS1	Chalcone synthase
B02_TRINITY_DN40753_c4_g1_i7	0,0000	0,9979	2,3522	CHS1	Chalcone synthase
C10_R3522663	0,0000	1,0594	2,2542	CHS1	Chalcone synthase
C06_R6809052	0,0000	0,9912	2,1125	CHS1	Chalcone synthase
B03_TRINITY_DN43873_c3_g2_i1	0,0000	1,0541	1,9019	CHS1	Chalcone synthase
A07_TRINITY_DN53880_c2_g1_i9	0,5426	1,1155	1,5694	CHS1	Chalcone synthase

B04_TRINITY_DN51541_c1_g1_i6	0,0000	-0,7797	-2,2131	CHS1	Chalcone synthase
ETHYLENE					
C08_S4036761	0,0000	4,2438	6,3059	ACO1	1-aminocyclopropane-1-carboxylate oxidase
B02_TRINITY_DN43656_c0_g1_i1	0,0000	2,6992	2,6710	ACO1	1-aminocyclopropane-1-carboxylate oxidase
C09_R2663204	0,0000	-1,0979	-2,6133	ACO2	1-aminocyclopropane-1-carboxylate oxidase
A10_TRINITY_DN45792_c0_g2_i2	0,0000	4,2144	6,4817	ACO1	1-aminocyclopropane-1-carboxylate oxidase
C09_R2661077	0,0000	0,0000	0,6866	ACS	1-aminocyclopropane-1-carboxylate synthase
C12_S78734	1,3262	3,7642	5,3557	ACS5	1-aminocyclopropane-1-carboxylate synthase
C06_S7158192	0,5418	0,8530	2,8001	ACS1	1-aminocyclopropane-1-carboxylate synthase
C04_R10660361	3,1149	7,7545	9,7239	ACS5	1-aminocyclopropane-1-carboxylate synthase
C09_R2683142	1,5999	6,1627	6,9689	ACS5	1-aminocyclopropane-1-carboxylate synthase
C08_R3955118	0,0000	6,0624	7,6629	ACS1	1-aminocyclopropane-1-carboxylate synthase
C09_R2693663	4,2835	8,1844	8,3340	ACS5	1-aminocyclopropane-1-carboxylate synthase
A03_TRINITY_DN30727_c1_g1_i1	0,0000	1,8032	2,5338	ETR2	ethylene sensor
C11_R1219272	0,0000	0,0000	-0,5625	ETR1	ethylene sensor
C12_R676892	0,0000	0,5552	0,5383	ERF/AP2	transcription factor
C11_R1177098	0,0000	1,6694	3,1513	ERF/AP2	transcription factor
C08_R3930482	2,5928	6,2942	7,6201	ERF/AP2	Transcription factor
C07_S1827741	0,0000	4,9713	8,5862	ERF/AP2	Transcription factor
C09_R2660126	0,0000	2,4174	4,9446	ERF/AP2	Transcription factor
C06_R6934388	5,1288	7,5581	10,8762	ERF/AP2	Transcription factor
C06_S6972650	1,5550	2,8250	4,4909	ERF/AP2	Transcription factor
C12_R635751	0,0000	0,0000	0,5990	ERF/AP2	Transcription factor
A02_TRINITY_DN23152_c0_g2_i3	3,1867	4,1327	8,6498	ERF/AP2	Transcription factor
C10_R3507856	0,0000	2,8465	4,2728	ERF/AP2	Transcription factor
A11_TRINITY_DN52708_c0_g1_i6	0,0000	9,1020	10,6221	ERF/AP2	Transcription factor
C09_R2670165	3,4650	10,6447	10,7176	ERF/AP2	Transcription factor

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A02_TRINITY_DN24588_c0_g1_i1	3,0506	7,5733	9,0699	ERF/AP2	Transcription factor
C08_R3908101	0,0000	-0,5977	-1,2122	ERF/AP2	transcription factor
C12_R647330	0,0000	1,0141	2,1253	ERF/AP2	Transcription factor
A01_TRINITY_DN17595_c3_g1_i2	3,9646	7,2582	9,3633	ERF/AP2	Transcription factor
A02_TRINITY_DN22532_c0_g1_i8	0,0000	5,8233	5,2257	ERF/AP2	Transcription factor
C06_S6422834	0,0000	6,6365	7,1954	ERF/AP2	Transcription factor
A05_TRINITY_DN50666_c1_g1_i1	2,8218	7,1535	9,2537	ERF/AP2	Transcription factor
A05_TRINITY_DN39992_c0_g1_i3	-0,6974	-0,6468	-2,3178	ERF/AP2	transcription factor
C09_R2671317	0,0000	6,3373	6,4684	ERF/AP2	Transcription factor
A03_TRINITY_DN27045_c0_g1_i3	5,0924	6,8040	8,4381	ERF/AP2	Transcription factor
B05_TRINITY_DN46947_c0_g4_i2	0,0000	0,0000	1,3577	ERF/AP2	Transcription factor
C08_R3923800	1,6901	4,8409	9,1342	ERF/AP2	Transcription factor
A06_TRINITY_DN51598_c1_g2_i2	0,0000	0,5349	1,4485	ERF/AP2	Transcription factor
C12_S684257	1,5514	3,5202	5,1971	ERF/AP2	Transcription factor
C08_R3891272	0,0000	1,1141	3,0550	ERF/AP2	Transcription factor
A11_TRINITY_DN52465_c0_g2_i2	0,0000	5,1835	6,2463	ERF/AP2	Transcription factor
C07_R5261051	0,0000	0,9415	3,1347	ERF/AP2	Transcription factor
B05_TRINITY_DN55164_c0_g1_i6	0,0000	1,5604	2,2186	ERF/AP2	Transcription factor
B02_TRINITY_DN33910_c0_g4_i1	0,0000	1,6112	3,0542	ERF/AP2	Transcription factor
A05_TRINITY_DN45799_c1_g2_i2	0,0000	1,1052	1,3326	ERF/AP2	transcription factor
C09_R2680557	0,0000	1,8965	2,5371	ERF/AP2	Transcription factor
A03_TRINITY_DN21931_c0_g1_i2	0,0000	-0,5890	0,0000	EBF1	EIN3-binding F-box protein
A02_TRINITY_DN22171_c0_g5_i3	0,0000	0,0000	0,5079	EIN3-LIKE1	protein ethylene insensitive
C10_S3454121	0,0000	0,6968	0,0000	EIN3-LIKE1	protein ethylene insensitive
C09_R2664125	0,0000	-0,5598	-0,8080	EIN2	ethylene-insensitive protein
SALICYLIC ACID					
A02_TRINITY_DN19059_c0_g2_i3	0,0000	0,0000	0,9414	ICS	Isochorismatase family

A05_TRINITY_DN47850_c1_g1_i9	0,0000	-0,5591	-1,7029	ICS	Isochorismatase family
A02_TRINITY_DN21697_c3_g2_i4	0,0000	1,6605	1,4524	EDS1	enhanced disease susceptibility 1
C09_R2653403	0,0000	0,0000	0,6811	EDS1	enhanced disease susceptibility 1
C08_R3885703	0,0000	0,0000	0,5990	EDS1	enhanced disease susceptibility 1
C08_R3978326	0,0000	-1,1159	-2,0407	EDS1	enhanced disease susceptibility 1
C08_S3882031	0,0000	4,0577	5,7572	PAD4	Lipase (class 3)
C07_R5256852	1,8270	4,2489	5,6674	PAD4	Lipase (class 3)
C09_S2708280	1,5534	5,3447	4,8655	PAD4	Lipase (class 3)
C07_R5446730	0,0000	2,3576	4,4623	PAD4	Lipase (class 3)
C09_S2754742	0,0000	4,2209	4,1982	PAD4	Lipase (class 3)
C07_R5299813	0,0000	3,2489	4,0546	PAD4	Lipase (class 3)
C11_R1203531	1,1328	2,1725	3,1067	PAD4	Lipase (class 3)
C08_S4015250	0,0000	3,0741	2,8668	PAD4	Lipase (class 3)
C06_J6918951	0,0000	1,9450	2,7296	PAD4	Lipase (class 3)
C12_S685705	0,0000	2,2489	2,6019	PAD4	Lipase (class 3)
A02_TRINITY_DN24739_c1_g1_i17	0,0000	1,6614	2,4120	PAD4	Lipase (class 3)
C09_R2713681	0,0000	0,6045	2,2425	PAD4	Lipase (class 3)
C09_R2666250	0,0000	1,7772	2,1908	PAD4	Lipase (class 3)
C09_S2662752	0,0000	1,8082	2,1679	PAD4	Lipase (class 3)
C11_S1167945	0,0000	1,8536	2,1649	PAD4	Lipase (class 3)
C06_R6812861	0,0000	1,4052	1,9882	PAD4	Lipase (class 3)
A06_TRINITY_DN44866_c1_g2_i5	0,0000	0,0000	1,6808	PAD4	Lipase (class 3)
C11_S1279418	0,0000	0,7043	1,6605	PAD4	Lipase (class 3)
C09_R2684604	0,5740	1,1147	1,6182	PAD4	Lipase (class 3)
C10_S3490257	0,0000	2,3552	1,5980	PAD4	Lipase (class 3)
C11_R1162376	0,0000	1,8895	1,5785	PAD4	Lipase (class 3)
C06_S6937406	0,0000	1,3582	1,4563	PAD4	Lipase (class 3)

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C12_R639211	0,0000	0,8856	1,4217	PAD4	Lipase (class 3)
C07_S5366068	0,0000	0,8655	1,3122	PAD4	Lipase (class 3)
C11_R1159641	0,0000	1,2563	1,0909	PAD4	Lipase (class 3)
C06_R7005335	0,0000	1,4625	0,0000	PAD4	Lipase (class 3)
C09_R2685621	0,0000	0,7201	0,0000	PAD4	Lipase (class 3)
C09_R2712320	0,0000	1,2706	0,0000	PAD4	Lipase (class 3)
C08_R3961219	0,0000	0,0000	-0,7119	PAD4	Lipase (class 3)
C09_R2652389	0,0000	0,0000	-0,8670	PAD4	Lipase (class 3)
C06_S7178009	0,0000	0,0000	-1,6373	PAD4	Lipase (class 3)
A09_TRINITY_DN35652_c0_g1_i8	0,0000	0,0000	1,1502	TGA4	Transcription factor
A06_TRINITY_DN46081_c0_g1_i2	0,0000	0,0000	0,6966	TGA2	Transcription factor
A08_TRINITY_DN27846_c0_g3_i1	1,4762	5,6248	6,5956	PAL	Phenylalanine ammonialyase
A03_TRINITY_DN28638_c1_g1_i9	0,0000	3,0237	3,8908	PAL	Phenylalanine ammonialyase
C10_R3565086	0,6137	2,4536	3,4304	PAL	Phenylalanine ammonialyase
C11_R1161283	0,7842	1,5412	2,7441	PAL	Phenylalanine ammonialyase
C06_R6821623	0,0000	0,0000	-1,0518	PAL	Phenylalanine ammonialyase
C09_R2725446	0,0000	-1,1158	-2,0355	PAL	Phenylalanine ammonialyase
A11_TRINITY_DN54438_c0_g2_i1	0,0000	0,0000	-2,4022	PAL	Phenylalanine ammonialyase
AUXINS					
B05_TRINITY_DN50617_c0_g2_i10	0,0000	0,0000	-0,9106	ARF19	auxin response factor
A05_TRINITY_DN42544_c0_g2_i7	0,0000	0,0000	-0,5259	ARF10	auxin response factor
B02_TRINITY_DN35936_c4_g1_i1	0,0000	0,0000	-0,5980	ARF2	auxin response factor
C11_R1164910	0,0000	0,0000	-0,6049	ARF2	auxin response factor
B02_TRINITY_DN40071_c1_g1_i16	0,0000	0,0000	-0,6844	ARF6	auxin response factor
B05_TRINITY_DN63564_c1_g1_i5	0,0000	0,0000	-0,9489	ARF2	auxin response factor
C09_R2667317	0,0000	-0,6624	-0,9840	ARF4	auxin response factor
A05_TRINITY_DN49280_c0_g2_i2	0,0000	-0,5596	-1,1749	ARF6	auxin response factor

A05_TRINITY_DN40837_c0_g1_i8	0,0000	-0,5232	-1,2040	ARF2	auxin response factor
A06_TRINITY_DN42254_c0_g1_i12	0,0000	2,0613	2,1744	IAA16	auxin-responsive protein
C09_S2547440	0,0000	0,9429	1,4684	IAA16	auxin-responsive protein
A01_TRINITY_DN15343_c3_g1_i3	0,0000	0,6507	0,5171	IAA13	auxin-responsive protein
C09_R2761854	0,0000	-0,9621	0,0000	IAA13	auxin-responsive protein
C10_R3573141	0,0000	0,0000	-0,6690	IAA8	auxin-responsive protein
A10_TRINITY_DN43099_c0_g1_i7	0,0000	0,0000	-0,5212	IAA33	AUX/IAA family
A01_TRINITY_DN17940_c4_g1_i1	0,0000	0,0000	-3,9391	IAA33	AUX/IAA family
C12_R648731	0,0000	0,0000	0,8561	TIR1	transport inhibitor response
A03_TRINITY_DN26829_c0_g1_i4	0,0000	0,5634	0,6197	GH3.6	Indole-3-acetic acid-amido synthetase
C11_J1167612	0,0000	0,0000	0,6104	GH3	Indole-3-acetic acid-amido synthetase
C08_S3881418	0,0000	-0,9214	-2,3263	GH3.17	Indole-3-acetic acid-amido synthetase
B04_TRINITY_DN37490_c0_g1_i1	0,0000	-0,6833	-0,9662	PGP10	ABC transporter B family member
A03_TRINITY_DN25437_c0_g2_i1	0,0000	0,0000	-1,6420	PGP10	ABC transporter B family member
A02_TRINITY_DN23109_c0_g1_i24	0,0000	-0,6327	-2,3746	PGP1	ABC transporter B family member
C05_R23765752	0,0000	0,0000	-1,0876	PIN4	auxin efflux carrier
A05_TRINITY_DN43671_c0_g2_i8	0,0000	-0,5775	-1,7605	PIN7	auxin efflux carrier
A02_TRINITY_DN21343_c0_g1_i5	1,8593	3,9182	3,2170	AUX3	auxin transporter-like protein
A09_TRINITY_DN39184_c0_g2_i1	0,0000	0,0000	-1,1247	AUX2	auxin transporter-like protein
A11_TRINITY_DN39722_c0_g2_i4	0,0000	0,0000	-1,4398	PIN7	auxin efflux carrier
C12_R654277	0,0000	-0,9205	-2,0203	PIN1	auxin efflux carrier
A08_TRINITY_DN25032_c2_g2_i2	0,0000	0,0000	-2,7663	YUCCA8	indole-3-pyruvate monooxygenase
C05_R24217077	0,0000	0,0000	2,4340	YUCCA8	indole-3-pyruvate monooxygenase
C12_S569876	-1,5757	-2,0698	0,0000	YUCCA4	indole-3-pyruvate monooxygenase
C10_J3579869	0,0000	0,0000	-2,0802	SAUR	SAUR family protein source
C10_S3707284	0,0000	0,0000	1,2606		auxin-induced protein
C09_S102071	0,0000	1,4587	0,0000		Auxin responsive protein

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C09_R2791291	0,0000	2,4431	9,3351	OSI_21546	Auxin-induced protein
C12_S602667	0,0000	2,1332	6,2157		Auxin responsive protein
C08_R4112252	0,0000	2,7727	5,1124		auxin-induced protein
C07_J5241658	0,0000	0,9398	1,2957	SAUR72	Auxin-responsive family
A02_TRINITY_DN37717_c0_g1_i1	0,0000	-1,7198	-4,6956	ARG7-like	auxin-induced protein
B05_TRINITY_DN62746_c1_g1_i10	0,0000	0,0000	-2,0540	POPTR_0009s12890g	auxin-induced protein
A03_TRINITY_DN24097_c0_g1_i4	5,3297	6,5038	9,2048		Auxin-responsive family
B02_TRINITY_DN35313_c1_g3_i1	0,0000	0,0000	3,4258	SAUR36	Auxin-induced protein
C09_S2580574	0,0000	2,1338	3,8803		Auxin-induced protein
A03_TRINITY_DN23316_c0_g1_i1	0,0000	0,0000	-2,8923		auxin-induced protein
C10_R3610130	0,0000	0,0000	2,0184		Auxin-induced protein
C11_R1178362	0,0000	0,0000	0,7971		Auxin-induced protein
C09_S2637108	0,0000	0,0000	-1,6288		auxin-induced protein
C09_R2691033	0,0000	0,0000	0,8549		Auxin-induced protein
C04_J10918852	0,0000	0,0000	0,7594		auxin-induced protein
A02_TRINITY_DN26919_c0_g1_i2	0,0000	0,0000	-0,8332	POPTR_0009s12890g	auxin-induced protein
A03_TRINITY_DN30855_c1_g1_i11	0,0000	0,0000	-1,6735		auxin-induced protein
A01_TRINITY_DN23043_c6_g3_i2	0,0000	0,0000	-1,2371	POPTR_0009s12890g	auxin-induced protein
A01_TRINITY_DN17831_c1_g1_i1	0,0000	0,0000	-1,0171	SAUR	Auxin-responsive SAUR gene
C07_R5330809	0,0000	-0,7626	-1,4905		auxin-induced protein
B01_TRINITY_DN35611_c2_g1_i1	0,0000	0,7383	1,1313		Auxin-induced protein
B03_TRINITY_DN35366_c0_g1_i1	0,9734	3,2912	5,0102		Auxin-responsive family
C11_R1286181	0,0000	-0,9214	-3,8670	IAMT1	indole-3-acetate O-methyltransferase 1-like
A03_TRINITY_DN10417_c0_g1_i1	3,3226	4,5812	7,1338	IAMT1	indole-3-acetate O-methyltransferase 1-like
A02_TRINITY_DN27562_c0_g1_i6	0,0000	2,5647	4,3239	IAMT1	indole-3-acetate O-methyltransferase 1-like
C12_R670253	0,0000	2,0639	5,3036	IAMT1	indole-3-acetate O-methyltransferase 1-like
A11_TRINITY_DN57218_c1_g1_i3	0,0000	2,3119	4,5721	IAMT1	indole-3-acetate O-methyltransferase 1-like

ABSCISIC ACID					
A07_TRINITY_DN43695_c0_g1_i6	0,0000	0,0000	-0,7677	ZEP	zeaxanthin epoxidase
C07_J5233552	0,0000	-1,5648	-3,0492	NCED4	9-cis-epoxy-carotenoid dioxygenase
A08_TRINITY_DN25735_c3_g6_i1	0,0000	0,0000	0,9502	NCED5	9-cis-epoxy-carotenoid dioxygenase
C08_R4009912	0,9092	3,3219	4,8205	NCED3	9-cis-epoxy-carotenoid dioxygenase
C09_S2625242	0,0000	5,0975	6,0843	PP2C	phosphatase 2C
C12_R679347	0,0000	-0,8897	-1,6346	PP2C	phosphatase 2C
A05_TRINITY_DN46375_c0_g1_i8	0,0000	0,0000	0,7180	PP2C	phosphatase 2C
C04_R10656255	0,0000	0,0000	0,6654	PP2C	phosphatase 2C
C05_R23822321	0,0000	0,0000	-2,5968	CYP707A1	Abscisic acid 8'-hydroxylase
C11_S1128402	0,0000	2,1688	1,3993	CYP707A1	Abscisic acid 8'-hydroxylase
GIBBERELIC ACID					
C08_R3924593	0,0000	-0,5725	-1,5257	KS1	Ent-kaurene oxydase
C08_S1743650	0,0000	0,0000	-4,0464	KO1	Ent-kaurene synthase
C08_R3940182	0,0000	0,0000	-2,4700	GA20OX1	GA20-oxidase
A06_TRINITY_DN49632_c0_g2_i1	0,9680	4,7862	5,8964	GA2ox	GA2-oxidase
CYTOKININS					
A07_TRINITY_DN52497_c3_g1_i5	0	2,834780 152	3,84460 7987		Cytokinin-O-glucosyltransferase
A11_TRINITY_DN47116_c3_g3_i1	0,0000	0,0000	0,8461	HKA	His Kinase A
C11_R1190420	-0,5181	-0,5261	-0,6507	HK1	Histidine kinase 1
C06_R6968699	0,0000	-0,6604	-0,7405	HK	Histidine kinase
A05_TRINITY_DN40516_c1_g1_i10	0,0000	-0,6395	-0,8048	HK3	Histidine kinase 3
A05_TRINITY_DN50680_c1_g2_i2	0,0000	0,0000	-0,9239	HK	Histidine kinase
C08_S4056064	0,0000	-0,6985	-1,4521	HK1	Histidine kinase
C12_R633703	0,0000	-0,9395	-2,8393		CHASE domain containing histidine kinase protein
B03_TRINITY_DN37767_c0_g1_i2	0,0000	-1,1631	-3,1616		CHASE domain containing histidine kinase protein

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A11_TRINITY_DN53476_c1_g6_i10	0,0000	-1,1817	-3,1855		CHASE domain containing histidine kinase protein
C09_R2691117	0,0000	0,9699	0,9542	HK	Histidine kinase
C11_R1219272	0,0000	0,0000	-0,5625	HK	Histidine kinase
B03_TRINITY_DN30504_c0_g1_i5	0,0000	-0,6762	-0,6895	HK	Histidine kinase
BRASSINOSTEROIDS					
C09_S2634498	0,0000	1,5743	1,8983	DET2	Brassinosteroid biosynthesis
A02_TRINITY_DN22292_c2_g3_i2	0,0000	2,1693	2,0626	DET2	Brassinosteroid biosynthesis
C08_S1911137	0,0000	0,0000	-3,3885	DWF4	Brassinosteroid biosynthesis
C11_R1158527	0,0000	0,7125	0,8306	BR6OX1	Brassinosteroid biosynthesis
C11_S884850	0,0000	0,0000	0,6732		Brassinosteroid biosynthesis; cytochrome P450
C08_R3905662	0,0000	-1,0179	-2,7762		Brassinosteroid biosynthesis; cytochrome P451
C11_S1143396	0,0000	0,0000	-0,9367	CYP866B10	Brassinosteroid biosynthesis; cytochrome P452
C12_R633470	0,0000	0,0000	-1,6387	CYP866A17	Brassinosteroid biosynthesis; cytochrome P453
A08_TRINITY_DN23162_c0_g1_i2	0,0000	1,2126	0,0000		Brassinosteroid biosynthesis; cytochrome P454
A10_TRINITY_DN42922_c0_g1_i6	0,0000	0,0000	1,3924		Brassinosteroid biosynthesis; cytochrome P455
C07_R5485259	0,0000	0,0000	-3,3089		Brassinosteroid biosynthesis; cytochrome P456
A03_TRINITY_DN29905_c0_g1_i2	0,0000	0,0000	-1,0615	cytochrome P450 90D2	Brassinosteroid biosynthesis; cytochrome P457
C12_J632668	0,0000	0,0000	-0,7192	DWF1	Brassinosteroid biosynthesis
A09_TRINITY_DN35511_c0_g1_i1	0,0000	0,0000	0,8063	STYKc	Brassinosteroid LRR receptor kinase BRL1-like
A08_TRINITY_DN19416_c0_g1_i1	0,0000	-0,8700	-1,7800	BRI1	BRI1 like
A01_TRINITY_DN21643_c3_g5_i2	0,0000	1,3283	2,6629		BES1 BZR1 homolog protein
B03_TRINITY_DN34334_c0_g3_i1	3,2684	7,1955	7,6878	BRH1	Brassinosteroid-responsive RING-H2; E3 ubiquitin-protein ligase
A05_TRINITY_DN40301_c0_g2_i2	5,1282	7,2137	9,3531	BRH1	Brassinosteroid-responsive RING-H2; E3 ubiquitin-protein ligase
A06_TRINITY_DN43800_c1_g1_i4	0,0000	6,5713	9,1382	BRH1	Brassinosteroid-responsive RING-H2; E3 ubiquitin-protein ligase

Supplementary Table 6. Pathogenesis related (PR) proteins related genes differentially expressed (DE) in *Pinus pinaster*. (Chapter 6).

	log2 (Fold Change)				
Sequence ID	3 dpi	5 dpi	10 dpi	Predicted Gene	Description
PR1 family					
A02_TRINITY_DN25849_c0_g2_i1	5,75955189	9,90987477	12,659027	PR1	Pathogenesis-related protein
A10_TRINITY_DN44957_c0_g5_i1	1,06333004	4,21520901	6,20157071	PR1	Pathogenesis-related protein
C04_R11174555	0	0	1,95466362	PR1	Pathogenesis-related protein
PR2 family					
A03_TRINITY_DN31059_c1_g1_i7	1,82243009	6,98132835	8,6363428		beta-1,3 glucanase 3
PR3 family					
C10_R3622106	0	7,27538372	12,2530929		class VII chitinase
C07_R5224799	4,36375481	7,04749828	10,6489999		class VII chitinase
A07_TRINITY_DN51074_c0_g2_i3	2,61312319	6,58391011	10,0841353		class VII chitinase
A03_TRINITY_DN29632_c0_g1_i2	1,05454288	6,4775292	9,3761443	RC24	chitinase
C07_R5227920	1,26239537	5,86141774	9,27359934	RC24	chitinase
A05_TRINITY_DN50237_c1_g1_i4	1,35194148	6,24727847	9,22239866	RC24	chitinase
A11_TRINITY_DN59373_c4_g1_i5	2,63763493	7,22851974	9,09782581		chitinase
C12_R628833	1,96727075	4,80708584	8,91275007		chitinase
C10_S3712202	3,63603092	7,02964819	8,55366434		chitinase
C05_R23812722	0	3,86276882	6,94366122		chitinase
C09_R2661824	1,53307786	3,3027661	5,56603493		chitinase
C08_S3965548	0	3,26280694	5,48944781		chitinase
A08_TRINITY_DN28748_c0_g1_i11	1,21029094	2,79070848	4,63286921		chitinase
A06_TRINITY_DN43915_c2_g1_i17	0	1,16190395	4,44087952		chitinase
C04_R10957630	0	2,09470785	4,41472619		chitinase
PR5 family					

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C08_S3964471	3,71854541	11,0210678	12,0878382		thaumatin-like protein
B05_TRINITY_DN60136_c4_g1_i8	3,15389766	7,12156905	10,7462251		thaumatin-like protein
A05_TRINITY_DN51664_c1_g1_i2	3,16390151	7,77842326	10,6774728		thaumatin-like protein
C04_S5132467	3,59028057	7,1569982	10,3358099		thaumatin-like protein L4
A07_TRINITY_DN52676_c1_g1_i7	3,06733286	7,76814092	10,2817564		thaumatin-like protein
C07_S5242570	2,54807332	5,26265463	8,64040852		thaumatin-like protein
A07_TRINITY_DN51256_c0_g1_i1	1,26909576	5,29174795	8,60265181		thaumatin-like protein
B05_TRINITY_DN57850_c2_g1_i5	2,99015359	4,98319298	8,00182816		thaumatin-like protein
C05_R23741266	2,28133132	4,91823799	7,85619217		thaumatin-like protein
B02_TRINITY_DN41415_c2_g1_i3	0,5739368	1,01435511	2,13613645		thaumatin-like protein
C10_R3592356	0	-0,66903581	0		thaumatin-like protein
C05_R24220847	0	-0,76442006	-1,88993587		thaumatin-like
PR9 family					
B04_TRINITY_DN57832_c1_g1_i2	5,17428785	7,97888569	10,2541248	PRX20	peroxidase
A09_TRINITY_DN43570_c0_g1_i5	3,98160419	9,36733916	9,95719138		peroxidase
C09_S2684103	0	6,6469424	8,64879317		peroxidase
A07_TRINITY_DN50410_c0_g1_i6	2,36151208	4,5583201	8,60935762		peroxidase
A02_TRINITY_DN23535_c1_g2_i11	2,0070456	5,29711239	8,47627902	PRX20	peroxidase
A06_TRINITY_DN41789_c0_g1_i2	5,12537167	9,91232082	8,33448347		peroxidase
A10_TRINITY_DN43364_c0_g1_i22	2,39761397	5,23892726	7,93399142	PRX20	peroxidase
B01_TRINITY_DN29562_c3_g1_i3	5,16090273	9,65200872	7,56079391		peroxidase
C06_R7103250	0	5,8028481	7,55086569		peroxidase
A02_TRINITY_DN23848_c0_g3_i6	2,11436187	4,549225	6,65365731	PRX20	peroxidase
A09_TRINITY_DN41597_c0_g2_i4	1,93985306	4,3241729	6,36347101	PRX20	peroxidase
A05_TRINITY_DN37982_c0_g1_i6	0	3,07140559	6,18816941	PRX20	peroxidase
C10_R3589587	1,36804391	2,87475715	5,87223516	PRX20	peroxidase
A05_TRINITY_DN49981_c0_g1_i6	0,50643638	3,19374099	5,44019041	PRX20	peroxidase

C07_R5395352	1,48335086	4,00335549	5,36328915	OSI_34838	peroxidase
A03_TRINITY_DN28873_c1_g1_i1	0	3,62088511	5,29697615	OSI_34838	peroxidase
A02_TRINITY_DN21625_c1_g2_i6	0	2,4108992	5,27095609		peroxidase
C12_R626021	1,19041848	3,68086642	5,16689168		peroxidase
C10_R3435785	0	1,67945442	4,65321838	OSI_34838	peroxidase
A08_TRINITY_DN27816_c0_g1_i16	0	2,58698682	3,96560989	PRX20	peroxidase
C11_R1182875	0	1,95196789	3,82082482		peroxidase
C09_R2654888	0	0	3,61272329		peroxidase
B05_TRINITY_DN55347_c2_g1_i5	0	1,12329907	3,58196581		peroxidase
A07_TRINITY_DN27955_c0_g1_i1	0	2,22472748	3,44985812	OSI_34838	peroxidase
C09_R2692092	0	1,82531918	3,44619383		peroxidase
A10_TRINITY_DN42866_c1_g3_i2	0	1,14991499	3,39062327		peroxidase
A06_TRINITY_DN52330_c0_g2_i3	0	0	3,19073194	OSI_34838	peroxidase
A01_TRINITY_DN19128_c10_g1_i5	1,07801311	1,49481134	2,55926768		peroxidase
A02_TRINITY_DN27516_c4_g2_i8	1,66073547	2,4459771	2,52829728		peroxidase
C09_R2706114	0	0	2,37685641		peroxidase
C10_R3549304	0	1,20509554	2,33546784		Peroxidase
A06_TRINITY_DN53662_c1_g2_i1	0	0	2,32936977		peroxidase
A03_TRINITY_DN22364_c0_g1_i2	0	0	2,16940272		peroxidase
A03_TRINITY_DN29887_c0_g1_i7	0	0	2,02015929		peroxidase
C12_R625412	0,64499851	0	1,93790517		peroxidase
C09_R2715356	0	0	1,90597948		peroxidase
C09_R2677601	0	0	1,86032187		peroxidase
C12_R639930	0	0	1,64783198	OSI_34838	peroxidase
C10_R3461200	0	1,51640237	1,60635174	OSI_34838	peroxidase
C10_S3747365	1,28838249	0	1,55599385		peroxidase
C07_R5254366	0	0	1,38223472		peroxidase

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C07_S1165358	0	0,75721885	1,09726289		peroxidase
A06_TRINITY_DN47250_c0_g1_i8	0	0,55858538	1,025935		peroxidase
B05_TRINITY_DN52135_c0_g3_i1	0	0	0,87080222		peroxidase
A06_TRINITY_DN44748_c0_g1_i3	0	1,53411757	0	OSI_34838	peroxidase
C08_R3945622	0	1,06809654	0	OSI_34838	peroxidase
C09_R2682379	0	-1,26307332	0	OSI_34838	peroxidase
C10_R3612931	0	1,70515155	0		peroxidase
C11_R1208490	1,23730228	1,24163321	0	OSI_34838	peroxidase
C11_S1122936	0	1,48641294	0		peroxidase
A01_TRINITY_DN19578_c3_g2_i5	0	0	-1,08855287	PRX20	peroxidase
A07_TRINITY_DN51795_c1_g1_i4	0	0	-1,1075641		peroxidase
C12_R654359	0	0	-1,36736354		peroxidase
A08_TRINITY_DN26481_c0_g1_i8	0	-0,71029061	-1,44376892		peroxidase
C09_S2634122	0	0	-1,66454296		peroxidase
C12_R631330	0	-0,90927352	-1,69699804		peroxidase
C07_R5255587	0	0	-1,71712544		peroxidase
A09_TRINITY_DN36307_c0_g3_i2	0	-1,42985319	-1,77611743		peroxidase
A05_TRINITY_DN36776_c0_g2_i1	0	0	-2,43583401		peroxidase
A06_TRINITY_DN40963_c1_g1_i4	0	-0,7865008	-2,50411746		peroxidase
A06_TRINITY_DN49235_c0_g1_i17	0	0	-2,68193653		peroxidase
A08_TRINITY_DN6960_c0_g1_i1	0	0	-3,99594428		peroxidase
A11_TRINITY_DN55226_c0_g1_i10	0	-2,80992498	-4,30131266		peroxidase

Supplementary Table 7. Cell wall related differential expressed (DE) genes in *Pinus pinaster* dataset. (Chapter 6).

Xyloglucan galactosyltransferase					
	3dpi	5dpi	10dpi	Predicted Gene	Description
A11_TRINITY_DN54162_c0_g1_i1	1.56715169	4.27523492	4.9632862		xyloglucan galactosyltransferase
A02_TRINITY_DN22074_c0_g1_i12	0	4.21894567	4.91847782		xyloglucan galactosyltransferase
B01_TRINITY_DN33360_c2_g2_i6	0	4.1572395	4.46844839		xyloglucan galactosyltransferase
A01_TRINITY_DN15913_c8_g1_i1	0	0	1.21386974		xyloglucan galactosyltransferase
C11_R193800	0	0.62442685	0.7579429		xyloglucan galactosyltransferase
Xyloglucan endotransglucosylase hydrolase					
C08_R3964450	1.66465881	6.74453726	8.82893974		Xyloglucan endotransglucosylase hydrolase protein
A10_TRINITY_DN46586_c0_g1_i14	2.1646184	5.79446418	8.52395137		Xyloglucan endotransglucosylase hydrolase protein
C06_R7137955	0	3.76857123	7.33631593		xyloglucan endotransglucosylase/hydrolase 12
A02_TRINITY_DN24373_c1_g1_i17	0	3.76753643	4.32117549		Xyloglucan endotransglucosylase hydrolase protein
B03_TRINITY_DN38998_c0_g1_i2	0	-1.47276795	3.55643283		Xyloglucan endotransglucosylase hydrolase protein
B01_TRINITY_DN28990_c0_g2_i1	0	-1.19927736	3.11539127		Xyloglucan endotransglucosylase hydrolase protein
A02_TRINITY_DN24373_c1_g1_i9	0	2.25397443	2.34569656		Xyloglucan endotransglucosylase hydrolase protein
C10_R3632750	0	0	1.68451249		Xyloglucan endotransglucosylase hydrolase protein
C12_R682742	0	1.49138248	1.57521577		Xyloglucan endotransglucosylase hydrolase protein
A02_TRINITY_DN24225_c1_g1_i5	0	0	1.13518383		Xyloglucan endotransglucosylase hydrolase protein
A06_TRINITY_DN40837_c4_g1_i13	0	1.34491228	1.89347774		Xyloglucan endotransglucosylase hydrolase protein
C09_R2670433	0	1.22423423	0.54935886		Xyloglucan endotransglucosylase hydrolase protein
A05_TRINITY_DN51167_c1_g1_i12	0.73862664	0	0		Xyloglucan endotransglucosylase hydrolase protein
C06_R6963969	0	-1.1618719	0		Xyloglucan endotransglucosylase hydrolase protein
C09_R2651194	0	-0.63562371	0		Xyloglucan endotransglucosylase hydrolase protein
C12_R657476	0.54633334	0	0		xyloglucan endotransglucosylase/hydrolase 10
B05_TRINITY_DN57642_c0_g1_i5	0	0	-0.66397218		Xyloglucan endotransglucosylase hydrolase protein
C08_R3968611	0	0.93872735	-0.69395924		Xyloglucan endotransglucosylase hydrolase protein

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A03_TRINITY_DN28544_c0_g1_i1	0	-0.78711957	-0.71567217		Xyloglucan endotransglucosylase hydrolase protein
A01_TRINITY_DN21294_c7_g1_i1	0	-0.76514443	-1.98554937	PGUG_02516	xyloglucan endotransglucosylase/hydrolase protein 22
C06_R6838662	0	0	-1.45735882		Xyloglucan endotransglucosylase hydrolase protein
A02_TRINITY_DN24541_c1_g3_i5	0	-0.72382364	-1.45826791		Xyloglucan endotransglucosylase hydrolase protein
A07_TRINITY_DN49958_c0_g1_i2	0	-1.28588376	-1.47845982		Xyloglucan endotransglucosylase hydrolase protein
A11_TRINITY_DN49040_c0_g1_i3	0	0	-1.49923374		Xyloglucan endotransglucosylase hydrolase protein
C11_R1166101	0	0	-1.69312777	XTH7	xyloglucan endotransglucosylase/hydrolase 6
C09_R2735578	-0.75766771	-1.51146252	-1.72493817		Xyloglucan endotransglucosylase hydrolase protein
C10_R3584382	0	-1.21649254	-2.86963565		xyloglucan endotransglucosylase/hydrolase 32
C11_R1169290	0	-1.51685115	-2.13386413		Xyloglucan endotransglucosylase hydrolase protein
A06_TRINITY_DN52976_c0_g1_i11	0	0	-2.12532832		Xyloglucan endotransglucosylase hydrolase protein
C08_R3920894	0.89765472	0	-2.2318219		Xyloglucan endotransglucosylase hydrolase protein
A10_TRINITY_DN44781_c0_g2_i3	0	-1.43939649	-2.24393432		Xyloglucan endotransglucosylase hydrolase protein
C09_S2628952	0	0	-2.33852347	OSI_34534	Xyloglucan endotransglucosylase hydrolase protein
C05_J23786999	0	-1.61476323	-2.73593838		Xyloglucan endotransglucosylase hydrolase protein
C04_R10921292	0	-1.23866279	-3.24452988		Xyloglucan endotransglucosylase hydrolase protein
C12_R677771	0	-0.84692366	-3.51543618		xyloglucan:xyloglucosyl transferase 33
Expansins					
A06_TRINITY_DN47814_c0_g1_i10	0	0	-1.26737504		expansin B1
B03_TRINITY_DN41146_c1_g1_i4	0	0	-1.28756929	EXLA1	expansin-like
C11_S1278061	0	0	-2.0221216	EXPA5	expansin A3
B02_TRINITY_DN59053_c0_g1_i1	2.23235733	9.68882802	11.9514613		expansin-like
A05_TRINITY_DN50800_c0_g1_i7	0	4.94117167	9.26892104		expansin-like
C08_S4087992	0	5.58999518	7.8832701		expansin A1
C09_S2775448	0	3.54400934	7.77850913		expansin A1
C08_S4048066	0	3.58566194	7.76173961		expansin A1
C09_R2670362	0	2.65171611	5.54955076		expansin A1
A07_TRINITY_DN45924_c0_g1_i3	0	2.15955774	5.41292253		expansin B1

C11_R1186126	0	2.92235364	4.24705004		expansin-like B1
C09_R2691346	0	1.22338802	3.71781859		expansin A
C09_S2643429	0	2.07775865	2.72475628		expansin
A06_TRINITY_DN46820_c0_g1_i1	0	0	2.04840193	EXLA1	Expansin-like A1
B02_TRINITY_DN43687_c0_g1_i1	0	0.69790744	1.07286083		expansin A4
C07_R5232269	0.6211222	0	0.80525487		expansin A9
A08_TRINITY_DN27827_c0_g1_i13	0	-0.92627409	0		expansin A1
B03_TRINITY_DN37741_c1_g1_i8	0	-0.85870212	0		expansin A1
B05_TRINITY_DN57170_c2_g1_i2	0	-0.55195024	0		expansin B1
C07_R5352538	0	1.09471193	0		expansin
C08_R3886521	0	-0.85333198	0	EXLA1	expansin A1
C08_S3960693	0	-0.92317553	0		expansin A1
C10_S3746944	0.78022769	0	0	EXPA5	expansin A9
C11_R1227549	0	-3.73790781	0		expansin B1
cellulose synthase					
A06_TRINITY_DN39665_c3_g1_i9	0	1.36859438	1.82850932		cellulose synthase-like protein
C11_S1149114	0	0	1.42291325		cellulose synthase-like protein
C05_S24037911	0	0	1.17402754		cellulose synthase-like protein
A06_TRINITY_DN46233_c0_g3_i2	0	0	-0.64891568		cellulose synthase A2
C11_R1211910	0	0	-0.75611038		cellulose synthase
B02_TRINITY_DN37839_c2_g2_i5	0	0	-0.79329889	CESA1	cellulose synthase
B02_TRINITY_DN37932_c0_g1_i3	0	-1.40562668	-0.96807682		cellulose synthase-like protein
C11_R1241622	0	0	-1.09632386	CSLD2	cellulose synthase-like protein
B03_TRINITY_DN38610_c0_g1_i19	0	-1.37853676	-1.10517442		cellulose synthase-like protein
A08_TRINITY_DN28373_c2_g1_i25	0	-0.61307941	-1.10695314		cellulose synthase-like protein
A01_TRINITY_DN20745_c3_g3_i2	0	0	-1.42311981	ATCSLA09	cellulose synthase A2
C12_S438133	0	-0.86619909	-1.63137513	CSLD2	cellulose synthase-like protein
C12_R639484	0	-0.64034302	-1.8298517	ATCSLA09	cellulose synthase A1

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C06_J6977865	0	-0.8681639	-2.01780065	CESA1	cellulose synthase-like protein
C04_R10833326	0	0	-2.2176696		cellulose synthase A
A08_TRINITY_DN27273_c2_g2_i5	0	-1.29488222	-3.76048085		cellulose synthase A2
A07_TRINITY_DN50247_c0_g3_i1	-0.89474422	-1.60896326	-4.41503543		cellulose synthase-like protein
C09_R2760682	0	-1.76471088	-4.84875914		cellulose synthase-like protein
B02_TRINITY_DN37839_c2_g2_i5	0	0	-0.79329889	CESA1	cellulose synthase, partial
C06_J6977865	0	-0.8681639	-2.01780065	CESA1	cellulose synthase A catalytic subunit 2
Cinnamyl alcohol dehydrogenase					
A10_TRINITY_DN31534_c0_g2_i1	8.96364251	9.87500595	13.7599117	FG01686.1	cinnamyl alcohol dehydrogenase homolog 2
C08_S3862566	0	0	12.6028656		cinnamyl alcohol dehydrogenase homolog 3
B05_TRINITY_DN61652_c0_g1_i4	0.63092997	3.4114732	5.97269112		Cinnamyl alcohol dehydrogenase
C05_S23770481	0	0.52560659	1.73280597		Cinnamyl alcohol dehydrogenase
A11_TRINITY_DN52049_c0_g1_i10	0	0	1.44209222		Cinnamyl alcohol dehydrogenase
C11_S1150888	0	0	0.7947955	CAD	Cinnamyl alcohol dehydrogenase
C11_R1184664	0	0	-1.46386452	ELI3-2	Cinnamyl alcohol dehydrogenase
A05_TRINITY_DN48193_c0_g1_i8	0	0	-3.04671033	ELI3-2	Cinnamyl alcohol dehydrogenase
Polygalacturonase					
B05_TRINITY_DN50035_c1_g1_i2	0	11.5956731	12.5377348	ADPG1	polygalacturonase
A11_TRINITY_DN54934_c0_g1_i4	2.89799001	6.07148777	8.78312396		Polygalacturonase non-catalytic subunit
B02_TRINITY_DN37367_c1_g1_i2	2.75659361	6.25126785	8.50455379		Polygalacturonase non-catalytic subunit
A10_TRINITY_DN45953_c0_g1_i2	2.4210129	5.59572997	8.38275324	OSI_25331	polygalacturonase
A03_TRINITY_DN30543_c0_g1_i14	0	4.31129461	7.48076449	ADPG1	polygalacturonase
A01_TRINITY_DN22587_c0_g3_i5	1.35763419	5.1864212	6.2364449		polygalacturonase QRT3-like
A06_TRINITY_DN46578_c0_g2_i4	1.86702804	4.99109447	5.39886662		Polygalacturonase non-catalytic subunit
C11_J1174288	0	0.95043248	3.06445221		Polygalacturonase
C12_R632916	0	0	1.4526143		polygalacturonase
A02_TRINITY_DN26065_c0_g1_i14	0	0	1.15415779		Polygalacturonase-like
C07_J5234815	0	-0.50675863	0		polygalacturonase QRT3-like

C10_R3472801	0	0	-0.73134211		polygalacturonase
C10_R3449663	0	0	-0.74164547		polygalacturonase
C06_J6906945	0	0	-0.96758154		polygalacturonase QRT3-like
C07_R5304461	0	0	-1.35613594		Polygalacturonase-like
C09_R2688450	0	0	-1.54572122		polygalacturonase
B03_TRINITY_DN42929_c2_g1_i4	0	-0.7161365	-1.80632365		polygalacturonase
Pectinesterase					
C08_R3903894	10.0483432	11.6572254	12.2598871	FG03406.1	pectinesterase 11
A06_TRINITY_DN53074_c1_g1_i2	4.75699949	11.0284809	12.0793174		pectin methylesterase
A02_TRINITY_DN21565_c4_g1_i3	2.79468069	5.80301685	8.82313468		pectinesterase
A06_TRINITY_DN53409_c1_g2_i4	2.22163603	7.32579149	8.05212341		pectinesterase
C09_R2666646	1.32254457	4.79569705	7.02459985		pectinesterase
C11_R1187310	0	3.52980304	6.17976728		pectinesterase
A06_TRINITY_DN40792_c0_g1_i10	0	2.86154523	5.57362514		pectinesterase
C09_R2678186	1.12505543	2.62929457	4.28854631		pectinesterase
A01_TRINITY_DN18371_c4_g3_i1	0	0	4.14088895		pectinesterase 11
C12_S608539	0	1.89141603	2.97380187		pectinesterase
C09_J2652547	0	1.1555773	2.44365078		pectinesterase 3-like protein
C07_S5485002	0	0	2.14832333		pectinesterase
A05_TRINITY_DN35198_c0_g1_i13	0	0.89387081	2.02396086		probable pectinesterase 53 isoform X1
A10_TRINITY_DN46858_c0_g1_i1	0	1.70667562	1.47738456		pectinesterase
C09_S2654066	0	0	1.39413801		pectin methylesterase-like protein
A11_TRINITY_DN45132_c0_g1_i3	0	0	1.29378812		probable pectinesterase 53 isoform X2
B01_TRINITY_DN32957_c3_g4_i3	0	0	1.0082998		pectinesterase
B01_TRINITY_DN37534_c0_g1_i4	0	0	0.97283262		pectinesterase-like isoform X2
C12_S299819	0	0.65295495	0.7751012		pectinesterase
A06_TRINITY_DN52004_c0_g1_i6	0	-0.54548106	0		pectinesterase
A07_TRINITY_DN41318_c0_g1_i3	0	1.16720576	0		pectinesterase

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C11_R1162157	0	-0.67934557	0	pectinesterase
C12_R636769	0	-0.73425769	0	pectinesterase
A11_TRINITY_DN50855_c0_g1_i3	0	-0.5698331	-0.58176165	pectinesterase
B04_TRINITY_DN55881_c2_g2_i1	0	0	-0.63083852	pectinesterase
A10_TRINITY_DN46858_c1_g1_i3	0	-0.54669907	-0.83106073	pectinesterase
C08_S3341940	0	0	-0.90557726	probable pectinesterase 8
A11_TRINITY_DN44911_c0_g1_i2	0	0	-1.24170112	pectinesterase
A05_TRINITY_DN51602_c1_g1_i5	0	-0.65063308	-1.25426959	pectinesterase
B02_TRINITY_DN35422_c4_g1_i6	0	-0.83283525	-1.26586705	pectinesterase
C06_R6988767	0	-0.77962379	-1.41003801	pectinesterase
A07_TRINITY_DN53652_c0_g1_i2	0	0	-1.42503099	pectinesterase
C12_S512494	0	-0.96758275	-2.05119652	pectinesterase
C09_S950131	0	-0.59128538	-2.12832211	pectinesterase
C09_S1021204	0	-0.98233921	-3.89892034	pectinesterase
Pectate lyase				
C08_S4125779	0	3.9940567	3.54777284	Pectate lyase
C11_R1182792	0	0	2.39749502	Pectate lyase
A05_TRINITY_DN51980_c2_g1_i1	0	1.72828658	2.09327667	Pectate lyase
C08_S3902146	0	2.18211203	1.81226641	Pectate lyase
C11_R1157670	0	1.0121358	1.72868044	Pectate lyase
C09_R2665942	0	1.39804547	1.68085771	Pectate lyase
C11_S1164094	0	1.97776196	1.41657183	Pectate lyase
B05_TRINITY_DN58319_c2_g1_i1	0	0.93237124	1.25608396	Pectate lyase
A08_TRINITY_DN26597_c7_g1_i10	0	1.59148633	1.01652635	Pectate lyase
A10_TRINITY_DN44589_c1_g1_i2	0	-1.17861787	0	probable pectate lyase 8 isoform X1
C09_R2666645	0	-1.21988723	0	Pectate lyase
C12_J628710	0	-0.86420374	0	Pectate lyase
C07_R5477221	0	0	-0.57799076	Pectate lyase

C11_S1174512	0	0	-0.64620184	Pectate lyase
A07_TRINITY_DN42688_c2_g1_i3	0	0	-0.65672795	Pectate lyase
A05_TRINITY_DN52282_c2_g1_i1	0	0	-0.6768834	Pectate lyase
A10_TRINITY_DN45133_c0_g2_i1	0	-0.63630863	-0.71415917	Pectate lyase
C12_S604760	0	-0.51293607	-0.73725664	Pectate lyase
C08_R3963603	0	0	-1.9051546	Pectate lyase
C12_R664139	0	0	-2.60405832	Pectate lyase
C11_R1188090	0	0	-2.72542174	Pectate lyase
C06_R6856096	0	-0.84825992	-3.14137301	Pectate lyase
C07_S5274047	0	0	-4.39842851	Pectate lyase

Supplementary Table 8: Oleoresin related terpenoids differential expressed (DE) genes in *Pinus pinaster* dataset. (Chapter 6).

	3dpi	5dpi	10dpi	Predicted Gene	Description
TERPENOIDS					
A06_TRINITY_DN53246_c0_g1_i12	0	0	-0.82430755		alpha-pinene synthase
C05_R23786221	0	0	-2.23587098		(+)-alpha-pinene synthase
C10_R3565726	0	0	-0.69800146		(-)-alpha-pinene synthase
C11_R1161309	0	0	-2.62170655		(-)-alpha-pinene synthase
C07_S5253718	0	0	-1.57710114		(+)-alpha-pinene synthase
C09_R2668160	0	1.06154343	3.98599896		(-)-alpha-pinene synthase
C08_R3920981	1.32778397	2.25066957	7.31037345	CPS1	Monofunctional isopimaradiene synthase
C11_S1164207	0	0	2.9245078	CPS1	Monofunctional isopimaradiene synthase
C09_R2654356	0	0	-1.13842875	CPS1	Monofunctional isopimaradiene synthase
C09_R2652402	0	0	-1.84470953	CPS1	Monofunctional isopimaradiene synthase
C09_R2710042	3.63779578	4.2207906	3.67616952		Alpha-bisabolene synthase
C09_R2660084	1.28606588	1.25593535	2.13160581		Alpha-bisabolene synthase
C12_R625237	0	0	-1.66601882		Delta-selinene synthase
A06_TRINITY_DN52549_c0_g1_i19	0	0	-3.28233791		longifolene synthase
C04_R10799926	0	0	-1.79840141		(+)-3-carene synthase

Supplementary Table 9. Hormone related differential expressed (DE) genes in *Fusarium circinatum*. (Chapter 6).

	Log2 (Fold Change) value				
Sequence ID	3v5 dpi	3v10 dpi	5v10 dpi	Predicted Gene	Description
Enzymes-related to GA biosynthesis					
FCIRG_05400T1	-6,4482	-8,5149	0,0000		Gibberellin cluster-kauren synthase
FCIRG_05399T1	-7,1855	-9,6374	-2,4519		Gibberellin cluster-C13-oxidase
FCIRG_05404T1	-5,5082	-7,1938	0,0000		Gibberellin cluster-GA14-synthase
FCIRG_05401T1	-6,7532	-9,1462	-2,3930		Gibberellin cluster-GGPP-synthase
Enzymes-related to Ethylene biosynthesis					
FCIRG_14291T1	-4,0340	-7,6199	-3,5859		2-oxoglutarate-dependent ethylene succinate-forming enzyme
FCIRG_01209T1	0,0000	-2,6599	-1,7377	ADI1	Catalyzes the formation of formate and 2-keto-4- methylthiobutyrate (KMTB) from 1,2-dihydroxy-3-keto-5- methylthiopentene (DHK-MTPene)
Auxin efflux carrier					
FCIRG_12316T1	-2,0638	-3,9266	-1,8628	PGUG_01990	auxin efflux carrier
ICSH					
FCIRG_14838T1	-2,9379	-4,4853	0,0000	FG07259.1	Isochorismatase
FCIRG_03234T1	-3,4771	-4,4394	0,0000	FG01746.1	Isochorismatase family hydrolase

Supplementary Table 10. Cell wall degrading enzymes (CWDE) differentially expressed (DE) related genes in *Fusarium circinatum* dataset. (Chapter 6).

	3v5	3v10	5v10	Description	Predicted Gene
Beta-glucosidase					
FCIRG_07911T1	-1.92	-2.84	0.00	Beta-glucosidase	BTGE
Xylanolytic enzymes					
FCIRG_14051T1	0.00	-1.85	0.00	xylanolytic enzymes	XLNR
Endoglucanase					
FCIRG_04966T1	-5.00	-8.59	-3.59	endoglucanase B	FG06397.1
FCIRG_13074T1	0.00	-3.31	-2.68	endoglucanase	
Polygalacturonase					
FCIRG_02737T1	0.00	2.24	0.00	polygalacturonase	PGAD
FCIRG_02825T1	0.00	-1.22	0.00	endopolygalacturonase 1	PGAB
FCIRG_08336T1	0.00	-2.24	0.00	Exo-polygalacturonase	PGAX
FCIRG_05295T1	0.00	-3.51	-2.64	Polygalacturonase	FG03194.1
FCIRG_02825T1	0.00	-1.22	0.00	Polygalacturonase 1	PGAB
FCIRG_08336T1	0.00	-2.24	0.00	Exo-polygalacturonase	PGAX
FCIRG_05295T1	0.00	-3.51	-2.64	Polygalacturonase	FG03194.1
Pectate lyase					
FCIRG_07063T1	-4.03	-8.45	-4.42	pectate lyase	FG03713.1
Cutinase					
FCIRG_05606T1	0.00	-4.30	-3.29	cutinase palindrome-binding protein	WC2
FCIRG_04964T1	-1.55	-2.61	0.00	cutinase transcription factor 1 alpha	CTF1
FCIRG_14021T1	-1.55	-2.97	0.00	cutinase transcription factor 1 beta	FG07192.1

Supplementary Table 11. Ergosterol and chitin biosynthesis differentially expressed (DE) related genes in *Fusarium circinatum* dataset.

	3v5	3v10	5v10	Predicted Gene	Description
ERGOSTEROL BIOSYNTHESIS					
FCIRG_12182T1	0.00	-3.92	-2.71	ERG1	squalene monooxygenase
FCIRG_14456T1	-1.62	-4.79	-3.17	ERG11	Cytochrome p450
FCIRG_14702T1	-1.73	-3.92	-2.18	ERG11	Cytochrome p451
FCIRG_08916T1	-1.66	-4.05	-2.39	ERG12	mevalonate kinase
FCIRG_06661T1	-2.33	-4.29	-1.96	ERG2	C-8 sterol isomerase
FCIRG_14873T1	-1.96	-3.41	0.00	ERG24	Delta(14)-sterol reductase
FCIRG_00844T1	-1.71	-4.57	-2.86	ERG25	C-4 methylsterol oxidase
FCIRG_07221T1	-1.56	-3.63	-2.06	ERG26	C-3 sterol dehydrogenase
FCIRG_02056T1	-1.76	-4.81	-3.05	ERG3	C-5 sterol desaturase
FCIRG_03131T1	0.00	-2.27	0.00	ERG4	delta24(24(1))-sterol reductase
FCIRG_13663T1	-1.95	-4.11	-2.16	ERG5	C-22 sterol desaturase
FCIRG_03557T1	-2.32	-4.38	-2.06	ERG6	Sterol 24-c-methyltransferase
FCIRG_10915T1	-1.76	-2.76	0.00	ERG8	Phosphomevalonate kinase
FCIRG_11234T1	-2.41	-3.88	-1.47	ERG9	squalene synthase
FCIRG_13580T1	-1.51	0.00	0.00	FG03443.1	Ergosterol biosynthesis ERG4/ERG24 family
FCIRG_09472T1	-2.29	-3.53	0.00	HMG1	1 3-hydroxy-3-methylglutaryl-coenzyme A reductase
CHITIN BIOSYNTHESIS					
FCIRG_06618T1	-2.42	-2.81	0.00	CHS2	chitin synthase
FCIRG_09316T1	0.00	-3.76	-2.97	CHS2	chitin synthase
FCIRG_11072T1	-1.62	-2.66	0.00	CHS2	chitin synthase
FCIRG_10502T1	-1.66	-2.86	0.00	CHS5	chitin biosynthesis protein
FCIRG_10891T1	-1.79	-3.02	0.00	CHS3	chitin synthase
FCIRG_11404T1	-1.80	-4.35	-2.55	PGUG_02718	chitinase

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FCIRG_06852T1	-1.83	-3.91	-2.08	0	chitin synthase
FCIRG_06851T1	-1.93	-3.86	-1.93	CHSV	Chitin Synthase
FCIRG_05393T1	-2.03	-3.71	0.00	FG02354.1	chitinase
FCIRG_01091T1	-2.05	-3.36	0.00	CHS7	chitin synthase export chaperone
FCIRG_08401T1	-2.39	-3.48	0.00	FG05969.1	Class III chitinase
FCIRG_04837T1	-2.86	-3.81	0.00	CHS1	chitin synthase
FCIRG_13807T1	0.00	-1.93	0.00	PGUG_02007	Chitin synthase
FCIRG_13292T1	0.00	-2.68	-1.88	FG06506.1	chitin binding protein
FCIRG_10583T1	0.00	-3.93	0.00	FG08673.1	chitin synthase activator
FCIRG_03959T1	0.00	-5.27	-3.97	CHS2	chitin synthase
FCIRG_09259T1	0.00	-6.86	-6.12	FG07072.1	Chitin synthesis regulation
FCIRG_11508T1	-1.73	-2.10	0.00	FG00952.1	chitinase
FCIRG_01050T1	-1.95	-3.48	-1.53	FG07937.1	chitin synthase

Supplementary Table 12. *Fusarium circinatum* DE genes related to hormone production with hits in the Pathogen Host Interaction (PHI) database. (Chapter 6).

Query ID	Gene description	Gene name	PHI accession	Species	% identity	E-value	PHI phenotype
FCIRG_03 234T1	Isochorismatase family hydrolase (ICSH)	EntB	6565	<i>Klebsiella pneumoniae</i>	26.000	2,85E-05	unaffected pathogenicity, reduced virulence
FCIRG_14 291T1	Catalyzes the formation of formate and 2-keto-4-methylthiobutyrate (KMTB) from 1,2-dihydroxy-3-keto-5-methylthiopentene (DHK-MTPene)	FTF1	5483	<i>Fusarium oxysporum</i>	90.909	6,31E-55	reduced virulence
FCIRG_05 399T1	Gibberellin cluster-C13-oxidase	Akt7	4194	<i>Alternaria alternata</i>	22.794	5,95E-06	increased virulence (hypervirulence), reduced virulence
		Cyp51a	2907	<i>Fusarium graminearum</i>	25.248	4,55E-05	unaffected pathogenicity, reduced virulence
		Cyp51a	1155	<i>Zymoseptoria tritici</i>	22.330	6,82E-04	chemistry target: resistance to chemical
		Hic-15	7173	<i>Verticillium dahliae</i>	25.366	8,41E-04	reduced virulence
FCIRG_05 404T1	Gibberellin cluster-GA14-synthase	o-methylsterigmatocystin_oxidoreductase	2393	<i>Fusarium graminearum</i>	27.485	4,39E-10	increased virulence (hypervirulence)
		Mocyp51a	2190	<i>Magnaporthe oryzae</i>	23.767	4,16E-07	reduced virulence
		Mocyp51b	2191	<i>Magnaporthe oryzae</i>	24.878	5,87E-07	unaffected pathogenicity
		Fgerg5a	3037	<i>Fusarium graminearum</i>	27.950	3,90E-06	reduced virulence
		Akt7	4194	<i>Alternaria alternata</i>	27.273	1,90E-05	increased virulence (hypervirulence), reduced

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					virulence
Cyp51b	2908	<i>Fusarium graminearum</i>	24.752	3,26E-05	unaffected pathogenicity, reduced virulence
Fgerg5b	3038	<i>Fusarium graminearum</i>	29.747	1,09E-04	reduced virulence
Hic-15	7173	<i>Verticillium dahliae</i>	23.529	1,11E-04	reduced virulence
Bxcyp33c9	4607	<i>Bursaphelenchus xylophilus</i>	24.675	1,57E-04	reduced virulence
C14dm	3258	<i>Leishmania major</i>	26.490	2,99E-04	reduced virulence
Erg11	8030	<i>Candida albicans</i>	21.285	5,69E-04	loss of pathogenicity

Supplementary Table 10. *Fusarium circinatum* potential virulence and pathogenicity differentially expressed (DE) genes with hits in the Pathogen Host Interaction (PHI) database. (Chapter 6).

Query ID	Access ID	Pathogen gene	Gene function	Pathogen species	Host	Mutant phenotype	% identity	E-value
FCIRG_05404T1	PHI: 2190	MoCYP51A	Sterol 14 a-demethylase; sterol biosynthesis	<i>Magnaporthe oryzae</i>	Oryza sativa	reduced virulence	23,767	8,18E-08
FCIRG_05404T1	PHI: 3037	Fgerg5a	cytochrome P450 61; ergosterol biosynthesis	<i>Fusarium graminearum</i>	Triticum aestivum	reduced virulence	27,95	7,66E-07
FCIRG_05404T1	PHI: 4194	Akt7	Cytochrome P450 monooxygenase; ergosterol biosynthesis	<i>Alternaria alternata</i>	Fragaria x ananassa	reduced virulence	27,273	3,73E-06
FCIRG_05404T1	PHI: 2908	Cyp51b	P450 sterol 14a –sterol biosynthesis	<i>Fusarium graminearum</i>	Triticum aestivum	reduced virulence	24,752	6,40E-06
FCIRG_05404T1	PHI: 3038	Fgerg5b	cytochrome P450 61; ergosterol biosynthesis	<i>Fusarium graminearum</i>	Triticum aestivum	reduced virulence	29,747	2,13E-05
FCIRG_05404T1	PHI: 4607	Bxcyp33c9	Cytochrome P450 Genes	<i>Bursaphelenchus xylophilus</i>	Pinus massoniana	reduced virulence	24,675	3,08E-05
FCIRG_05404T1	PHI: 3258	C14dm	Sterol Biosynthesis	<i>Leishmania major</i>	Mus musculus	reduced virulence	26,49	5,88E-05
FCIRG_05404T1	PHI: 8030	Erg11	Lanosterol 14-alpha demethylase; ergosterol biosynthesis	<i>Candida albicans</i>	Mus musculus	loss of pathogenicity	21,285	1,12E-04
FCIRG_05404T1	PHI: 2909	Cyp51c	P450 sterol 14a; sterol biosynthesis	<i>Fusarium graminearum</i>	Triticum aestivum	reduced virulence	23,039	3,15E-04
FCIRG_0357T1	PHI: 7788	Aldb_(pspto_2673)	Indole-3-acetaldehyde dehydrogenase; indole-3-acetic acid biosynthesis	<i>Pseudomonas syringae</i>	Arabidopsis thaliana	reduced virulence	30,238	1,39E-68
FCIRG_0357T1	PHI: 7787	Alda_(pspto_0092)	Indole-3-acetaldehyde dehydrogenase; indole-3-acetic acid biosynthesis	<i>Pseudomonas syringae</i>	Arabidopsis thaliana	reduced virulence	31,878	1,55E-64
FCIRG_05402T1	PHI: 3037	Fgerg5a	cytochrome P450 61; ergosterol biosynthesis	<i>Fusarium graminearum</i>	Triticum aestivum	reduced virulence	29,688	1,03E-08
FCIRG_05402T1	PHI: 3037	Erg11	Lanosterol 14-alpha demethylase; ergosterol biosynthesis	<i>Candida</i>	Mus	loss of	23,041	5,50E-07

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5402T1	8030		biosynthesis	<i>albicans</i>	musculus	pathogenicity		
FCIRG_05402T1	PHI: 2908	Cyp51b	cytochrome P450 61; ergosterol biosynthesis	<i>Fusarium graminearum</i>	Triticum aestivum	reduced virulence	24,752	6,04E-05
FCIRG_05402T1	PHI: 3038	Fgerg5b	cytochrome P450 61; ergosterol biosynthesis	<i>Fusarium graminearum</i>	Triticum aestivum	reduced virulence	23,932	2,10E-04
FCIRG_05402T1	PHI: 2907	Cyp51a	cytochrome P450 61; ergosterol biosynthesis	<i>Fusarium graminearum</i>	Triticum aestivum	reduced virulence	22,886	4,81E-04
FCIRG_14029T1	PHI: 6872	Atrr	Zn2-Cys6 Transcription Factor; ergosterol biosynthesis	<i>Aspergillus fumigatus</i>	Mus musculus	reduced virulence	24,138	1,54E-07
FCIRG_00355T1	PHI: 325	Ace1	Effector protein, polyketide synthase	<i>Magnaporthe oryzae</i>	Oryza sativa	plant avirulence determinant	37,445	0
FCIRG_00358T1	PHI: 6331	Vdcyp1	Cyt P450 monooxygenase	<i>Verticillium dahliae</i>	Gossypium hirsutum	loss of pathogenicity	25,056	1,72E-25
FCIRG_00358T1	PHI: 438	Bcbot1_(cnd5)	Cyt P450 monooxygenase	<i>Botrytis cinerea</i>	Phaseolus vulgaris	reduced virulence	22,391	1,62E-24
FCIRG_00358T1	PHI: 4194	Akt7	Cytochrome P450 monooxygenase; ergosterol biosynthesis	<i>Alternaria alternata</i>	Fragaria x ananassa	reduced virulence	22,757	3,90E-24
FCIRG_00358T1	PHI: 4608	Bxcyp33c4	Cytochrome P450 Genes	<i>Bursaphelenchus xylophilus</i>	Pinus massoniana	reduced virulence	30,857	7,51E-18
FCIRG_00358T1	PHI: 4609	Bxcyp33d3	Cytochrome P450 Genes	<i>Bursaphelenchus xylophilus</i>	Pinus massoniana	reduced virulence	26,316	2,55E-14
FCIRG_00358T1	PHI: 4607	Bxcyp33c9	Cytochrome P450 Genes	<i>Bursaphelenchus xylophilus</i>	Pinus massoniana	reduced virulence	30,286	1,38E-13
FCIRG_00358T1	PHI: 2908	Cyp51b	cytochrome P450 61; ergosterol biosynthesis	<i>Fusarium graminearum</i>	Triticum aestivum	reduced virulence	22,961	1,90E-12
FCIRG_00358T1	PHI: 2357	Cyp52x1	Fatty Acid Hydroxylase Activity; Cytochrome P450 Subfamily	<i>Beauveria bassiana</i>	Galleria mellonella	reduced virulence	26,984	5,51E-10
FCIRG_00358T1	PHI: 8030	Erg11	Lanosterol 14-alpha demethylase; ergosterol biosynthesis	<i>Candida albicans</i>	Mus musculus	loss of pathogenicity	22,667	6,12E-10

FCIRG_0 0358T1	PHI: 2190	MoCYP51 A	Sterol 14 a-demethylase; sterol biosynthesis	<i>Magnaporthe oryzae</i>	Oryza sativa	reduced virulence	21,642	6,17E-10
FCIRG_0 0358T1	PHI: 2535	Erg11b	14 alpha-demethylase	<i>Aspergillus fumigatus</i>	Mus musculus	loss of pathogenicity	21,849	5,07E-09
FCIRG_0 0358T1	PHI: 2534	Erg11a	14 alpha-demethylase	<i>Aspergillus fumigatus</i>	Mus musculus	loss of pathogenicity	22,594	2,69E-08
FCIRG_0 0358T1	PHI: 3038	Fgerg5b	cytochrome P450 61; ergosterol biosynthesis	<i>Fusarium graminearum</i>	Triticum aestivum	reduced virulence	24,096	5,95E-06
FCIRG_0 0358T1	PHI: 3037	Fgerg5a	cytochrome P450 61; ergosterol biosynthesis	<i>Fusarium graminearum</i>	Triticum aestivum	reduced virulence	27,273	8,36E-06
FCIRG_0 0358T1	PHI: 2909	Cyp51c	P450 sterol 14a -demethylase, an enzyme essential for sterol biosynthesis and the target of azole fungicides	<i>Fusarium graminearum</i>	Triticum aestivum	reduced virulence	21,212	2,06E-05
FCIRG_1 3784T1	PHI: 2896	Bec1005	Effector protein	<i>Blumeria graminis</i>	Hordeum vulgare	reduced virulence	34,043	1,62E-45
FCIRG_0 5542T1	PHI: 2321	Sidi	Acyl-CoA ligase; ergosterol biosynthesis	<i>Aspergillus fumigatus</i>	Mus musculus	reduced virulence	23,801	1,88E-26
FCIRG_0 3748T1	PHI: 5082	Moacat1	Acetyl-CoA acetyltransferase	<i>Magnaporthe oryzae</i>	Oryza sativa	reduced virulence	83,333	0
FCIRG_1 3990T1	PHI: 96	Fas2	Fatty acid synthetase	<i>Candida albicans</i>	Mus musculus	loss of pathogenicity	62,929	0
FCIRG_1 2409T1	PHI: 4602	Fdb2	N-malonyltransferase encoding gene	<i>Fusarium graminearum</i>	Triticum aestivum	reduced virulence	96,878	0
FCIRG_0 0380T1	PHI: 7788	Aldb (pspto 2673)	Indole-3-acetaldehyde dehydrogenase; Indole-3-acetic acid biosynthesis	<i>Pseudomonas_s yiringae</i>	Arabidopsis thaliana	reduced virulence	40,586	3,53E-119
FCIRG_0 6602T1	PHI: 3226	Pnl1	pectin lyase	<i>Penicillium digitatum</i>	Citrus sinensis	reduced virulence	57,643	1,02E-119
FCIRG_0 6602T1	PHI: 4618	Pcpl15	pectate lyase	<i>Phytophthora capsici</i>	Capsicum annuum	reduced virulence	41,562	8,29E-77

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FCIRG_0 6602T1	PHI: 4619	Pcpl16	pectate lyase	<i>Phytophthora capsici</i>	Capsicum annuum	reduced virulence	39,701	1,04E-75
FCIRG_0 6602T1	PHI: 4617	Pcpl1	pectate lyase	<i>Phytophthora capsici</i>	Capsicum annuum	reduced virulence	40	1,79E-68
FCIRG_1 4617T1	PHI: 120	Pth2__Cra t1_(pth2)	carnitine-dependent acetyl unit transport from peroxisomes	<i>Magnaporthe oryzae</i>	Hordeum vulgare	loss of pathogenicity	82,09	4,25E-34
FCIRG_1 4617T1	PHI: 2911	Ss-pth2	Peroxisomal Carnitine Acetyl Transferase	<i>Sclerotinia sclerotiorum</i>	Glycine max	reduced virulence	80,597	5,56E-33
FCIRG_0 4837T1	PHI: 236	Wdchs2	Chitin Synthase	<i>Exophiala dermatitidis</i>	Mus musculus	reduced virulence	61,497	0
FCIRG_0 4837T1	PHI: 1111	Chs4	Chitin Synthase	<i>Ustilago maydis</i>	Zea mays	reduced virulence	54,873	0
FCIRG_0 4837T1	PHI: 1110	Chs3	Chitin Synthase	<i>Ustilago maydis</i>	Zea mays	reduced virulence	48,953	0
FCIRG_0 4837T1	PHI: 336	Chs2	Chitin Synthase	<i>Fusarium oxysporum</i>	Solanum lycopersicum	reduced virulence	50,631	0
FCIRG_0 4837T1	PHI: 1109	Chs2	Chitin Synthase	<i>Ustilago maydis</i>	Zea mays	reduced virulence	46,762	0

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Herbaceous plants in the understory of a pitch canker-affected *Pinus radiata* plantation are endophytically infected with *Fusarium circinatum*

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ABSTRACT

Fusarium circinatum was recently detected as an endophyte in grasses causing no apparent damage. Our goal was to describe the endophytic colonization of herbaceous host plants growing in a plantation of *Pinus radiata* with symptoms of pitch canker disease, which may act as a reservoir of inoculum. We detected the fungus in five species of dicot families (Asteraceae, Lamiaceae, Rosaceae), in addition to two species in the Poaceae. The fungus was found in the aerial part of non-symptomatic hosts, so we describe *F. circinatum* as an endophyte that is mainly transmitted by spores through the air. It was also detected in *Hypochaeris radicata* seeds, suggesting the potential occurrence of vertical transmission. An analysis of microsatellite markers showed a unique haplotype regardless of whether the isolates' origin was pine cankers or non-symptomatic herbaceous plants. Thus, the same genotype can adopt a pathogenic or endophytic lifestyle. We conclude that non-symptomatic plants can act as reservoirs of inoculum; pine seedlings can be infected from senescent tissue of non-symptomatic hosts colonized by the fungus.

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1. Introduction

In plant-fungal interaction research, emphasis has been placed on plant pathogens and their outcome (Stergiopoulos and Gordon, 2014), as well as on arbuscular mycorrhizas and on endophytes (Zabalgogezcoa, 2008; Rodríguez et al., 2009; Hardoim et al., 2015). Traditionally, a fungus that causes symptoms in the host has been defined exclusively as a pathogen, not taking into account

the possibility of an endophytic lifestyle in other hosts (i.e., colonizing tissue of living plants without causing symptoms). However, there are a growing number of examples in the literature where a fungal species can be pathogenic to some plants while being endophytic in others (Zabalgogezcoa, 2008; Hardoim et al., 2015). The terms 'symptomatic host plant' and 'non-symptomatic host plant' have been proposed to refer to these two situations in a specific plant-microbe interaction (Malcolm et al., 2013). The former term involves plants showing symptoms of disease, while the latter implies an endophytic relationship between the plant and fungus. However, these terms do not account for the fact that some fungi can infect endophytically for a period of time, and then cause symptoms (Bacon and Hinton, 1996; Phoitá et al., 2004; Hardoim

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Article

Root Infection of Canker Pathogens, *Fusarium circinatum* and *Diplodia sapinea*, in Asymptomatic Trees in *Pinus radiata* and *Pinus pinaster* Plantations

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Abstract: The existence of a latent stage within host tissue of the pine pathogens *Fusarium circinatum* and *Diplodia sapinea*, the causal agents of pitch canker and shoot blight disease respectively, has previously been cited. However, studies on this cryptic phase in each disease lifecycle has only been focused on the host aerial parts but not on the roots. Therefore, our objective was to analyze the presence of both pathogens in roots of non-symptomatic mature trees in plantations where the pathogens are known to be causing canker symptoms. For that, we sampled roots from ten non-symptomatic and ten symptomatic trees in three *Pinus radiata* and one *Pinus pinaster* plantations in Basque Country, Spain. Both pathogens were isolated from roots of non-symptomatic trees in a higher frequency than from roots of symptomatic trees, 23.3% and 6.6% respectively for *D. sapinea* and 16.6% and 3.3% respectively for *F. circinatum*. Neither pathogens was detected in the *P. pinaster* plantation. The two pathogens were never isolated from the same tree. A high molecular variability was observed for *D. sapinea* isolates with six different haplotypes and two mating types for the eleven characterized isolates, but only one haplotype and mating type was found for *F. circinatum*, with all isolates of both fungi being proved pathogenic. These results evidence the importance root infection may have in the disease lifecycle and, therefore, disease management.

Keywords: pine pitch canker; pine shoot blight; Monterey pine; maritime pine; root; latent; lifecycle; non-symptomatic

1. Introduction

Fusarium circinatum Nirenberg and O'Donnell 1998 [1] and *Diplodia sapinea* (Fr.) Fuckel [2] are two of the most important pathogens that affect *Pinus* species causing canker diseases, and can result in significant economic losses in nurseries, plantations and natural forests worldwide [3–8].