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TRANSCRIPTOMICS TECHNOLOGIES AND THEIR POTENTIAL FOR PINE PITCH CANKER MANAGEMENT

Presented by/Presentada por:

Cristina Zamora Ballesteros

Supervisors/Directores:

Dr. Julio J. Diez Casero
Dr. Jorge Martín García

Transcriptomics technologies and their potential for pine pitch canker management

Tecnologías transcriptómicas y su potencial en la gestión del chancro resinoso del pino

PhD Student:
Cristina Zamora Ballesteros

Supervisor:
Dr. Julio Javier Diez Casero

Co-supervisor:
Dr. Jorge Martín García
Sustainable Forest Management Research Institute
Department of Vegetal Production and Forest
Resources, University of Valladolid
Palencia, Spain

Supervisor of the International Stay:
Dr. Alex Douglas
School of Biological Sciences
Zoology Building
Tillydrone Avenue
Aberdeen AB24 2TZ
Scotland, United Kingdom

External reviewers:
Dr. Carmen Morales Rodriguez
Department of Innovation of Biological Systems, Food and Forestry (DIBAF)
University of Tuscia
Viertbo, Italy

Dr. Maria Helena Pires Bragança
Instituto Nacional de Investigação Agrária e Veterinária (INIAV)
Oeiras, Portugal

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“The output of your PhD is not your thesis.
The output is YOU”.

@DrNishaKThakur

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Cristina Zamora Ballesteros

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Abstract

Pine pitch canker (PPC) is a serious disease of *Pinus* spp. and *Pseudotsuga menziesii* globally. The infection of its causal agent, *Fusarium circinatum*, causes pitch or resin-soaked cankers on trunks and lateral branches of mature hosts, which may eventually die due to girdling or stem breakage. In nurseries, the main symptoms are damping-off and tip dieback of seedlings. However, the pathogen, with a hemibiotrophic nature, can remain endophytic in pine seedlings that do not show symptoms of infection and even inconspicuous in some herbaceous species. Since the first report in 1945 in North America, the presence of *F. circinatum* has been notified in 14 countries in America, Asia, Africa and Europe. Several factors have contributed to the spread of the disease to all these continents, the most important being globalization in terms of trade in reproductive plant material. Wind, raindrops and forest insects associated with pines contribute to the local dispersion of the pathogen. Despite its importance, no effective measures are available to eradicate or control PPC disease either in nurseries or in the field. The main objective of this doctoral thesis was to shed light on effective regulatory mechanisms for the control of PPC disease. For this purpose, firstly, a review focused on collecting current information on pathways of pathogen spread and proposing preventive mechanisms to avoid its introduction into disease-free areas was elaborated. The multiple pathways of spread make *F. circinatum* challenging to prevent, exacerbated by the recent discovery of its endophytic colonization of non-reported host species that illustrates the importance of the biological and ecological knowledge for the design of effective intervention strategies. In addition, eradication of the disease may be feasible only if its entry is detected at a very early stage. In this regard, new methods for detection and diagnosis for the prompt detection of *F. circinatum* in seeds, plants, and vector insects are urgently needed. For that, it is essential the collaboration between phytosanitary authorities and researchers through interdisciplinary networks that allows increasing knowledge of the disease and raising awareness of the risks and mitigation measures among crucial target groups. The review also identified weak points in current regulations and provided suggestions for implementation.

Secondly, to understand the molecular mechanisms involving *F. circinatum* pathogenicity, its transcriptome was explored using next generation sequencing technologies. The following scientific research aimed to investigate the residual effect on the *F. circinatum* transcriptome of the loss of a putative mycovirus. Our results showed a slight acceleration of the host metabolism, possibly due to positive regulation of genes involved in functions essential for fungal development. For that, obtaining isogenic lines with and without mycovirus infections for an accurate study of virus-fungus interactions, avoiding altered isolates that have undergone mycovirus losses or have been subjected to invasive treatments to eliminate them is of particular importance. Transfection methods with *F. circinatum* mycoviruses should be tested in order to understand the feasibility of virocontrol of this forest pathogen. In the third scientific research, the pathogen transcriptome was analysed during the initial phase of infection to *Pinus* spp. species with different degrees of susceptibility. When infecting a relatively resistant species (*Pinus pinea*), *F. circinatum* genes related to cell wall and lignin degradation were predominantly induced. Conversely, the pathogen had an active uptake of nutrients (such as nitrogen) during its infection to the highly susceptible species (*P. radiata*). This could provide *F. circinatum* with a competitive advantage in the plant-pathogen interaction.

Finally, the regulatory molecular mechanisms involved in the tree defense were examined by the study of *Pinus* spp. transcriptome using the RNA-Seq technology. In the third

scientific research, transcriptome profiling of *P. pinea* four days after the infection by the pathogen revealed an early perception of the pathogen infection together with a strong and coordinated defense activation through the reinforcement and lignification of the cell wall, the antioxidant activity, the induction of PR genes, and the biosynthesis of defense hormones. On the contrary, *P. radiata* had a weaker response, possibly due to impaired perception of the fungal infection that led to a reduced downstream defense signaling. As a result, highly expressed disease resistance genes in *P. pinea* that may be candidates with applications in breeding programs were identified. Although physiological parameters behind PPC susceptible and resistant host phenotypes were also recorded in this work, no significant changes were found upon pathogen infection. In the fouth scientific research, an in-depth transcriptomic study of *P. radiata-F. circinatum* interaction was carried out by screening of long non-coding RNA (lncRNAs) molecules in the host and identifying those responsive to the pathogen infection. Functional analysis of genes nearby these pathogen-responsive lncRNAs suggested their involvement of important defense processes including signal transduction and cell wall reinforcement. These results present a comprehensive map of lncRNAs in *P. radiata* under *F. circinatum* infection and provide a starting point to understand their regulatory mechanisms and functions in conifer defense. Overall, a thorough understanding of the mechanism of gene regulation will contribute to the improvement of breeding programs for future resistant pine commercialization, one of the most promising approaches for PPC management.

Resumen

El chancro resinoso del pino (PPC, por sus siglas en inglés) es una grave enfermedad que afecta a *Pinus* spp. y *Pseudotsuga menziesii* en todo el mundo. La infección por su agente causal, *Fusarium circinatum*, provoca chancros de resina en los troncos y ramas laterales de los huéspedes maduros, que pueden acabar muriendo por anillamiento o rotura del tronco. En los viveros, los principales síntomas son el puntisecado, marchitamiento y muerte de las plántulas. Sin embargo, el patógeno, de naturaleza hemibiotrófica, puede permanecer endofítico en plántulas de pino sin mostrar síntomas de infección e incluso ser inadvertido en algunas especies herbáceas. Desde el primer registro en 1945 en Norteamérica, se ha notificado la presencia de *F. circinatum* en 14 países de América, Asia, África y Europa. Varios factores han contribuido a la propagación de la enfermedad a todos estos continentes, siendo el más importante la globalización en lo que respecta al comercio de material vegetal de reproducción. El viento, las gotas de lluvia y los insectos forestales asociados a los pinos contribuyen a la dispersión local del patógeno. A pesar de su importancia, no se dispone de medidas eficaces para erradicar o controlar la enfermedad del PPC ni en los viveros ni en el monte. El objetivo principal de esta tesis doctoral fue esclarecer los mecanismos de regulación eficaces para el control de la enfermedad PPC. Para ello, en primer lugar, se llevó a cabo una recopilación de información actual sobre las vías de propagación del patógeno, proponiendo mecanismos preventivos para evitar su introducción en zonas libres de la enfermedad. Las múltiples vías de propagación hacen de *F. circinatum* un reto a prevenir, agravado por el reciente descubrimiento de su colonización endofítica de especies no contampladas previamente como hospedantes que ilustra la importancia del conocimiento biológico y ecológico para el diseño de estrategias de intervención eficaces. Además, la erradicación de la enfermedad puede ser factible sólo si se detecta de forma inmediata a su introducción. En este sentido, se necesitan urgentemente nuevos métodos de detección y diagnóstico para la pronta detección de *F. circinatum* en semillas, plantas e insectos vectores. Para ello, es fundamental la colaboración entre las autoridades fitosanitarias y los investigadores a través de redes interdisciplinarias que permitan aumentar el conocimiento de la enfermedad y sensibilizar a los agentes implicados sobre los riesgos y las medidas de mitigación. En este trabajo también se identificaron los puntos débiles de la normativa actual y se aportaron sugerencias para su aplicación.

En segundo lugar, para entender los mecanismos moleculares implicados en la patogenicidad de *F. circinatum*, su transcriptoma fue explorado utilizando tecnologías de secuenciación masiva. Dicho estudio tenía como objetivo investigar el efecto residual en el transcriptoma de *F. circinatum* de la pérdida de un micovirus. Nuestros resultados mostraron una ligera aceleración del metabolismo del hospedador, posiblemente debido a la regulación positiva de genes implicados en funciones esenciales para el desarrollo del hongo. Por ello, cobra especial importancia la obtención de líneas isogénicas con y sin infecciones por micovirus para un estudio preciso de las interacciones virus-hongo, evitando aislados alterados que hayan sufrido pérdidas de micovirus o hayan sido sometidos a tratamientos invasivos para eliminarlos. Los métodos de transfección con micovirus de *F. circinatum* deben ser probados para comprobar la viabilidad del virocontrol de este patógeno forestal. En el tercero de los estudios, se analizó el transcriptoma del patógeno durante la fase inicial de la infección en especies de *Pinus* spp. con diferentes grados de susceptibilidad. En una especie relativamente resistente (*Pinus pinea*), los genes de *F. circinatum* relacionados con la degradación de la pared celular y la lignina fueron predominantemente inducidos. Por el contrario, el patógeno tuvo una

absorción activa de nutrientes (como el nitrógeno) durante su infección en la especie altamente susceptible (*P. radiata*). Esto podría proporcionar a *F. circinatum* una ventaja competitiva en la interacción planta-patógeno.

Por último, se examinaron los mecanismos moleculares de regulación implicados en la defensa del árbol mediante el estudio del transcriptoma de *Pinus* spp. utilizando la tecnología RNA-Seq. En el tercer estudio, el perfil del transcriptoma de *P. pinea* cuatro días después de la inoculación de *F. circinatum* reveló una percepción temprana de la infección del patógeno junto con una fuerte y coordinada activación de la defensa a través del refuerzo y lignificación de la pared celular, la actividad antioxidante, la inducción de genes PR y la biosíntesis de hormonas de defensa. Por el contrario, *P. radiata* tuvo una respuesta más débil, posiblemente debido a una percepción deficiente de la infección fúngica que condujo a una señalización de defensa menor. Como resultado, se identificaron genes de resistencia a la enfermedad altamente expresados en *P. pinea* que pueden ser utilizados como indicadores de resistencia en programas de mejora genética. Aunque en este trabajo también se evaluaron los parámetros fisiológicos que subyacen a los fenotipos de huésped susceptible y resistente a PPC, no se encontraron cambios significativos tras la infección del patógeno. En el cuarto estudio, se llevó a cabo un estudio transcriptómico en profundidad de la interacción *P. radiata-F. circinatum* mediante la identificación de moléculas de ARN no codificantes de cadena larga (ARNlnc) en el huésped y, especificamente, aquellos que responden a la infección del patógeno. El análisis funcional de los genes próximos a estos ARNlnc de respuesta al patógeno sugirió su participación en importantes procesos de defensa, incluyendo la transducción de señales y el refuerzo de la pared celular. Estos resultados presentan un mapa completo de los ARNlnc en *P. radiata* tras la infección de *F. circinatum* y proporcionan un punto de partida para entender sus mecanismos de regulación y funciones en la defensa de las coníferas. En general, una comprensión profunda del mecanismo de regulación de los genes contribuirá a la optimización de los programas de mejora genética para una futura comercialización de pinos resistentes, una de las herramientas más prometedoras para la gestión de PPC.

List of original articles

This thesis is based on the following scientific articles, which will be referred to by their Roman numerals (I-IV) in the text.

I. **Zamora-Ballesteros, C.; Diez, J.J.; Martín-García, J.; Witzell, J.; Solla, A.; Ahumada, R.; Capretti, P.; Cleary, M.; Drenkhan, R.; Dvorák, M.; Elvira-Recuenco, M.; Fernández-Fernández, M.M.; Ghelardini, L.; Gonthier, P.; Hernández-Escribano, L.; Ioos, R.; Markovskaja, S.; Martínez-Álvarez, P.; Muñoz-Adalia, E.J.; Nowakowska, J.A.; Oszako, T.; Raposo, R.; Santini, A.; Hantula, J.** 2019. Pine pitch canker (PPC): Pathways of pathogen spread and preventive measures. *Forests* **10**, 1158. https://doi.org/10.3390/f10121158

II. **Zamora-Ballesteros, C.; Wingfield, B.D.; Wingfield, M.J.; Martín-García, J.; Diez, J.J.** 2021. Residual effects caused by a past mycovirus infection in *Fusarium circinatum*. *Forests* **12**, 11. https://dx.doi.org/10.3390/f12010011

III. **Zamora-Ballesteros, C.; Pinto, G.; Amaral, J.; Valledor, L.; Alves, A.; Diez, J.J.; Martín-García, J.** 2021. Dual RNA-Sequencing analysis of resistant (*Pinus pinea*) and susceptible (*Pinus radiata*) hosts during *Fusarium circinatum* challenge. *International Journal of Molecular Sciences* **22**, 5231. https://doi.org/10.3390/ijms22105231

IV. **Zamora-Ballesteros, C.; Martín-García, J.; Suárez-Vega, A.; Diez, J.J**. 2022. Genome-wide identification and characterization of *Fusarium circinatum*-responsive lncRNAs in *Pinus radiata*. *BMC Genomics* **23**, 194. https://doi.org/10.1186/s12864-022-08408-9

Chapter 1: Introduction

1.1. Pine pitch canker (PPC) disease: a global concern

Despite their important contribution to the benefit of all living species on Earth, trees and forests are under great threat from a variety of sources, including commodity driven deforestation, climate change and alien invasive species (Anderson *et al.*, 2004; Curtis *et al.*, 2018). The incidence of forest diseases is increasing at an unprecedented rate through globalization (Santini *et al.*, 2013; Wingfield *et al.*, 2015), a scenario aggravated by climate change that often improves conditions for the establishment of a pathogen once introduced (Sturrock *et al.*, 2011). The introduction of a potential pathogen from its center of origin (where it generally causes little or no disease in its plant host due to long-term co-evolution) to a new geographical location, where potential hosts become highly susceptible as they have not previously been exposed to it, results in new tree disease outbreaks (Stenlid and Oliva, 2016). Examples of devastating emerging forest diseases include Dutch elm disease (*Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier), chestnut blight (*Cryphonectria parasitica* (Murrill) M.E. Barr), sudden oak death (*Phytophthora ramorum* Werres, De Cock & Man in 't Veld), and ash dieback (*Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya) (Brasier and Buck, 2001; Grünwald *et al.*, 2012; Gross *et al.*, 2014; Rigling and Prospero, 2018). Pine pitch canker (PPC) is another destructive disease affecting pines (*Pinus* spp.) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) (Wingfield *et al.*, 2008). The pathogen responsible for this disease, the ascomycetous *Fusarium circinatum* Nirenberg & O'Donnell (teleomorph = *Gibberella circinata*), has been recorded in countries of North and South Hemisphere, causing serious problems in nurseries, pine plantations and natural forests (Wingfield *et al.*, 2008). Nowadays, *F. circinatum* is a quarantine organism included in the European and Mediterranean Plant Protection Organization (EPPO) A2 list and regulated in the European Union (Commission Decision 2007/433/EC). A comprehensive overview of the PPC disease is provided in **Annex I**.

Since the first record of PPC in 1945 affecting *Pinus virginiana* Mill. of the South-Eastern United States (Hepting and Roth, 1946; Figure 1.1), *F. circinatum* has spread to 14 states in the United States (EPPO, 2021). The pathogen is also present in Haiti since 1953 (Hepting and Roth, 1953) and in Mexico since 1989 (Santos and Tovar, 1991), where it is widespread with records from at least nine states (Drenkhan *et al.*, 2020). In South America, *F. circinatum* was detected for the first time in Chile in 2001 infecting mother plants (hedges) of *Pinus radiata* D.Don in nurseries (Wingfield *et al.*, 2002). Since then, the pathogen has been found in Uruguay (Alonso and Bettucci, 2009), Colombia (Steenkamp *et al.*, 2012), and Brazil (Pfenning *et al.*, 2014). In Chile and Brazil, *F. circinatum* remains restricted to nurseries so far, while in Uruguay it has been declared eradicated (EPPO, 2021). Japan was the first country to report the presence of the pathogen in Asia in 1981 (Kobayashi and Muramoto, 1989) and later, in the mid-1990s, it was notified in South Korea (Lee *et al.*, 2000). In relation to Africa, *F. circinatum* has only been reported from South Africa, where it appeared in 1990 on *Pinus patula* Schiede ex Schltdl. & Cham. seedlings (McCain *et al.*, 1987; Viljoen *et al.*, 1994) and later notified in 2005 affecting *P. radiata* plantations (Coutinho *et al.*, 2007). *F. circinatum* is considered the most important pathogen in forest nurseries in this country (Coutinho *et al.*, 2007).

In Europe, PPC was detected for the first time in 1995 causing mortality in *Pinus halepensis* Mill. and *P. radiata* seedlings in a nursery located in Galicia (NW Spain; Collar Urquijo, 1995). However, the official confirmation of the presence of the pathogen in this country

occurred during the winter of 2003-2004 on *P. radiata* and *P. pinaster* Aiton in pine nurseries in Asturias (North Spain). Later in the year, PPC was detected in a 20-year-old *P. radiata* plantation in Cantabria (North Spain; Landeras *et al.*, 2005). Therefore, the disease has presumably been present for a long time in Spain. The pathogen has also been reported in Portugal (Braganca *et al.*, 2009), France (EPPO, 2006) and Italy (Carlucci *et al.*, 2007), but eradication has been confirmed in the latter two countries. In a current study carried out in 28 European countries, no evidence of *F. circinatum* was found in 24 of them (http://bit.do/phytoportal). However, although the highest incidence in Europe occurs in Mediterranean and sub-tropical climates and temperate regions due to the strong dependence of the pathogen on climatic conditions, under future climate change scenarios, cooler latitudes with the presence of susceptible hosts would become suitable for the disease establishment (Ganley *et al.*, 2009; Watt *et al.*, 2011; Möykkynen *et al.*, 2014).

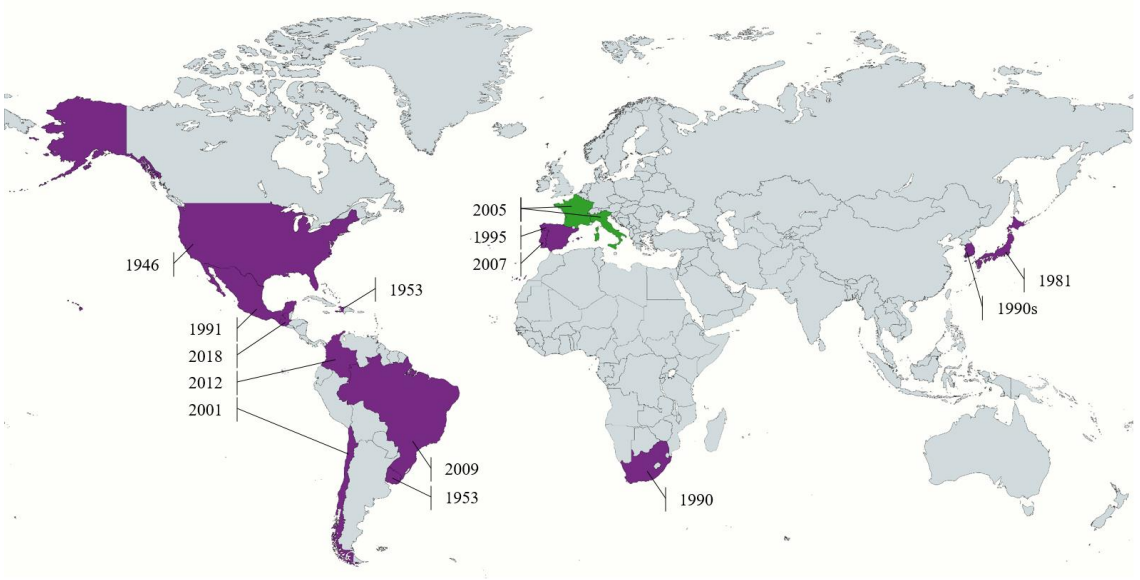

Figure 1.1. Current (2021) distribution range of *Fusarium circinatum* and dates of pathogen introduction (according to EPPO Global Database). Countries shaded in purple: Disease present, in green: Eradicated disease.

In mature trees, the PPC symptoms can be found in almost all parts of them and at any time of the year (EPPO, 2019; Kinkar and Vos, 2020). PPC is characterized by pitch resin-soaked cankers causing deformations in the trunk and large branches (Figure 1.2), sometimes affecting large surfaces of the cortical and subcortical tissue of the trunk (Storer *et al.*, 1994). These damages can girdle the trees leading to a uniform loss of color in the foliage, from dull green to yellow and finally brown, and dieback of the shoots (EPPO, 2005; COST, 2017). Deformations caused by the pathogen drastically reduce the value of timber since the logs are rendered unusable for the sawmill industry. When the trees are severely attacked, an extensive dieback in the canopy can be observed (Gordon *et al.*, 2001). At this point, the breakage of branches and even the stem can often occur due to loss of structural integrity or windstorms, and the tree eventually dies (Wingfield *et al.*, 2008; Martínez-Álvarez *et al.*, 2014). The fungus can also affect reproductive organs of the pine such as seeds, female strobili and mature cones that often abort before reaching maturity (Barrows-Broaddus, 1990). *F. circinatum* can cause root rot, visually recognized by brown discoloration and cortex disintegration in roots (EPPO, 2019). However, root infections are most often observed on seedlings in forest nurseries. Here, *F. circinatum* causes pre- and post-emergence damping-off of seedlings (Storer *et al.*, 1998), infected seeds being an important source of inoculum. Main symptoms in young plants and

seedlings are chlorosis, shoot and tip dieback, desiccation, and collar rot and wilting (Wingfield *et al.*, 2008; Figure 1.3).

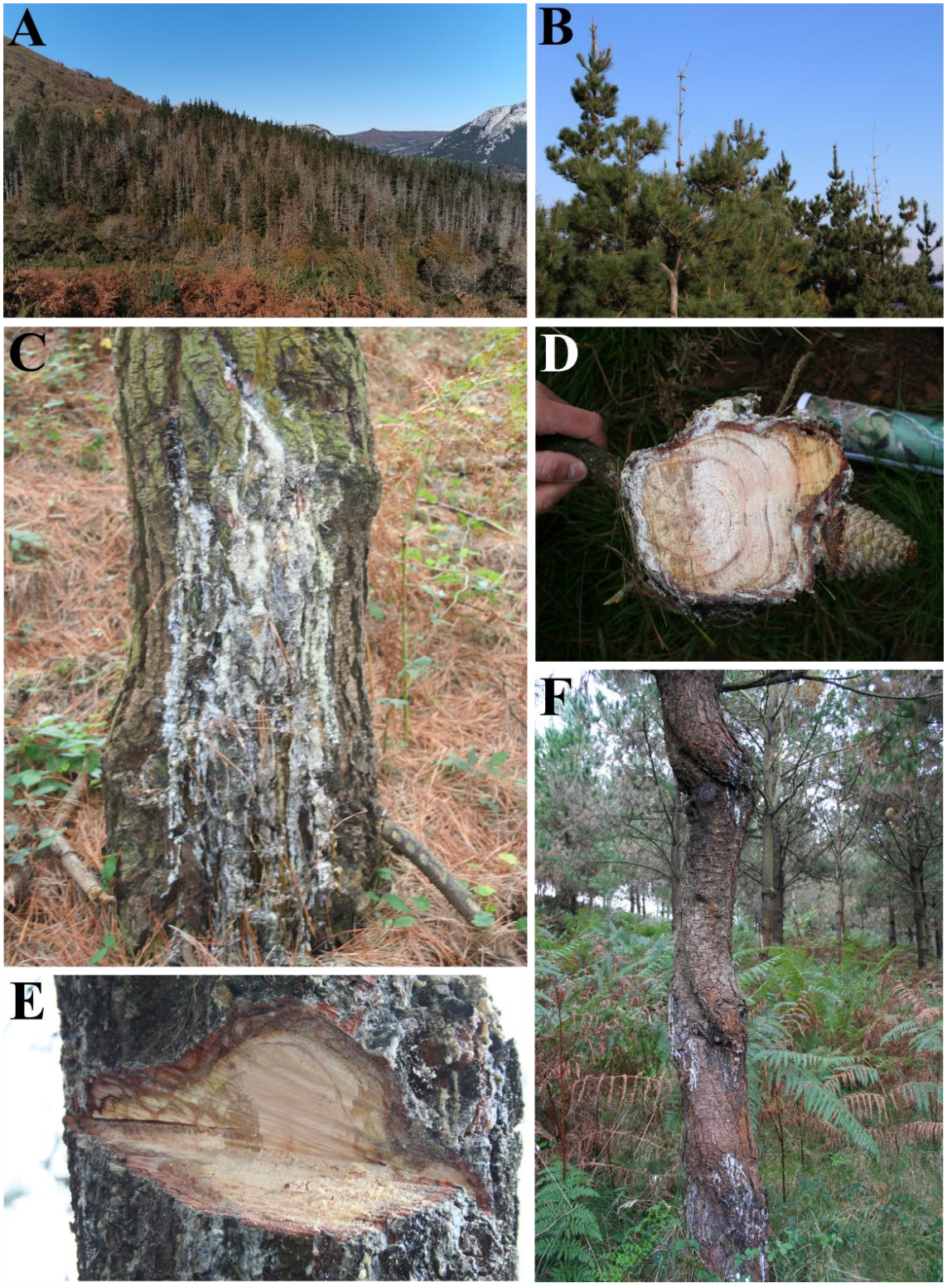

Figure 1.2. Symptoms of the PPC disease: (**A**) PPC damage in a *Pinus radiata* plantation; (**B**) crown dieback (*P. radiata*); (**C**) resin bleeding on the stem of *P. radiata*; (**D**) cross section of main stem showing deformation in the trunk due to the presence of a canker; (**E**) cut in main stem showing pathogen colonisation; (**F**) deformation in the trunk by *Fusarium circinatum* girdling.

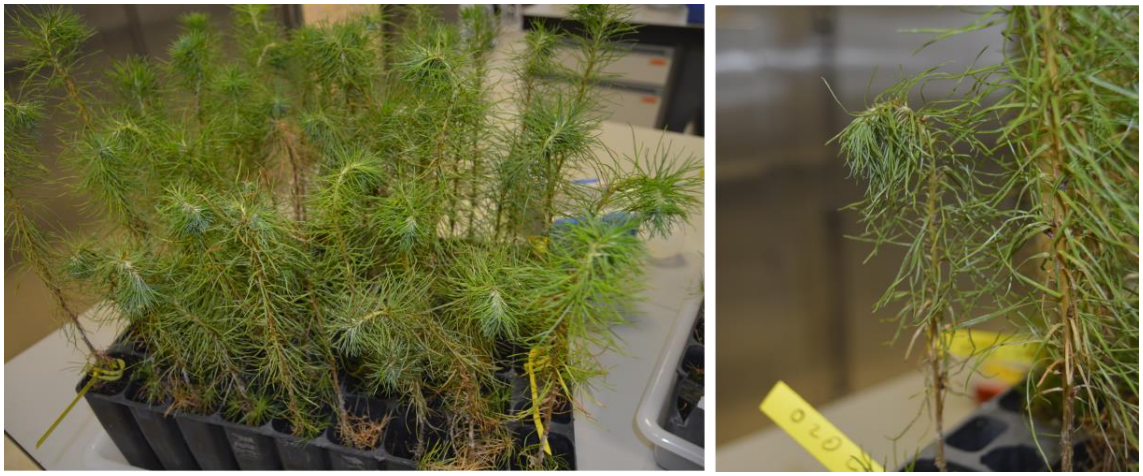

Figure 1.3. Pine pitch canker on *Pinus radiata* seedlings.

Fusarium circinatum is able to infect 91 different forest tree species, including 67 *Pinus* species, 18 *Pinus* hybrids, as well as 6 non-pine trees such as *P. menziesii* and species of *Picea*, *Larix*, *Abies* and *Libocedrus* (Martínez-Álvarez *et al.*, 2014; Martín-García *et al.*, 2017; Drenkhan *et al.*, 2020). Hence, the fungus poses a special threat to natural conifer forests, nurseries, and commercial plantations (Drenkhan *et al.*, 2020). Particularly, plantations of *P. radiata* have been extensively affected since it is not only the most susceptible species to the disease (Hodge and Dvorak, 2000; Gordon, 2006; Iturritxa *et al.*, 2012), but also one of the most widely planted pine species for commercial forestry in the world (Mead, 2013). Its high productivity and suitability for the construction timber, furniture, pulp and paper industries have made *P. radiata* the predominant species planted in Australia, Chile and Spain, and it accounts for 90% of the planted production forest area in New Zealand (MPI, 2019). Although *F. circinatum* is considered absent in Oceania, its introduction and establishment is of high concern (Ganley, 2007, 2011; Cook and Matheson, 2008). Strict border biosecurity regulations in New Zealand are preventing large plantations of *P. radiata* from being infected by the pathogen (Maxwell *et al.*, 2014). In countries where *P. radiata* is a well established species in the timber industry such as Chile and Spain, PPC has caused severe economic losses. The introduction of this invasive pathogen leads to direct damage to the forestry economy, as well as ecological impacts on the ecosystem services on which humans depend (Perrings *et al.*, 2002). However, the environmental and recreational impact of forest losses are difficult to quantify accurately. In Spain, the negative impact has been a consequence of reducing revenues due to the ban on planting susceptible species (*Pinus* spp. and *P. menziesii*) in infected areas (MAPAMA, 2006, 2010), the high costs invested in monitoring and control, and bans on the export of timber and other products. In addition, *Eucalyptus* spp. plantations that have a shorter rotation period, lower social acceptance, and a very narrow genetic pool, have become popular after PPC. This, together with the fact that these types of plantations have not coevolved with the native insects and fungi increase the probability of appearance of new pests and diseases (Branco *et al.*, 2015; Burgess and Wingfield, 2017).

1.2. Infection biology and dispersal pathways of the PPC pathogen

Fusarium circinatum can reproduce sexually and asexually. The sexual stage, involving perithecia, has never been observed in the field (Wingfield *et al.*, 2008), therefore, the pathogen spreads mainly asexually through the production of micro- and macro-conidia on infected host tissues (Gordon *et al.*, 1996; Britz *et al.*, 1998, 1999; Berbegal *et al.*, 2013). In fact, the local dispersion of the disease occurs by the airborne conidia of the pathogen,

driven by wind over midrange distances (Gordon, 2013; Dvorak *et al.*, 2017), raindrops (Deacon, 2006) and forest insects associated with pines (Fernández-Fernández *et al*., 2019). The airborne conidia can often naturally infect fresh wounds on trees, regardless of their natural or artificial origin (Barrows-Broaddus and Dwinell, 1983; Dwinell *et al.*, 1985), where, if the environmental conditions are suitable for the pathogen development, the inoculum germinates. The optimal temperature of *F. circinatum* germination and growth is around 25 ºC, dropping significantly at 10 ºC (Inman *et al.*, 2008). However, the viability of the inoculum is highly moisture-dependent (Sakamoto and Gordon, 2006; Garbelotto *et al.*, 2008; Gordon, 2013). For this reason, coastal areas represent the most suitable climate for disease development (Wikler *et al.*, 2003; Blank *et al.*, 2019; Drenkhan *et al.*, 2020). The role of forest insects associated with pines in the dissemination and the incidence of the disease is important not only for their capacity to carry and inoculate the pathogen to the hosts, but also for their behavior as wounding agents. Several bark beetle species damage the pine tissues when bore their breeding or feeding galleries, resulting in fresh wounds prone to infection by *F. circinatum* spores (Bezos *et al.*, 2017). The insect-PPC association depends on multiple factors such as the feeding habit of the insects, their mobility or their ability of transporting the spores on their exoskeletons (Fernández-Fernández *et al.*, 2019). In Europe, the pine shoot beetle species that has been proven to be able to transmit the disease and thus acting as a vector of PPC is *Tomicus piniperda* L. (Bezos *et al.*, 2015). In addition, some other insects considered as carriers (e.g. *Pityophthorus pubescens* Marsham) or wounding agents (e.g. *Pissodes validirostris* Sahlberg) have been associated with the incidence of the disease (Fernández-Fernández *et al.*, 2019).

The disease progress in *P. radiata* seedlings has been studied under laboratory conditions (Martín-Rodrigues *et al.*, 2013). *F. circinatum* colonizes the aerial tissues of the host, first radically towards the pitch of the stem, and then tangentially through the phloem and xylem. During its advance into host tissues, *F. circinatum* produces conidiophores in pith cavities that likely leads to the dispersal of conidia throughout different parts of the plant (Martín-Rodrigues *et al.*, 2013). The pathogen takes advantage of the formation of traumatic resin ducts, a conifer defense mechanism, for vertical colonization. The disruption of water flow due to collapse of the xylem by resin accumulation contributes to plant death (Gordon, 2011). With root infection, different types of hyphae (e.g., bulbous or narrow) are able to penetrate and colonize the root without causing apparent damage and switch to an active pathogenic phase when the pathogen colonizes the root collar (Martín-Rodrigues *et al.*, 2015; Swett *et al.*, 2016). Thus, *F. circinatum* displays hemibiotrophic behavior, since it first establishes a biotrophic interaction with the host plant followed by a necrotrophic lifestyle. Then, under certain conditions, infected seedlings can be asymptomatic (Swett and Gordon, 2012; Elvira-Recuenco *et al.*, 2015; Martín-García *et al.*, 2018), rendering visual identification useless. These infected but symptomless seedlings can be easily outplanted and, once in field, some of them will develop the disease after the stress produced during transplanting (Mitchell *et al.*, 2012; Jones *et al.*, 2014). Recent research has also revealed an endophytic behaviour of *F. circinatum* on alternative hosts making the disease more complex. Surveys in California (US), South Africa, and Spain have demonstrated natural colonization of 17 herbaceous species belonging to different families including Poaceae, Asteraceae, Lamiaceae or Rosaceae, by the PPC pathogen (Swett and Gordon, 2012, 2013, 2015; Hernández-Escribano *et al.*, 2018; Carter and Gordon, 2020; Herron *et al.*, 2020). The same genotype of the fungus able to colonize asymptomatically herbaceous plants was also capable of adopting a pathogenic lifestyle infecting *Pinus* species (Swett and Gordon, 2012; Hernández-Escribano *et al.*, 2018; Carter

and Gordon, 2020; Herron *et al.*, 2020). Most of these plants grew beneath the canopy of PPC-affected plantations, which represents a source reservoir of inoculum.

International trade is the major long-distance dispersal avenue for *F. circinatum*. In particular, the movement of infected seeds is one of the most important (Wingfield *et al.*, 2008). In fact, first PPC outbreaks in different countries such as South Africa, Chile or Spain have been associated with infected seeds (Britz *et al.*, 2001; Carey *et al.*, 2005; Coutinho *et al.*, 2007; Berbegal *et al.*, 2013). They also represent the major source of *F. circinatum* introduction into forest nurseries and can result in symptomatic but also asymptomatic seedlings. Therefore, the establishment of *F. circinatum* in nurseries may lead to an increasing proportion of asymptomatic seedlings transplanted in the field (Wingfield *et al.*, 2008; Elvira-Recuenco *et al.*, 2015).

1.3. Integrated approach towards PPC management

Problems arising from the indiscriminate use of pesticides, such as plant pest or pathogen resistance, harmful consequences to non-target organisms and adverse effects on humans and biodiversity, led scientists to shift the strategy for pest and disease control (Ehler, 2006). In the early 1970s, the concept 'Integrated Pest Management (IPM)' was coined to refer to a holistic approach to dealing with pests and pathogens that advocates reduced chemical control, increased economic benefits for growers and protection of both the environment and human health (Kogan, 1998). IPM uses available information on the lifestyle of pests and pathogens and their interaction with the environment to design a combination of common-sense control practices. These methods range from prevention of future problems to active suppression of current infestations. In addition, an adaptive forest management (AFM) strategy, a cyclic process that seeks continuous improvement and sustainability through planning, implementation, monitoring, evaluation outcomes and reviewing practices (Spittlehouse and Stewart, 2003), would be worthwhile to implement under the climate change.

Contemporary forest protection in the European Union is based on IPM strategy through the implementation of new and highly restrictive legislation known as Green Deal, which aims to reduce chemical pesticide inputs at least by 50% by 2030 (EU Commission, 2019). Although the efficacy of some fungicides to control *F. circinatum* has already been demonstrated (Allen *et al.*, 2004; Mitchell *et al.*, 2004; Carey *et al.*, 2005; Iturritxa *et al.*, 2011; Berbegal *et al.*, 2015; Serrano *et al.*, 2015), the current European legislative situation forces the search for environmentally friendly management methods. A number of challenges such as the limited availability of effective bio-based products, lack of knowledge of the forest managers and cost-effectiveness maintenance bring with this transition (Matyjaszczyk, 2019). Therefore, considerable research effort today is focused on seeking safe, eco-friendly, affordable and effective alternatives (Thambugala *et al.*, 2020). In this context, the European COST (European COoperation in Science and Technology) Action FP1406 "Pine pitch canker strategies for management of *Gibberella circinata* in green houses and forests" (PINESTRENGTH) has promoted co-operation and communication between research groups across the world in the fight against PPC disease. The main aim of this European project was to establish a European-focused network to increase knowledge of the biology, ecology and pathways of spread of *F. circinatum*, to examine the potential for the development of effective and environmentally-friendly

prevention and mitigation strategies and to transmit these outcomes to stakeholders and policy makers through science-based deliverables (COST 100/14, 2014).

Preventive measures are the key components for an IPM approach, followed by protection measures in which priority is given to eco-friendly tactics. Monitoring and correct pathogen identification is the first step for determining the best management strategy. In this regard, some studies have used spore traps for monitoring the presence of *F. circinatum* spores, as well as means of dispersal and temporal dynamics (Schweigkofler *et al.*, 2004; Garbelotto *et al.*, 2008; Fourie *et al.*, 2014; Dvorak *et al.*, 2017; Quesada *et al.*, 2018). On the other hand, the use of natural products for plant protection, including essential oils, propolis or monoterpenes, has been shown to inhibit mycelial growth of *F. circinatum* (Iturritxa *et al*., 2011, 2017; Slinski *et al*. 2015; López-López *et al*., 2016; Silva-Castro *et al*., 2018a,b). In addition, chitosan and phosphite have been also tested as resistance inducers against *F. circinatum*, demonstrating their capacity for *in vitro* inhibition of mycelial growth of the fungus and reduction of its pathogenicity in seedlings of different *Pinus* species (Reglinski *et al.*, 2004; Fitza *et al.*, 2013; Silva-Castro *et al.*, 2018a,b). Biological control, with a presumably higher level of safety and minimal negative environmental impacts, implies any reduction in the inoculum or pathogenic activity of the causal agent that is achieved through the use of natural enemies or compounds derived from its metabolism (Cook and Baker, 1983). The role of biocontrol approaches in combating *F. circinatum* is crucial within a framework of integrated management of PPC (Martín-García *et al.*, 2019). Several endophytic fungi and bacteria have demonstrated their antagonistic effect against *F. circinatum* (reviewed in Martín-García *et al.*, 2019). *Trichoderma* species are likely the most promising biological control agent against the PPC pathogen (Iturritxa *et al.*, 2011; Moraga-Suazo *et al.*, 2011; Martínez-Álvarez *et al.*, 2012, 2016; Morales-Rodríguez *et al.*, 2018). Nevertheless, whereas its implementation in nurseries could become a reality according to the greenhouse experiments (Lopez-Lopez *et al.*, 2016; Martín-García *et al.*, 2017), its application in the field needs further experimentation (Martín-García *et al.*, 2019).

The virocontrol has been recognized as a promising tool to control plant diseases (Nuss, 2005) and has been successfully implemented to control the chestnut blight fungus *C. parasitica* in the field (Rigling and Prospero, 2018). The infection of the mycovirus Cryphonectria hypovirus 1 (CHV1) induces hypovirulence in its hosts by reducing *in planta* virulence and disrupting colony growth and sporulation (Zamora *et al.*, 2017). Consequently, a growing body of literature has recently studied a large number of fungal viruses with the aim to achieve effective means of virocontrol of fungal diseases (Pearson *et al.*, 2009; Muñoz-Adalia *et al.*, 2016; García-Pedrajas *et al.*, 2019). However, besides inducing hypovirulence, the successful use of mycoviruses as effective biological control agents is dependent on multiple factors such as the ability to be transmitted horizontally among isolates in the natural fungal populations and vertically by sporogenesis (Son *et al.*, 2015). The horizontal transmission is strongly dependent on how efficiently mycoviruses are transmitted to uninfected isolates through hyphal anastomosis, converting them from virulent to hypovirulent (García-Pedrajas *et al.*, 2019). The anastomosis between fungi is determined by the vegetative incompatibility (vic), therefore a low proportion of vegetative compatibility groups (VCGs) is required for feasible biological control. Likewise, the vertical transmission rate must be high to ensure the viral dispersion to the progeny.

Three mycoviruses have been reported from *F. circinatum* (Martínez-Álvarez *et al.*, 2014; Vainio *et al.*, 2015). These mycoviruses belong to the genus *Mitovirus* in the family Narnaviridae and are predominantly found in the mitochondria of their hosts (Hillman

and Cai, 2013). Some species from this genus have been associated with hypovirulence in plant pathogenic fungi such as *O. novo-ulmi* (Hong *et al.*, 1999), *Sclerotinia sclerotiorum* (Lib.) de Bary (Xu *et al.*, 2015), *Fusarium graminearum* Schwabe (Darissa *et al.*, 2012) and *Botrytis cinerea* Pers. (Wu *et al.*, 2007, 2010). However, the *F. circinatum* mitoviruses have not showed a clear pattern of hypovirulence to date (Flores-Pacheco *et al.*, 2017; Muñoz-Adalia *et al.*, 2016).

1.4. Long-term solution to reduce the impact of PPC by genetic resistance

Development of resistant populations as a part of an IPM approach is essential to preserve the long-term value of healthy forests (Showalter *et al.*, 2018). Breeding programmes have produced successfully disease-resistant populations such as *Pinus monticola* Douglas ex D.Don. resistant to *Cronartium ribicola* J.C. Fisch, *Chamaecyparis lawsoniana* (A. Murray) Parl. resistant to *Phytophthora lateralis* Tucker & Milbrath, *Pinus taeda* L. resistant to fusiform rust, *Castanea dentata* (Marshall) Borkh. resistant to chestnut blight, and *Dothistroma pini* Hulbary resistance in *P. radiata* (Sniezko, 2006; Sniezko and Koch, 2017). Although genetic resistance may offer a more permanent solution once the pathogen is established, the implementation of a resistance program requires a long process, linked to long generation times of trees, of decision-making supported by a comprehensive knowledge of the full array of resistance mechanisms present in the tree (Woodcock *et al.*, 2018). Screening for natural resistance is one of the first steps to be taken in resistant tree programmes, the aim of which is to inform strategies for large-scale development of resistant plants. In this regard, different approaches such as surveying affected populations, inoculation trials or examining resistance in related species can be used for investigating resistant individuals (Woodcock *et al.*, 2018). In the case of PPC disease, the range of susceptibility to *F. circinatum* varies significantly between species (Gordon *et al.*, 1998; Iturritxa *et al.*, 2012; Iturritxa *et al.*, 2013; Martín-García *et al.*, 2017), offering an excellent opportunity for disease management. *Pinus radiata*, and to a lesser extent, *P. patula* and *P. elliottii* Engelm. are the most susceptible species to PPC (Viljoen *et al.*, 1995; Hodge and Dvorak, 2000) and, in turn, the most used at large-scale forestry in southern hemisphere countries (e.g. Chile, Colombia, South Africa, Uruguay). Their substitution for highly resistant *Pinus* species including *Pinus tecunumanii* Eguiluz & J.P.Perry, *P. densiflora* Siebold & Zucc., *P. oocarpa* Schiede ex Schltdl., *P. canariensis* C.Sm., *P. thunbergii* Parl. or *P. pinea* L. (Gordon *et al.*, 1998; Hodge and Dvorak, 2000; Kim *et al.*, 2008) could be a suitable alternative to manage PPC in these countries where no native pines occur.

The search for alternative species, however, should consider factors such as the environmental conditions and the requirements of the well-established forestry industry (Martín-García *et al.*, 2019). For this reason, selection of highly resistant populations within species or the use of tolerant hybrids is another promising strategy to reduce the impact of PPC. Artificial inoculations are invaluable in reducing the time and expense to seek and develop these resistant populations. Accordingly, the susceptibility level of provenances of several European conifers to *F. circinatum* have been recently evaluated through controlled inoculation studies (Martín-García *et al.*, 2017, 2018; Davydenko *et al.*, 2018), pointing out some interspecific genetic resistance. Other studies had also demonstrated this interspecific resistance in *P. patula*, *P. leiophylla* Schltdl. & Cham., *P.*

tecunumanii, *P. oocarpa* and *P. pinaster* to *F. circinatum* (Dvorak *et al.*, 2007, 2009; Hodge and Dvorak, 2007; Iturritxa *et al.*, 2012; Vivas *et al.*, 2012; Elvira-Recuenco *et al.*, 2014), being this variation sometimes related to geographical and environmental gradients (Hodge and Dvorak, 2007; Dvorak *et al.*, 2009; Steenkamp *et al.*, 2012). In addition, hybridizations between *P. patula* and any other tolerant species, especially low elevation *P. tecunumanii* populations, have shown improved resistance to *F. circinatum* and successfully implemented in South Africa (Roux *et al.*, 2007; Mitchell *et al.*, 2013; Kanzler *et al.*, 2014).

Variation in response to disease within a host population results from a combination of genetics, evolved immunity, plasticity and interaction with environmental conditions (Naidoo *et al.*, 2014; Telford *et al.*, 2015). However, the extent to which the variation in these resistance mechanisms is genetically encoded, and the degree of environmental influence are difficult to determine. Genetic variation in *F. circinatum* resistance appears to be quantitative since it is attributed to the integrated action of many genes (Kayihan *et al.*, 2005; Quesada *et al.*, 2010). This quantitative resistance is caused by relatively small contributions of several genes, or by one or two genes with large effects and several additional genes with small effects (Flint and Mackay, 2009). Although this complexity can pose a challenge for breeding programmes, the multi-gene basis and the weaker selection pressure imposed on the pathogen in turn increases the durability of the resistance traits (Telford *et al.*, 2015). Thus, the identification of these genes is essential for the process of PPC resistance development through selection and breeding.

Different defense mechanisms have emerged in conifer lineages, but the general strategy is the overlap of constitutive mechanical and chemical defenses together with the induction of additional defenses (Franceschi *et al.*, 2005). The complex plant immune response consists of a two-layered approach: a constitutive or non-specific response and an inducible defense mechanism (Jones and Dangl, 2006; Bent and Mackey, 2007). Proteins known as pattern recognition receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs) and their interaction is called PAMP-triggered immunity (PTI). Successful pathogens express a suite of effector proteins that are able to suppress PTI but, in turn, plants have evolved other proteins (R) that detect them and activate the inducible response called effector-triggered immunity (ETI; Jones and Dangl, 2006; Bent and Mackey, 2007). R protein-mediated recognition is faster and stronger defense response (Dodds and Rathjen, 2010). Disease resistance is observed if the product of any particular R gene has recognition specificity for a particular effector secreted by the pathogen (Bent and Mackey, 2007).

Both PTI and ETI lead to activation of various signaling transduction pathways that comprises Ca^{2+} ion flux across the membrane, ROS production and MAPK phosphorylation (Ng *et al.*, 2018). These signaling cascades target proteins involved in cellular protection or transcription factors controlling sets of defense-regulated genes in the nucleus (Dodds and Rathjen, 2010). The reception of the signal at the nucleus results in a severe transcriptional reprogramming that leads to diverse cellular responses, and, consequently, physiological alterations in the plant (Pedley and Martín, 2005). *F. circinatum* infection has been shown to induce the expression of genes involved in secondary metabolite synthesis and pathogenesis-related genes, as well as changes in antioxidant activity, needle gas

exchanges, water status and hormonal dynamics (Vivas *et al.*, 2014; Cerqueira *et al.*, 2017; Amaral *et al.*, 2019a,b). However, the response may be dependent on genetic or physiological differences among the different *Pinus* species. In fact, PPC-susceptible species such as *P. radiata* and *P. pinaster* experience a water obstruction that led to stomata closure and abscisic acid (ABA) accumulation, as well as photosynthesis impairment, induction of sink metabolism and activation of jasmonic acid-dependent signalling pathway (Amaral *et al.*, 2019a,b, 2020; Cerqueira *et al.*, 2017). In contrast, the resistant species *P. pinea* was able to maintain the stomatal opening, the ABA levels and the energy production through photosynthesis after inoculation with *F. circinatum* (Amaral *et al.*, 2019a, 2020). Additionally, the jasmonic acid levels decreased in the resistant species upon pathogen infection (Amaral *et al.*, 2019a). A recent proteomics study has also shown that membrane trafficking, ABA responses and maintenance of redox homeostasis seems to play a key role in *P. pinea* response against PPC (Amaral *et al.*, 2021). Therefore, studies on the plethora of genes and the physiological traits involved in the mechanisms underlying the plant-pathogen interaction in different hosts are crucial to understand the disease and to further select resistant genotypes. Furthermore, the knowledge gained by studying hormonal responses in the host plant could be applied to trigger the induced resistance through pre-treatment of plants with a suitable phytohormone prior to pathogen infection, activating their defense mechanisms (Eyles *et al.*, 2010).

1.5. Transcriptomics technologies and PPC management

Next-generation sequencing (NGS) and bioinformatics tools have revolutionised the biological sciences at an unprecedented level. NGS refers to non-Sanger-based high-throughput nucleic acid sequencing technologies that gives researchers the opportunity to address a large number of biological questions at a genome-wide scale (Schuster, 2007). Bioinformatics is essential for interpreting these biological queries using computer software, since the handling and analysis of the massive amount of data generated by this technology requires this interdisciplinary field (Yang *et al.*, 2021). Optimizations in protocols and sequencing platforms of NGS have allowed an exponential growth of applications reflected in the development of omics technologies such as genomics, metagenomics, transcriptomics and proteomics, among others. These omics-based approaches have contributed enormously to research in plant pathology disease management. The applications in this field are innumerable, including the discovery of new resistance and defense-related genes, sensitive and precise pathogen diagnostics, identification of pathogen effectors and quantitative trait loci (QTLs) for plant tolerance to pathogens, and the development of small molecules that target virulence genes (Klosterman *et al.*, 2016).

The omics approaches with high throughput techniques are playing an important role in expediting the understanding of interactions in biocontrol strategies. The interest in the microbiome associated with plants to predict the spread and impact of pathogens as a tool in biocontrol has increased during recent decades (Gopal *et al.*, 2013; Koskella *et al.*, 2017). In a recent study, although rhizobiomes of the *F. circinatum*-susceptible *P. radiata* and the resistant *P. pinea* species did not show significant changes after the stem inoculation of the pathogen, differences in the non-inoculated plants were found. In particular, the resistant species hosted higher abundance of bacterial taxa associated to disease protection (Leitão *et al.*, 2021). Thus, microbial detection powered by the

metagenomics technology could easily be employed to engineer microbiomes to reduce disease incidence or biopesticides to control specific pathogens (Klosterman *et al.*, 2016). On the other hand, many RNA mycoviruses have been identified by short-read NGS and using transcriptomics approaches, which concern the study and quantification of the complete set of transcripts (RNA) in a cell (Figure 1.4; Marzano *et al.*, 2016; Muñoz-Adalia *et al.*, 2018; Zhang *et al.*, 2018). Virus discovery using these technologies has the advantage of detecting them independently of the viral titre of the sample and without the need for prior knowledge of the genomic sequences of candidate viruses (Adams and Fox, 2016).

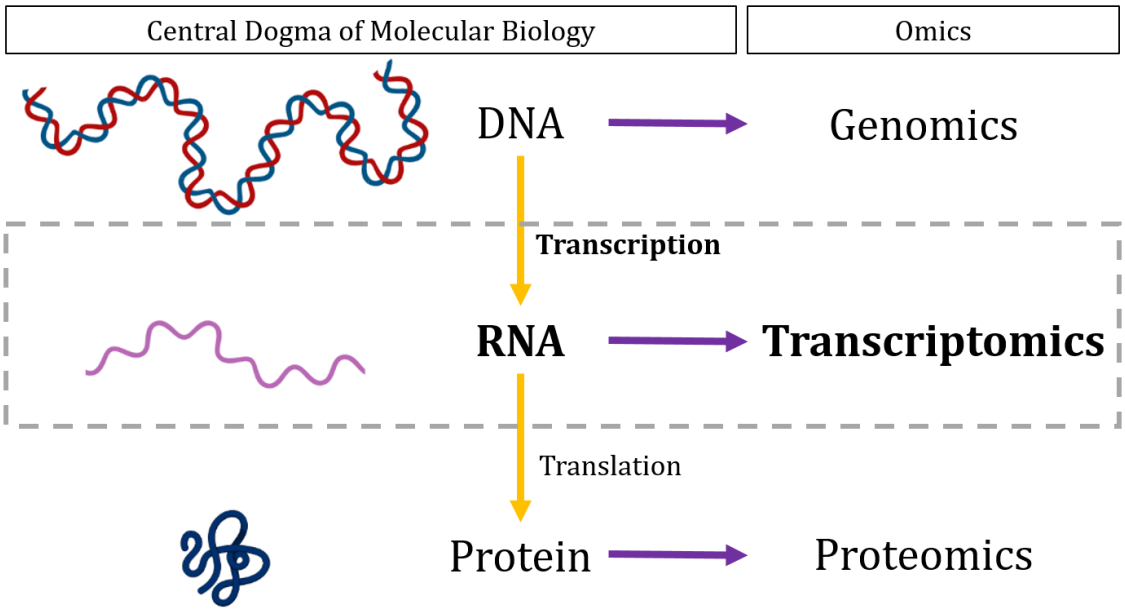

Figure 1.4. The central dogma of molecular biology and its corresponding omics disciplines.

The study of host-parasite interaction has also been greatly benefited from NGS and associated omics. Transcriptomics not only allows the detection of RNA virus sequences, but also the examination of the effect of these viruses on host gene expression by analysing their messenger RNA (mRNA). By exploiting the transcriptomics in the study of differential gene expression, the interaction of mycoviruses with several pathogenic fungi such as *F. graminearum* (Lee *et al.*, 2014; Bormann *et al.*, 2018), *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not. (Wang *et al.*, 2018), *Phytophthora infestans* (Mont.) de Bary (Cai *et al.*, 2019) and *Heterobasidion annosum* (Fr.) Bref. (Vainio *et al.*, 2018) have been elucidated using RNA sequencing (RNA-Seq) technology.

RNA-Seq is a recent and revolutionary technique that uses deep-sequencing technologies for sequencing of transcripts and subsequent mapping and quantifying transcriptomes (Wang *et al.*, 2009). This technology has clear advantages over previous approaches, such as serial analysis of gene expression (SAGE) or microarray methods, which require existing knowledge about genome sequence or are unable to analyse the entire set of transcripts or distinguish different isoforms (Wang *et al.*, 2009). This made it particularly challenging to study non-model organisms without their genome being sequenced, as is the case for many forest trees. The RNA-Seq technique overcomes these limitations with an improved sensitivity and a steadily decreasing cost, render previous technologies obsolete (Figure 1.5). The large amounts of RNA-Seq data generated per run, the protocols

for paired end reads and the low sequencing error rate in the reads obtained enable the transcriptome assembly for non-model plants. Likewise, if a genome sequence for the studied plant or a closely related species is available, it should be possible to identify transcripts by mapping RNA-Seq reads onto the genome (Conesa *et al.*, 2016). However, in contrast to the rapid progress in the availability of angiosperm genomes, the number of sequenced gymnosperm genomes is increasing slowly. This is mainly due to the fact that the most important and widely distributed group of gymnosperm plants, the conifers, has extremely large and complex genomes that hinder the sequencing and assembly of a reference genome (Zimin *et al.*, 2014). In fact, the genome of *Pinus lambertiana* Douglas is one of the largest ever sequenced at 31.6 Gb, more than ten times larger than the human genome (Stevens *et al.*, 2016). The complexity of the conifer genomes is reflected in the high representation of repetitive DNA (~75%), with transposable elements and large gene families generated by gene duplication (Ahuja and Neale, 2005; Nystedt *et al.*, 2013; Wegrzyn *et al.*, 2013, 2014). Other woody plant genomes, such as those of *P. taeda* (Neale *et al.*, 2014), *Picea abies* (L.) H.Karst. (Nystedt *et al.*, 2013), *Picea glauca* (Moench) Voss (Birol *et al.*, 2013) or *Sequoiadendron giganteum* (Lindl.) J.Buchholz (Scott *et al.*, 2020), are also publicly available to date.

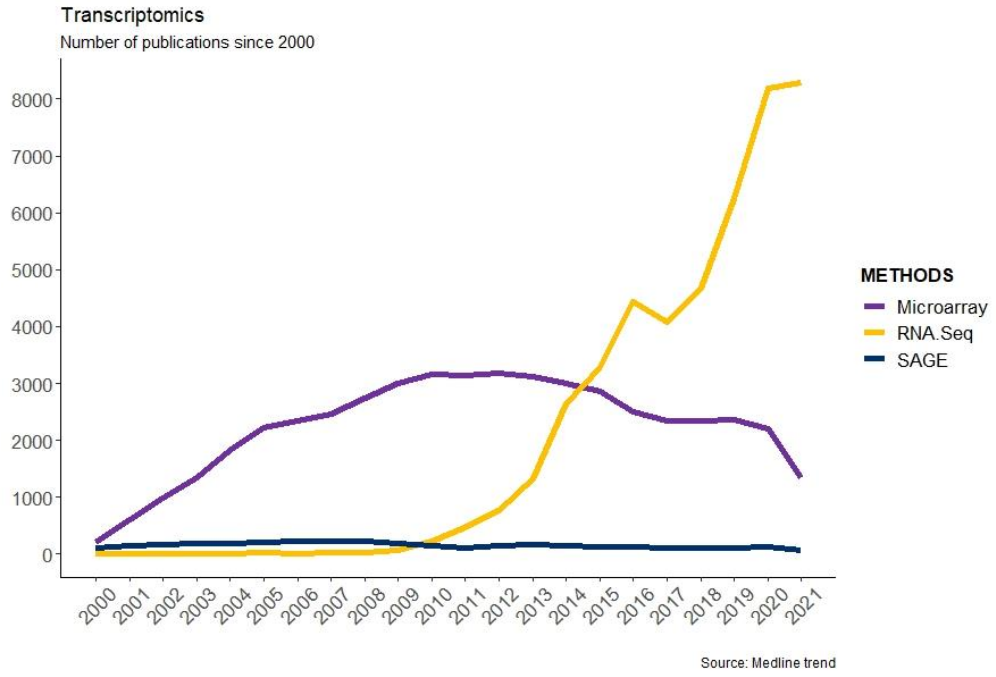

Figure 1.5. Number of articles published according to the transcriptomics method used over time (Dan Corlan, n.d.).

RNA-Seq technology has a wide variety of applications and, consequently, many variations in the analysis process. In addition to allowing the discovery of new genes, the detection of single nucleotide polymorphisms (SNPs) and the analysis of alternative splicing (synthesis of different isoforms), the main application of RNA-Seq is the genome-wide expression profile (coding and non-coding transcripts) and functional analysis in different tissues, conditions, or time points (Conesa *et al.*, 2016). In general, there are two different pipelines to perform a comparative analysis of gene expression data: aligning to a reference genome or transcriptome (if available) or *de novo* assembly without genomic sequence to produce a genome-scale transcription map (if the reference genome is unavailable or incomplete; Figure 1.6).

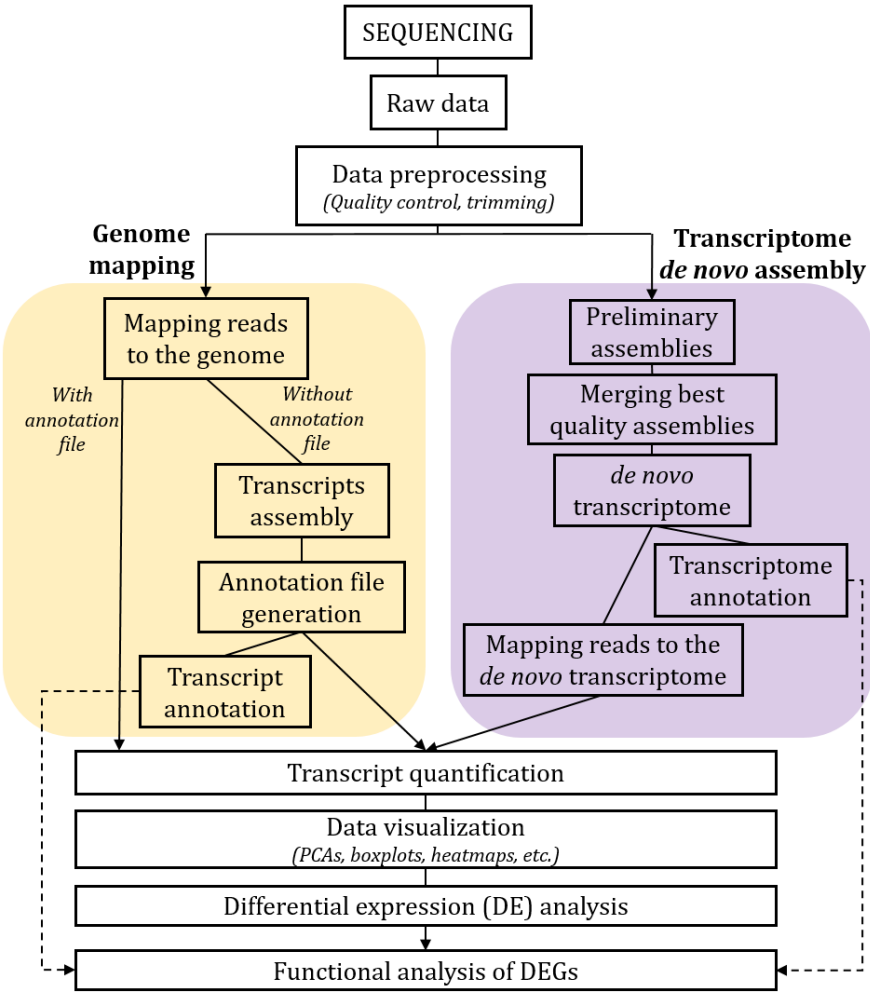

Figure 1.6. Different strategies of a comparative analysis of gene expression data.

In a context of science guiding forest pest management, the study of the plant-pathogen system helps to accelerate the discovery and understanding of the molecular mechanisms underlying disease resistance and offers the opportunity to improve the design of plant breeding programmes. The use of RNA-Seq technology together with the availability of tree and pathogen genomic resources is contributing to this purpose allowing the identification of plant key functional genes in susceptible and resistant responses, and the understanding of the molecular basis of compatible interactions during pathogen attack. In this regard, the dual-RNA sequencing approach during a pathogen infection provides a comprehensive picture of the interaction since it allows the simultaneous analysis of the differences in transcript expression of both plant and pathogen (Naidoo *et al.*, 2017; Westermann *et al.*, 2017). Therefore, the study of the transcriptional reprogramming for defense as response of the host tree and the identification of avirulence effector genes of the pathogen can be carried out in the same sample. This approach has been already employed in a comparative transcriptomic analysis between two contrasting genotypes of *Pinus pinaster* (Hernández-Escribano *et al.*, 2020) and between the susceptible species *P. patula* and the resistant *P. tecunumanii* after *F. circinatum* infection (Visser *et al.*, 2019). Moreover, unlike the genomes of conifers, the small and simple genomes (37-75 Mb) of fungi have allowed the straightforward sequencing of multiple forest pathogens, as in the case of *Hymenoscyphus* spp. (Stenlid *et al.*, 2017), *Phytophthora* spp. (Feau *et al.*, 2016), *Heterobasidion* spp. (Choi *et al.*, 2017), *C. parasitica* (Crouch *et al.*, 2020) or *F. circinatum*

(Wingfield *et al.*, 2012). All this information facilitates the selection of candidate genes for disease resistance and may inform strategies for resistance-breeding programmes.

According to the central dogma of molecular biology, the main role of the RNA is to passively convert information stored in DNA into proteins. However, the development of transcriptomics technologies has revealed that the vast majority of eukaryotic genomes is transcribed into non-coding RNAs (ncRNAs), which have minimal or no protein-coding capacity, but are functional (Kapranov *et al.*, 2007; Lander, 2011). Among the ncRNAs, the well-known housekeeping RNAs (transfer and ribosomal RNA) or small regulatory molecules including microRNAs (miRNAs), small nuclear RNAs (snRNAs) and small silencing RNAs (siRNAs) can be found (Bonnet *et al.*, 2006). An heterogeneous class of ncRNA, long non-coding RNA (lncRNA), has recently emerged as another eukaryotic non-coding transcript class that participate in many cellular processes by regulating gene expression at the transcriptional and post- transcriptional levels (Quan *et al.*, 2015). These transcripts of length above 200 nt have aroused intense interests due to their significant roles in many biological processes including the response to biotic stress. For example, in *Arabidopsis thaliana* (L.) Heynh., the lncRNA ELENA1 has been identified as a factor enhancing resistance against the pathogen *Pseudomonas syringae* van Hall by regulating positively the expression of the defense-related PR1 gene (Seo *et al.*, 2017). Likewise, 35 lncRNAs responsive to *Fusarium oxysporum* Schltdl. infection, some of them associated with genes that have a potential function in disease resistance (Zhu *et al.*, 2014). In cotton plants, the silencing of two lncRNAs (GhlncNAT- ANX2 and GhlncNAT-RLP7) led to increased resistance to *Verticillium dahliae* Klebahn and *B. cinerea*, possibly due to the transcriptional induction of two lipoxygenases involved in the jasmonic acid defense signaling pathway (Zhang *et al.*, 2018). In addition, overexpression of lncRNA ALEX1 in rice increased JA levels enhancing resistance to the bacteria *Xanthomonas oryzae* pv. *oryzae* (Yu *et al.*, 2020). These studies highlight the important role of lncRNAs in plant immunity, thus their screening can promote the development of better approaches for breeding disease-resistant trees.

References

Adams, I. and Fox, A. (2016) Diagnosis of Plant Viruses Using Next-Generation Sequencing and Metagenomic Analysis. In Current Research Topics in Plant Virology. (Wang, A. and Zhou, X., eds), pp. 323–335. Springer, Cham.

Ahuja, M.R. and Neale, D.B. (2005) Evolution of genome size in conifers. *Silvae Genet.* **54**, 126–137.

Allen, T.W., Enebak, S.A. and Carey, W.A. (2004) Evaluation of fungicides for control of species of *Fusarium* on longleaf pine seed. *Crop Prot.* **23**, 979–982.

Alonso, R. and Bettucci, L. (2009) First report of the pitch canker fungus *Fusarium circinatum* affecting *Pinus taeda* seedlings in Uruguay. *Australas. Plant Dis. Notes* **4**, 91–92.

Amaral, J., Correia, B., António, C., Rodrigues, A.M., Gómez-Cadenas, A., Valledor, L., Hancock, R.D., Alves, A. and Pinto, G. (2019a) Pinus Susceptibility to Pitch Canker Triggers Specific Physiological Responses in Symptomatic Plants: An Integrated Approach. *Front. Plant Sci.* **10**, 509.

Amaral, J., Pinto, G., Flores-Pacheco, J.A., Díez-Casero, J.J., Cerqueira, A., Monteiro, P., Gómez-Cadenas, A., Alves, A. and Martín-García, J. (2019b) Effect of *Trichoderma viride* pre-inoculation in pine species with different levels of susceptibility to *Fusarium circinatum*: physiological and hormonal responses. *Plant Pathol.* **68**, 1645–1653.

Amaral, J., Correia, B., Escandón, M., *et al*. (2020) Temporal physiological response of pine to *Fusarium circinatum* infection is dependent on host susceptibility level: the role of ABA catabolism. *Tree Physiol.* **tpaa143**.

Amaral, J., Lamelas, L., Valledor, L., Castillejo, M.Á., Alves, A. and Pinto, G. (2021) Comparative proteomics of *Pinus–Fusarium circinatum* interactions reveal metabolic clues to biotic stress resistance. *Physiol. Plant.* **173**, 1–13.

Anderson, P.K., Cunningham, A.A., Patel, N.G., Morales, F.J., Epstein, P.R. and Daszak, P. (2004) Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends Ecol. Evol.* **19**, 535–544.

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

Barrows-Broaddus, J.B. (1990) Colonization of Cones and Seed of Loblolly Pine Following Inoculation with *Fusarium subglutinans*. *Plant Dis.* **74**, 1002–1005.

Bent, A.F. and Mackey, D. (2007) Elicitors, Effectors, and R Genes: The New Paradigm and a Lifetime Supply of Questions. *Annu. Rev. Phytopathol.* **45**, 399–436.

Berbegal, M., Landeras, E., Sánchez, D., Abad-Campos, P., Pérez-Sierra, A. and Armengol, J. (2015) Evaluation of *Pinus radiata* seed treatments to control *Fusarium circinatum*: Effects on seed emergence and disease incidence. *For. Pathol.* **45**, 525–533.

Berbegal, M., Pérez-Sierra, A., Armengol, J. and Grünwald, N.J. (2013) Evidence for multiple introductions and clonality in Spanish populations of *Fusarium circinatum*. *Phytopathology* **103**, 851–861.

Bezos, D., Martínez-Alvarez, P., Diez, J.J. and Fernández, M.M. (2015) The pine shoot beetle *Tomicus piniperda* as a plausible vector of *Fusarium circinatum* in northern Spain. *Ann. For. Sci.* **72**, 1079–1088.

Bezos, D., Martínez-Alvarez, P., Fernández, M. and Diez, J.J. (2017) Epidemiology and management of pine pitch canker disease in Europe - A review. *Balt. For.* **23**, 279–293.

Birol, I., Raymond, A., Jackman, S.D., *et al*. (2013) Assembling the 20 Gb white spruce (*Picea glauca*) genome from whole-genome shotgun sequencing data. *Bioinformatics* **29**, 1492–1497.

Blank, L., Martín-García, J., Bezos, D., *et al*. (2019) Factors Affecting the Distribution of Pine Pitch

Canker in Northern Spain. *Forests* **10**, 305.

Bonnet, E., Peer, Y. Van de and Rouzé, P. (2006) The small RNA world of plants. *New Phytol.* **171**, 451–468.

Bormann, J., Heinze, C., Blum, C., *et al*. (2018) Expression of a structural protein of the mycovirus FgV-ch9 negatively affects the transcript level of a novel symptom alleviation factor and causes virus infection-like symptoms in *Fusarium graminearum*. *J. Virol.* **92**, 326–344.

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

Branco, M., Brockerhoff, E.G., Castagneyrol, B., Orazio, C. and Jactel, H. (2015) Host range expansion of native insects to exotic trees increases with area of introduction and the presence of congeneric native trees. *J. Appl. Ecol.* **52**, 69–77.

Brasier, C.M. and Buck, K.W. (2001) Rapid evolutionary changes in a globally invading fungal pathogen (Dutch elm disease). *Biol. Invasions* **3**, 223–233.

Britz, H., Couhnho, T.A., Gordon, T.R. and Wingfield, M.J. (2001) Characterisation of the pitch canker fungus, *Fusarium circinatum*, from Mexico. *South African J. Bot.* **67**, 609–614.

Britz, H., Coutinho, T.A., Wingfield, M.J., Marasas, W.F.O.O., Gordon, T.R. and Leslie, J.F. (1999) *Fusarium subglutinans* f. sp. *pini* represents a distinct mating population in the *Gibberella fujikuroi* species complex. *Appl. Environ. Microbiol.* **65**, 1198–1201.

Britz, H., Wingfield, M.J., Coutinho, T.A., Marasas, W.F.O. and Leslie, J.F. (1998) Female fertility and mating type distribution in a south african population of *Fusarium subglutinans* f. sp. *pini*. *Appl. Environ. Microbiol.* **64**, 2094–5.

Burgess, T.I. and Wingfield, M.J. (2017) Pathogens on the move: A 100-Year global experiment with planted eucalypts. *Bioscience* **67**, 14–25.

Cai, G., Fry, W.E. and Hillman, B.I. (2019) PiRV-2 stimulates sporulation in *Phytophthora infestans*. *Virus Res.* **271**.

Carey, W.A., Oak, S.W. and Enebak, S.A. (2005) Pitch canker ratings of longleaf pine clones correlate with *Fusarium circinatum* infestation of seeds and seedling mortality in containers. *For. Pathol.* **35**, 205–212.

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

Carter, J.W. and Gordon, T.R. (2020) Infection of the native California grass, bromus carinatus, by *Fusarium circinatum*, the cause of pitch canker in pines. *Plant Dis.* **104**, 194–197.

Cerqueira, A., Alves, A., Berenguer, H., Correia, B., Gómez-Cadenas, A., Diez, J.J., Monteiro, P. and Pinto, G. (2017) Phosphite shifts physiological and hormonal profile of Monterey pine and delays *Fusarium circinatum* progression. *Plant Physiol. Biochem.* **114**, 88–99.

Choi, J., Lee, G.W., Kim, K.T., Jeon, J., Détry, N., Kuo, H.C., Sun, H., Asiegbu, F.O. and Lee, Y.H. (2017) Comparative analysis of genome sequences of the conifer tree pathogen, *Heterobasidion annosum* s.s. *Genomics Data* **14**, 106.

Conesa, A., Madrigal, P., Tarazona, S., *et al*. (2016) A survey of best practices for RNA-seq data analysis. *Genome Biol.* **17**, 1–19.

Cook, D.C. and Matheson, A.C. (2008) An estimate of the potential economic impact of pine pitch canker in Australia. *Aust. For.* **71**, 107–112.

Cook, R.J. and Baker, K.F. (1983) The nature and practice of biological control of plant pathogens, St. Paul, Minnesota, USA: APS Press.

COST (2017) *Pine pitch canker Field Guide COST Action FP1406 PINESTRENGTH*. Available online: http://pinestrength.eu/2018/04/12/disseminationpitch-canker-field-guide-wg-1-pamphlet/

COST 100/14 (2014) *Memorandum of Understanding for the implementation of a European Concerted Research Action designated as COST Action FP1406: Pine pitch canker - strategies for management of Gibberella Circinata in greenhouses and forests (PINESTRENGTH)*, Brussels.

Coutinho, T.A., Steenkamp, E.T., Mongwaketsi, K., Wilmot, M. and Wingfield, M.J. (2007) First outbreak of pitch canker in a South African pine plantation. *Australas. Plant Pathol.* **36**, 256–261.

Crouch, J.A., Dawe, A., Aerts, A., *et al*. (2020) Genome sequence of the chestnut blight fungus *Cryphonectria parasitica* EP155: A fundamental resource for an archetypical invasive plant pathogen. *Phytopathology* **110**, 1180–1188.

Curtis, P.G., Slay, C.M., Harris, N.L., Tyukavina, A. and Hansen, M.C. (2018) Classifying drivers of global forest loss. *Science* **361**, 1108–1111.

Dan Corlan, A. Medline trend: automated yearly statistics of PubMed results for any query.

Darissa, O., Adam, G. and Schäfer, W. (2012) A dsRNA mycovirus causes hypovirulence of *Fusarium graminearum* to wheat and maize. *Eur. J. Plant Pathol.* **134**, 181–189.

Davydenko, K., Nowakowska, J.A., Kaluski, T., Gawlak, M., Sadowska, K., Martín-García, J., Diez, J.J., Okorski, A. and Oszako, T. (2018) A comparative study of the pathogenicity of *Fusarium circinatum* and other *Fusarium* species in Polish provenances of *Pinus sylvestris* L. *Forests* **9**, 560.

Deacon, J.W. (2006) Fungal Biology, Malden, MA: Blackwell Pub.

Dodds, P.N. and Rathjen, J.P. (2010) Plant immunity: towards an integrated view of plant–pathogen interactions. *Nature* **11**, 539–548.

Drenkhan, R., Ganley, B., Martín-García, J., *et al*. (2020) Global geographic distribution and host range of *Fusarium circinatum*, the causal agent of pine pitch canker. *Forests* **11**, 724.

Dvorak, M., Janos, P., Botella, L., Rotkova, G. and Zas, R. (2017) Spore dispersal patterns of *Fusarium circinatum* on an infested Monterey pine forest in North-Western Spain. *Forests* **8**, 432.

Dvorak, W.S., Hodge, G.R. and Kietzka, J.E. (2007) Genetic variation in survival, growth, and stem form of *Pinus leiophylla* in Brazil and South Africa and provenance resistance to pitch canker. *South. Hemisph. For. J.* **69**, 125–135.

Dvorak, W.S., Potter, K.M., Hipkins, V.D. and Hodge, G.R. (2009) Genetic Diversity and Gene Exchange in *Pinus oocarpa* , a Mesoamerican Pine with Resistance to the Pitch Canker Fungus (*Fusarium circinatum*). *Int. J. Plant Sci.* **170**, 609–626.

Dwinell, L.D., Barrows-Broaddus, J.B. and Kuhlman, G. (1985) Pitch canker: a disease complex of southern pines. *Plant Dis.* **69**, 270–276.

Ehler, L.E. (2006) Perspective Integrated pest management (IPM): definition, historical development and implementation, and the other IPM. *Pest Manag. Sci.* **62**, 787–789.

Elvira-Recuenco, M., Iturritxa, E., Majada, J., Alia, R. and Raposo, R. (2014) Adaptive Potential of Maritime Pine (*Pinus pinaster*) Populations to the Emerging Pitch Canker Pathogen, *Fusarium circinatum*. *PLoS One* **9**, e114971.

Elvira-Recuenco, M., Iturritxa, E. and Raposo, R. (2015) Impact of seed transmission on the infection and development of pitch canker disease in *Pinus radiata*. *Forests* **6**, 3353–3368.

EPPO (2005) Data sheets on quarantine pests: *Gibberella circinata*. *EPPO Bull.* **35**, 383–386.

EPPO (2006) First report of *Gibberella circinata* in France. *EPPO* **5**.

EPPO (2021) *Fusarium circinatum* (GIBBCI) [World distribution]| EPPO Global Database.

EPPO (2019) PM 7/91 (2) *Fusarium circinatum* (formerly Gibberella circinata). *EPPO Bull.* **49**, 228–247.

EU Commission (2019) *The European Green Deal*, Brussels.

Eyles, A., Bonello, P., Ganley, R. and Mohammed, C. (2010) Induced resistance to pests and pathogens in trees. *New Phytol.* **185**, 893–908.

Feau, N., Taylor, G., Dale, A.L., Dhillon, B., Bilodeau, G.J., Birol, I., Jones, S.J.M. and Hamelin, R.C. (2016) Genome sequences of six *Phytophthora* species threatening forest ecosystems. *Genomics Data* **10**, 85.

Fernández-Fernández, M., Naves, P., Witzell, J. *et al*. (2019) Pine Pitch Canker and Insects: Relationships and Implications for Disease Spread in Europe. *Forests* **10**, 627.

Fitza, K.N.E., Payn, K.G., Steenkamp, E.T., Myburg, A.A. and Naidoo, S. (2013) Chitosan application improves resistance to *Fusarium circinatum* in *Pinus patula*. *South African J. Bot.* **85**, 70–78.

Flint, J. and Mackay, T.F.C. (2009) Genetic architecture of quantitative traits in mice, flies, and humans. *Genome Res.* **19**, 723–733.

Flores-Pacheco, J.A., Muñoz-Adalia, E.J., Martinez-Alvarez, P., Pando, V., Diez, J.J. and Martin-Garcia, J. (2017) Effect of mycoviruses on growth, spore germination and pathogenicity of the fungus *Fusarium circinatum*. *For. Syst.* **26**.

Fourie, G., Wingfield, M.J., Wingfield, B.D., Jones, N.B., Morris, A.R. and Steenkamp, E.T. (2014) Culture-independent detection and quantification of *Fusarium circinatum* in a pine-producing seedling nursery. *South. For. a J. For. Sci.* **76**, 137–143.

Franceschi, V.R., Krokene, P., Christiansen, E. and Krekling, T. (2005) Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytol.* **167**, 353–376.

Ganley, R. (2007) Pitch canker: Risk of establishment in New Zealand based on a global perspective. *NZ J. For.* **52**, 26–30.

Ganley, R.J., Watt, M.S., Kriticos, D.J., Hopkins, A.J.M. and Manning, L.K. (2011) Increased risk of pitch canker to Australasia under climate change. *Australas. Plant Pathol.* **40**, 228–237.

Ganley, R.J., Watt, M.S., Manning, L. and Iturritxa, E. (2009) A global climatic risk assessment of pitch canker disease. *Can. J. For. Res.* **39**, 2246–2256.

Garbelotto, M., Smith, T. and Schweigkofler, W. (2008) Variation in rates of spore deposition of *Fusarium circinatum*, the causal agent of pine pitch canker, over a 12-month-period at two locations in Northern California. *Phytopathology* **98**, 137–143.

García-Pedrajas, M.D., Cañizares, M.C., Sarmiento-Villamil, J.L., Jacquat, A.G. and Dambolena, J.S. (2019) Mycoviruses in biological control: From basic research to field implementation. *Phytopathology* **109**, 1828–1839.

Gopal, M., Gupta, A. and Thomas, G. V. (2013) Bespoke microbiome therapy to manage plant diseases. *Front. Microbiol.* **4**, 355.

Gordon, T.R. (2011) Biology and management of *Gibberella circinata*, the cause of pitch canker in pines. In Control of *Fusarium* diseases. (Álves- Santos, F.M. and Diez, J.J., eds), pp. 217–232. Kerala, India: Research Sign Post.

Gordon, T.R. (2013) Pitch Canker. In Infectious Forest Diseases. (Gonthier, P and Nicolotti, G., ed), pp. 376–391.

Gordon, T.R. (2006) Pitch canker disease of pines. *Phytopathology* **96**, 657–659.

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

Gordon, T.R., Storer, A.J. and Wood, D.L. (2001) The Pitch Canker Epidemic in California. *Plant Dis.* **85**, 1128–1139.

Gordon, T.R., Wikler, K.R., Clark, S.L., Okamoto, D., Storer, A.J. and Bonello, P. (1998)

Resistance to pitch canker disease, caused by *Fusarium subglutinans* f.sp. *pini*, in Monterey pine (*Pinus radiata*). *Plant Pathol.*

Gross, A., Holdenrieder, O., Pautasso, M., Queloz, V. and Sieber, T.N. (2014) *Hymenoscyphus pseudoalbidus*, the causal agent of European ash dieback. *Mol. Plant Pathol.* **15**, 5–21.

Grünwald, N.J., Garbelotto, M., Goss, E.M., Heungens, K. and Prospero, S. (2012) Emergence of the sudden oak death pathogen *Phytophthora ramorum*. *Trends Microbiol.* **20**, 131–138.

Hepting, G.H. and Roth, E.R. (1953) Host relations and spread of the pine pitch canker disease. *Phytopathology* **43**, 475.

Hepting, G.H. and Roth, E.R. (1946) Pitch canker, a new disease of some southern pines. *J. For.* **44**, 742–744.

Hernández-Escribano, L., Iturritxa, E., Elvira-Recuenco, M., Berbegal, M., Campos, J.A., Renobales, G., Garcia, I. and Raposo, R. (2018) Herbaceous plants in the understory of a pitch canker-affected *Pinus radiata* plantation are endophytically infected with *Fusarium circinatum*. *Fungal Ecol.* **32**, 65–71.

Hernández-Escribano, L., Visser, E.A., Iturritxa, E., Raposo, R. and Naidoo, S. (2020) The transcriptome of *Pinus pinaster* under *Fusarium circinatum* challenge. *BMC Genomics* **21**, 1–18.

Herron, D.A., Wingfield, M.J., Fru, F., Wingfield, B.D. and Steenkamp, E.T. (2020) Grasses as a refuge for *Fusarium circinatum* L. - evidence from South Africa. *J. For. Sci.* **82**, 253–262.

Hillman, B.I. and Cai, G. (2013) The Family Narnaviridae. Simplest of RNA Viruses. In Advances in Virus Research. (Said, A.G., ed), pp. 149–176. Academic Press Inc.

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

Hodge, G.R. and Dvorak, W.S. (2007) Variation in pitch canker resistance among provenances of *Pinus patula* and *Pinus tecunumanii* from Mexico and Central America. *New For.* **33**, 193–206.

Hong, Y., Dover, S.L., Cole, T.E., Brasier, C.M. and Buck, K.W. (1999) Multiple mitochondrial viruses in an isolate of the Dutch elm disease fungus *Ophiostoma novo-ulmi*. *Virology* **258**, 118–127.

Inman, A.R., Kirkpatrick, S.C., Gordon, T.R. and Shaw, A.V. (2008) Limiting effects of low temperature on growth and spore germination in *Gibberella circinata*, the cause of pitch canker in pine species. *Plant Dis.* **92**, 542–545.

Iturritxa, E., Desprez-Loustau, M.L., García-Serna, I., Quintana, E., Mesanza, N. and Aitken, J. (2011) Effect of alternative disinfection treatments against fungal canker in seeds of *Pinus radiata*. *Seed Technol.* **33**, 88–110.

Iturritxa, E., Ganley, R.J., Raposo, R., García-Serna, I., Mesanza, N., Kirkpatrick, S.C. and Gordon, T.R. (2013) Resistance levels of Spanish conifers against *Fusarium circinatum* and *Diplodia pinea*. *For. Pathol.* **43**, 488–495.

Iturritxa, E., Mesanza, N., Elvira-Recuenco, M., Serrano, Y., Quintana, E. and Raposo, R. (2012) Evaluation of genetic resistance in *Pinus* to pitch canker in Spain. *Australas. Plant Pathol.* **41**, 601–607.

Iturritxa, E., Trask, T., Mesanza, N., Raposo, R., Elvira-Recuenco, M., Patten, C. (2017). Biocontrol of *Fusarium circinatum* infection of young *Pinus radiata* trees. *Forests* **8**, 32.

Jones, J.D.G. and Dangl, J.L. (2006) The plant immune system. *Nature* **444**, 323–329.

Jones, N.B., Ford, C.M., Light, M.E., Nadel, R.L., Greyling, I., Fourie, G., Wingfield, M.J. and Morris, A.R. (2014) Effect on nursery and field performance of *Pinus patula* seedlings after inoculation with *Fusarium circinatum*. *South. For.* **76**, 125–136.

Kanzler, A., Nel, A. and Ford, C. (2014) Development and commercialisation of the *Pinus patula* x

P. tecunumanii hybrid in response to the threat of *Fusarium circinatum*. *New For.* **45**, 417–437.

Kapranov, P., Cheng, J., Dike, S., *et al*. (2007) RNA maps reveal new RNA classes and a possible function for pervasive transcription. *Science* **316**, 1484–1488.

Kayihan, G.C., Huber, D.A., Morse, A.M., White, T.L. and Davis, J.M. (2005) Genetic dissection of fusiform rust and pitch canker disease traits in loblolly pine. *Theor. Appl. Genet.* **110**, 948–958.

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

Kinkar, M. and Vos, S. (2020) Pest survey card on *Fusarium circinatum*. *EFSA Support. Publ.* **17**.

Klosterman, S.J., Rollins, J.R., Sudarshana, M.R. and Vinatzer, B.A. (2016) Disease Management in the Genomics Era - Summaries of Focus Issue Papers. *Phytopathology* **106**, 1068–1070.

Kobayashi, T. and Muramoto, M. (1989) Pitch canker of *Pinus luchuensis*, a new disease of Japanese forests. *For. Pests* **40**, 169–173.

Kogan, M. (1998) Integrated Pest Management: Historical Perspectives and Contemporary Developments. *Annu. Rev. Entomol.* **43**, 243–270.

Koskella, B., Meaden, S., Crowther, W.J., Leimu, R. and Metcalf, C.J.E. (2017) A signature of tree health? Shifts in the microbiome and the ecological drivers of horse chestnut bleeding canker disease. *New Phytol.* **215**, 737–746.

Lander, E.S. (2011) Initial impact of the sequencing of the human genome. *Nature* **470**, 187–197.

Landeras, E., García, P., Fernández, M., Braña, M., Pérez-Sierra, A., León, M., Abad-Campos, P. and Armengol, J. (2005) Outbreak of Pitch Canker Caused by *Fusarium circinatum* on *Pinus* spp. in Northern Spain. *Plant Dis.* **89**, 1015.

Lee, J.K., Lee, S.-H., Yang, S.-I. and Lee, Y.-W. (2000) First Report of Pitch Canker Disease on *Pinus rigida* in Korea. *Plant Pathol. J.* **16**.

Lee, Y.-S., Kim, J., Shin, S.-C., Lee, S.-G., Park, I.-K. (2008). Antifungal activity of Myrtaceae essential oils and their components against three phytopathogenic fungi. *Flavour Fragr. J.* **23**, 23–28.

Lee, Y.-S., Kim, J., Lee, S.-G., Oh, E., Shin, S.-C., Park, I.-K. (2009). Effects of plant essential oils and components from Oriental sweetgum (*Liquidambar orientalis*) on growth and morphogenesis of three phytopathogenic fungi. *Pestic. Biochem. Physiol*. **93**, 138–143.

Lee, K.M., Cho, W.K., Yu, J., Son, M., Choi, H., Min, K., Lee, Y.W. and Kim, K.H. (2014) A comparison of transcriptional patterns and mycological phenotypes following infection of *Fusarium graminearum* by four mycoviruses. *PLoS One* **9**.

Leitão, F., Pinto, G., Amaral, J., Monteiro, P. and Henriques, I. (2021) New insights into the role of constitutive bacterial rhizobiome and phenolic compounds in two *Pinus* spp. with contrasting susceptibility to pine pitch canker. *Tree Physiol.* **tpab119***.*

López-López, N., Segarra, G., Vergara, O., López-Fabal, A. and Trillas, M.I. (2016) Compost from forest cleaning green waste and *Trichoderma asperellum* strain T34 reduced incidence of *Fusarium circinatum* in *Pinus radiata* seedlings. *Biol. Control* **95**, 31–39.

MAPAMA (2006) REAL DECRETO 637/2006, de 26 de mayo, por el que se establece el programa nacional de erradicación y control del hongo *Fusarium Circinatum* Niremberg et O'donnell. *BOE* **137**, 22069–22073.

MAPAMA (2010) Real Decreto 65/2010, de 29 de enero, por el que se modifica el Real Decreto 637/2006, de 26 de mayo, por el que se establece el programa nacional de erradicación y control del hongo de las coníferas "*Fusarium circinatum*" Niremberg et O´Donnell.

Martín-García, J., Lukacevicova, A., Asdrubal Flores-Pacheco, J., Diez, J.J. and Dvorak, M. (2018) Evaluation of the susceptibility of several Czech conifer provenances to *Fusarium*

circinatum. *Forests* **9**, 72.

Martín-García, J., Paraschiv, M., Flores-Pacheco, J.A., Chira, D., Diez, J.J. and Fernández, M. (2017) Susceptibility of Several Northeastern Conifers to *Fusarium circinatum* and Strategies for Biocontrol. *Forests* **8**, 318.

Martín-García, J., Zas, R., Solla, A., et al. (2019) Environmentally-friendly methods for controlling pine pitch canker. *Plant Pathol.* **68**, 843–860.

Martín-Rodrigues, N., Espinel, S., Sanchez-Zabala, J., Ortíz, A., González-Murua, C. and Duñabeitia, M.K. (2013) Spatial and temporal dynamics of the colonization of *Pinus radiata* by *Fusarium circinatum*, of conidiophora development in the pith and of traumatic resin duct formation. *New Phytol.* **198**, 1215–1227.

Martín-Rodrigues, N., Sánchez-Zabala, J., Salcedo, I., Majada, J., González-Murua, C. and Duñabeitia, M.K. (2015) New insights into radiata pine seedling root infection by *Fusarium circinatum*. *Plant Pathol.* **64**, 1336–1348.

Martínez-Álvarez, P., Fernandez-Gonzalez, R., Vicente Sanz-Ros, A., Pando, V. and Diez, J.J. (2016) Two fungal endophytes reduce the severity of pitch canker disease in *Pinus radiata* seedlings. *Biol. Control* **94**, 1–10.

Martínez-Álvarez, P., Alves-Santos, F. and Diez, J.J. (2012) *In vitro* and *in vivo* interactions between *Trichoderma viride* and *Fusarium circinatum*. *Silva Fenn.* **46**, 303–316.

Martínez-Álvarez, P., Pando, V. and Diez, J.J. (2014) Alternative species to replace Monterey pine plantations affected by pitch canker caused by *Fusarium circinatum* in northern Spain. *Plant Pathol.* **63**, 1086–1094.

Martínez-Álvarez, P., Vainio, E.J., Botella, L., Hantula, J. and Diez, J.J. (2014) Three mitovirus strains infecting a single isolate of *Fusarium circinatum* are the first putative members of the family Narnaviridae detected in a fungus of the genus *Fusarium*. *Arch. Virol.* **159**, 2153–2155.

Marzano, S.-Y.L., Nelson, B.D., Ajayi-Oyetunde, O., Bradley, C.A., Hughes, T.J., Hartman, G.L., Eastburn, D.M. and Domier, L.L. (2016) Identification of diverse mycoviruses through metatranscriptomics characterization of the viromes of five major fungal plant pathogens. *J. Virol.* **90**, 6846.

Matyjaszczyk, E. (2019) Problems of implementing compulsory integrated pest management. *Pest Manag. Sci.* **75**, 2063–2067.

Maxwell, A., Vettraino, A.M., Eschen, R. and Andjic, V. (2014) International Plant Trade and Biosecurity. *Hortic. Plants People Places* **3**, 1171–1195.

McCain, A.H., Koehler, C.S. and Tjosvold, S.A. (1987) Pitch canker threatens California pines. *Calif. Agric.* **41**, 22–23.

Mead, D.J. (2013) *Sustainable management of Pinus radiata plantations*, Rome (Italy).

Mitchell, R.G., Coutinho, T.A., Steenkamp, E., Herbert, M. and Wingfield, M.J. (2012) Future outlook for *Pinus patula* in South Africa in the presence of the pitch canker fungus (*Fusarium circinatum*). *South. For.* **74**, 203–210.

Mitchell, R.G., Wingfield, M.J., Hodge, G.R., Steenkamp, E.T. and Coutinho, T.A. (2013) The tolerance of *Pinus patula* × *Pinus tecunumanii*, and other pine hybrids, to *Fusarium circinatum* in greenhouse trials. *New For.* **44**, 443–456.

Mitchell, R.G., Zwolinski, J., Jones, N. and Coutinho, T. (2004) The effect of applying prophylactic measures on the post-planting survival of *Pinus patula* in South Africa. *South. African For. J.* **200**, 51–58.

Moraga-Suazo, P., Opazo, A., Zaldua, S., Gonzalez, G. and Sanfuentes, E. (2011) Evaluation of *Trichoderma* spp. and *Clonostachys* spp. strains to control *Fusarium circinatum* in *Pinus radiata* seedlings. *Chil. J. Agric. Res.* **71**, 412–417.

Morales-Rodríguez, C., Bastianelli, G., Aleandri, M., Chilosi, G. and Vannini, A. (2018) Application of *Trichoderma* spp. complex and biofumigation to control damping-off of *Pinus radiata* D.Don caused by *Fusarium circinatum* Nirenberg and O'Donnell. *Forests* **9**, 421.

Möykkynen, T., Capretti, P. and Pukkala, T. (2014) Modelling the potential spread of *Fusarium circinatum*, the causal agent of pitch canker in Europe. *Ann. For. Sci.* **72**, 169–181.

MPI (2019) *National Exotic Forest Description 2019*, Wellington: New Zealand.

Munoz-Adalia, E.J., Diez, J.J., Fernandez, M.M., Hantula, J. and Vainio, E.J. (2018) Characterization of small RNAs originating from mitoviruses infecting the conifer pathogen *Fusarium circinatum*. *Arch. Virol.* **163**, 1009–1018.

Muñoz-Adalia, E.J., Fernández, M.M. and Diez, J.J. (2016) The use of mycoviruses in the control of forest diseases. *Biocontrol Sci. Technol.* **26**, 577–604.

Muñoz-Adalia, E.J., Flores-Pacheco, J.A., Martínez-Álvarez, P., Martín-García, J., Fernández, M. and Diez, J.J. (2016) Effect of mycoviruses on the virulence of *Fusarium circinatum* and laccase activity. *Physiol. Mol. Plant Pathol.* **94**, 8–15.

Naidoo, S., Külheim, C., Zwart, L., Mangwanda, R., Oates, C.N., Visser, E.A., Wilken, F.E., Mamni, T.B. and Myburg, A.A. (2014) Uncovering the defence responses of *Eucalyptus* to pests and pathogens in the genomics age. *Tree Physiol.* **34**, 931–943.

Naidoo, S., Visser, E.A., Zwart, L., Toit, Y. Du, Bhadauria, V. and Shuey, L.S. (2017) Dual RNA-seq to elucidate the plant-pathogen duel. *Curr. Issues Mol. Biol* **27**, 127–142.

Neale, D.B., Wegrzyn, J.L., Stevens, K.A., *et al*. (2014) Decoding the massive genome of loblolly pine using haploid DNA and novel assembly strategies. *Genome Biol.* **15**, 1–13.

Ng, D.W.-K., Abeysinghe, J.K. and Kamali, M. (2018) Regulating the Regulators: The Control of Transcription Factors in Plant Defense Signaling. *Int. J. Mol. Sci.* **19**, 3737.

Nuss, D.L. (2005) Hypovirulence: Mycoviruses at the fungal-plant interface. *Nat. Rev. Microbiol.* **3**, 632–642.

Nystedt, B.., Street, N.R.., Wetterbom, A., *et al*. (2013) The Norway spruce genome sequence and conifer genome evolution. *Nature* **497**, 579–584.

Pearson, M.N., Beever, R.E., Boine, B. and Arthur, K. (2009) Mycoviruses of filamentous fungi and their relevance to plant pathology. *Mol. Plant Pathol.* **10**, 115–128.

Pedley, K.F. and Martin, G.B. (2005) Role of mitogen-activated protein kinases in plant immunity. *Curr. Opin. Plant Biol.* **8**, 541–547.

Perrings, C., Williamson, M., Barbier, E.B., Delfino, D., Dalmazzone, S., Shogren, J., Simmons, P. and Watkinson, A. (2002) Biological Invasion Risks and the Public Good: an Economic Perspective. *Conserv. Ecol.* **6**, art1.

Pfenning, L.H., Costa, S. da S., Melo, M.P. de, Costa, H., Ventura, J.A., Auer, C.G. and Santos, A.F. dos (2014) First report and characterization of *Fusarium circinatum*, the causal agent of pitch canker in Brazil. *Trop. Plant Pathol.* **39**, 210–216.

Quan, M., Chen, J. and Zhang, D. (2015) Exploring the secrets of long noncoding RNAs. *Int. J. Mol. Sci.* **16**, 5467–5496.

Quesada, T., Gopal, V., Cumbie, W.P., *et al*. (2010) Association Mapping of Quantitative Disease Resistance in a Natural Population of Loblolly Pine (*Pinus taeda* L.). *Genetics* **186**, 677-U336.

Quesada, T., Hughes, J., Smith, K., Shin, K., James, P. and Smith, J. (2018) A Low-Cost Spore Trap Allows Collection and Real-Time PCR Quantification of Airborne *Fusarium circinatum* Spores. *Forests* **9**, 586.

Reglinski, T., Taylor, J.T. and Dick, M.A. (2004) Chitosan induces resistance to pitch canker in *Pinus radiata*. *New Zeal. J. For. Sci.* **34**, 49–58.

Rigling, D. and Prospero, S. (2018) *Cryphonectria parasitica* , the causal agent of chestnut blight: invasion history, population biology and disease control. *Mol. Plant Pathol.* **19**, 7–20.

Roux, J., Eisenberg, B., Kanzler, A., Nel, A., Coetzee, V., Kietzka, E. and Wingfield, M.J. (2007) Testing of selected South African *Pinus* hybrids and families for tolerance to the pitch canker pathogen, *Fusarium circinatum*. *New For.* **33**, 109–123.

Sakamoto, J.M. and Gordon, T.R. (2006) Factors influencing infection of mechanical wounds by *Fusarium circinatum* on Monterey pines (*Pinus radiata*). *Plant Pathol.* **55**, 130–136.

Santini, A., Ghelardini, L., Pace, C. De, *et al*. (2013) Biogeographical patterns and determinants of invasion by forest pathogens in Europe. *New Phytol.* **197**, 238–250.

Santos, J. and Tovar, D. (1991) Algunos aspectos sobre el cancro resinoso de los pinos. In VI Simposio Nacional Sobre Parasitologia Forestal., p. 31. Mexico.

Schuster, S.C. (2007) Next-generation sequencing transforms today's biology. *Nat. Methods* **5**, 16–18.

Schweigkofler, W., O'Donnell, K. and Garbelotto, M. (2004) Detection and quantification of airborne conidia of *Fusarium circinatum*, the causal agent of pine pitch canker, from two California sites by using a real-time PCR approach combined with a simple spore trapping method. *Appl. Environ. Microbiol.* **70**, 3512–3520.

Scott, A.D., Zimin, A. V., Puiu, D., *et al*. (2020) A Reference Genome Sequence for Giant Sequoia. *G3 Genes, Genomes, Genet.* **10**, 3907–3919.

Seo, J.S., Sun, H.X., Park, B.S., Huang, C.H., Yeh, S.D., Jung, C. and Chua, N.H. (2017) ELF18-INDUCED LONG-NONCODING RNA associates with mediator to enhance expression of innate immune response genes in *Arabidopsis*. *Plant Cell* **29**, 1024–1038.

Serrano, Y., Elvira-Recuenco, M., Conde, M., Troya, M.T. and Raposo, R. (2015) *In vitro* evaluation of wood preservatives to prevent dispersal of pitch canker pathogen, *Fusarium circinatum*. *J. Phytopathol.* **163**, 783–786.

Showalter, D.N., Raffa, K.F., Sniezko, R.A., Herms, D.A., Liebhold, A.M., Smith, J.A. and Bonello, P. (2018) Strategic development of tree resistance against forest pathogen and insect invasions in defense-free space. *Front. Ecol. Evol.* **6**, 124.

Silva-Castro, I., Diez, J.J., Martín-Ramos, P., Pinto, G., Alves, A., Martín-Gil, J. and Martín-García, J. (2018a) Application of bioactive coatings based on chitosan and propolis for *Pinus* spp. protection against *Fusarium circinatum*. *Forests* **9**, 685.

Silva-Castro, I., Martín-García, J., Diez, J.J., Flores-Pacheco, J.A., Martín-Gil, J. and Martín-Ramos, P. (2018b) Potential control of forest diseases by solutions of chitosan oligomers, propolis and nanosilver. *Eur. J. Plant Pathol.* **150**, 401–411.

Slinski, S.L., Zakharov, F., Gordon, T.R. (2015). The effect of resin and monoterpenes on spore germination and growth in *Fusarium circinatum*. *Phytopathology* **105**, 119–125.

Sniezko, R.A. (2006) Resistance breeding against nonnative pathogens in forest trees-current successes in North America. *Can. J. Plant Pathol.* **28**, 270–279.

Sniezko, R.A. and Koch, J. (2017) Breeding trees resistant to insects and diseases: putting theory into application. *Biol. Invasions* **19**, 3377–3400.

Son, M., Yu, J. and Kim, K.H. (2015) Five questions about mycoviruses. *PLoS Pathog.* **11**, 5–11.

Spittlehouse, D.L. and Stewart, R.B. (2003) Adaptation to climate change in forest management. *BC J. Ecosyst. Manag.* **4**, 1–11.

Steenkamp, E.T., Rodas, C.A., Kvas, M. and Wingfield, M.J. (2012) *Fusarium circinatum* and pitch canker of *Pinus* in Colombia. *Australas. Plant Pathol.* **41**, 483–491.

Stenlid, J., Elfstrand, M., Cleary, M., Ihrmark, K., Karlsson, M., Davydenko, K. and Durling, M.B.

(2017) Genomes of *Hymenoscyphus fraxineus* and *Hymenoscyphus albidus* Encode Surprisingly Large Cell Wall Degrading Potential, Balancing Saprotrophic and Necrotrophic Signatures. *Balt. For.* **23**, 41–51.

Stenlid, J. and Oliva, J. (2016) Phenotypic interactions between tree hosts and invasive forest pathogens in the light of globalization and climate change. *Philos. Trans. R. Soc. B Biol. Sci.* **371**.

Stevens, K.A., Wegrzyn, J.L., Zimin, A., *et al*. (2016) Sequence of the Sugar Pine Megagenome. *Genetics* **204**, 1613–1626.

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

Storer, A.J., Gordon, T.R., Dallara, P.L. and Wood, D.L. (1994) Pitch canker kills pines, spreads to new species and regions. *Calif. Agric.* **48**, 9–13.

Sturrock, R.N., Frankel, S.J., Brown, A. V., Hennon, P.E., Kliejunas, J.T., Lewis, K.J., Worrall, J.J. and Woods, A.J. (2011) Climate change and forest diseases. *Plant Pathol.* **60**, 133–149.

Swett, C.L. and Gordon, T.R. (2013) Dualism in symbiosis: Growth and defense enhancement of symptomless infection by the pathogen *Fusarium circinatum* in *Pinus radiata* seedlings. *Phytopathology* **103**, 191.

Swett, C.L. and Gordon, T.R. (2015) Endophytic association of the pine pathogen *Fusarium circinatum* with corn (*Zea mays*). *Fungal Ecol.* **13**, 120–129.

Swett, C.L. and Gordon, T.R. (2012) First Report of Grass Species (Poaceae) as Naturally Occurring Hosts of the Pine Pathogen *Gibberella circinata*. *Plant Dis.* **96**, 908–908.

Swett, C.L. and Gordon, T.R. (2012) Latent Infection by *Fusarium circinatum* Influences Susceptibility of Monterey Pine Seedlings to Pitch Canker. In Proceedings of the fourth international workshop on the genetics of host-parasite interactions in forestry: Disease and insect resistance in forest trees. (Sniezko, R., Yanchuk, A.D., Kliejunas, J.T., Palmieri, K., Alexander, J., and Frankel, S., eds), pp. 159–161. Albany, CA: Pacific Southwest Research Station, Forest Service, U.S. Department of Agriculture.

Swett, C.L., Kirkpatrick, S.C. and Gordon, T.R. (2016) Evidence for a Hemibiotrophic Association of the Pitch Canker Pathogen *Fusarium circinatum* with *Pinus radiata*. *Plant Dis.* **100**, 79–84.

Telford, A., Cavers, S., Ennos, R.A. and Cottrell, J.E. (2015) Can we protect forests by harnessing variation in resistance to pests and pathogens? *Forestry* **88**, 3–12.

Thambugala, K.M., Daranagama, D.A., Phillips, A.J.L., Kannangara, S.D. and Promputtha, I. (2020) Fungi vs. Fungi in Biocontrol: An Overview of Fungal Antagonists Applied Against Fungal Plant Pathogens. *Front. Cell. Infect. Microbiol.* **10**, 604923.

Collar Urquijo, J. (1995) Informe de la reunión del grupo de trabajo de laboratorios de diagnóstico y prospecciones fitosanitarias. *Almería*, **7**.

Vainio, E.J., Jurvansuu, J., Hyder, R., *et al*. (2018) Heterobasidion Partitivirus 13 Mediates Severe Growth Debilitation and Major Alterations in the Gene Expression of a Fungal Forest Pathogen. *J. Virol.* **92**, 1–15.

Vainio, E.J., Martínez-Álvarez, P., Bezos, D., Hantula, J. and Diez, J.J. (2015) *Fusarium circinatum* isolates from northern Spain are commonly infected by three distinct mitoviruses. *Arch. Virol.* **160**, 2093–2098.

Viljoen, A., Wingfield, M.J., Kemp, G.H.J. and Marasas, W.F.O. (1995) Susceptibility of pines in South Africa to the pitch canker fungus *Fusarium subglutinans* f.sp. *pini*. *Plant Pathol.* **44**, 877–882.

Viljoen, A., Wingfield, M.J. and Marasas, W.F.O. (1994) First Report of *Fusarium subglutinans* f. sp. *pini* on Pine Seedlings in South Africa. *Plant Dis.* **78**, 309–312.

Visser, E.A., Wegrzyn, J.L., Steenkamp, E.T., Myburg, A.A. and Naidoo, S. (2019) Dual RNA-Seq analysis of the pine-*Fusarium circinatum* interaction in resistant (*Pinus tecunumanii*) and susceptible (*Pinus patula*) hosts. *Microorganisms* **7**, 7–9.

Vivas, M., Nunes, C., Coimbra, M.A. and Solla, A. (2014) Antioxidant activity of *Pinus pinaster* infected with *Fusarium circinatum* is influenced by maternal effects. *For. Pathol.* **44**, 337–340.

Vivas, M., Zas, R. and Solla, A. (2012) Screening of maritime pine (*Pinus pinaster*) for resistance to *Fusarium circinatum*, the causal agent of pitch canker disease. *Forestry* **85**, 185–192.

Wang, Lihua, Luo, H., Hu, W., Yang, Y., Hong, N., Wang, G., Wang, A. and Wang, L. (2018) *De novo* transcriptomic assembly and mRNA expression patterns of *Botryosphaeria dothidea* infection with mycoviruses chrysovirus 1 (BdCV1) and partitivirus 1 (BdPV1). *Virol. J.* **15**, 126.

Wang, Z., Gerstein, M. and Snyder, M. (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* **10**, 57–63.

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

Wegrzyn, J.L., Liechty, J.D., Stevens, K.A., *et al*. (2014) Unique features of the loblolly pine (*Pinus taeda* L.) megagenome revealed through sequence annotation. *Genetics* **196**, 891–909.

Wegrzyn, J.L., Lin, B.Y., Zieve, J.J., *et al*. (2013) Insights into the Loblolly Pine Genome: Characterization of BAC and Fosmid Sequences. *PLoS One* **8**, e72439.

Westermann, A.J., Barquist, L. and Vogel, J. (2017) Resolving host–pathogen interactions by dual RNA-seq. *PLoS Pathog.* **13**, e1006033.

Wikler, K., Storer, A.J., Newman, W., Gordon, T.R. and Wood, D.L. (2003) The dynamics of an introduced pathogen in a native Monterey pine (*Pinus radiata*) forest. *For. Ecol. Manage.* **179**, 209–221.

Wingfield, B.D., Steenkamp, E.T., Santana, Q.C., *et al*. (2012) First fungal genome sequence from Africa: A preliminary analysis. *S. Afr. J. Sci.* **108**, 104–112.

Wingfield, M.J., Brockerhoff, E.G., Wingfield, B.D. and Slippers, B. (2015) Planted forest health: The need for a global strategy. *Science (80-.).* **349**, 832–836.

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

Wingfield, M.J., Jacobs, A., Coutinho, T.A., Ahumada, R. and Wingfield, B.D. (2002) First report of the pitch canker fungus, *Fusarium circinatum*, on pines in Chile. *Plant Pathol.* **51**, 397.

Woodcock, P., Cottrell, J.E., Buggs, R.J.A. and Quine, C.P. (2018) Mitigating pest and pathogen impacts using resistant trees: a framework and overview to inform development and deployment in Europe and North America. *For. An Int. J. For. Res.* **91**, 1–16.

Wu, M., Zhang, L., Li, G., Jiang, D. and Ghabrial, S.A. (2010) Genome characterization of a debilitation-associated mitovirus infecting the phytopathogenic fungus *Botrytis cinerea*. *Virology* **406**, 117–126.

Wu, M.D., Zhang, L., Li, G.Q., Jiang, D.H. and Hou, M.S. (2007) Hypovirulence and double-stranded RNA in *Botrytis cinerea*. *Phytopathology* **97**, 1590–1599.

Xu, Z., Wu, S., Liu, L., Cheng, J., Fu, Y., Jiang, D. and Xie, J. (2015) A mitovirus related to plant mitochondrial gene confers hypovirulence on the phytopathogenic fungus *Sclerotinia sclerotiorum*. *Virus Res.* **197**, 127–136.

Yang, Y., Saand, M.A., Huang, L., Abdelaal, W.B., Zhang, J., Wu, Y., Li, J., Sirohi, M.H. and Wang, F. (2021) Applications of multi-omics technologies for crop improvement. *Front. Plant Sci.* **12**, 1846.

Yu, Y., Zhou, Y.F., Feng, Y.Z., He, H., Lian, J.P., Yang, Y.W., Lei, M.Q., Zhang, Y.C. and Chen, Y.Q. (2020) Transcriptional landscape of pathogen-responsive lncRNAs in rice unveils the role of ALEX1 in jasmonate pathway and disease resistance. *Plant Biotechnol. J.* **18**, 679–690.

Zamora, P., González Casas, A., Dueñas, M., San Martin, R. and Diez, J.J. (2017) Factors influencing growth, sporulation and virus transfer in *Cryphonectria parasitica* isolates from Castilla and León (Spain). *Eur J Plant Pathol* **148**, 65–73.

Zhang, L., Wang, M., Li, N., *et al*. (2018) Long noncoding RNAs involve in resistance to *Verticillium dahliae*, a fungal disease in cotton. *Plant Biotechnol. J.* **16**, 1172–1185.

Zhang, Y.Z., Shi, M. and Holmes, E.C. (2018) Using metagenomics to characterize an expanding virosphere. *Cell* **172**, 1168–1172.

Zhu, Q.-H., Stephen, S., Taylor, J., Helliwell, C.A. and Wang, M.-B. (2014) Long noncoding RNAs responsive to *Fusarium oxysporum* infection in *Arabidopsis thaliana*. *New Phytol.* **201**, 574–584.

Zimin, A., Stevens, K.A., Crepeau, M.W., *et al*. (2014) Sequencing and assembly of the 22-Gb loblolly pine genome. *Genetics* **196**, 875–890.

Chapter 2: Objectives

The serious economic, social and ecological implications of *Fusarium circinatum* establishment and the lack of effective measures to eradicate or control the PPC disease in nurseries or in the field, make the search for viable solutions imperative. New European guidelines for the reduction of chemical use generate the need to find sustainable methods to prevent and mitigate PPC disease. Promoting the use of environmentally friendly mechanisms with the help of intensive knowledge of the *F. circinatum* lifestyle and the molecular interactions of PPC pathosystem using next generation sequencing technologies will enhance the development of effective management methods. The overall aim of this doctoral research was to shed light on effective regulatory mechanisms for the control of PPC disease. In order to achieve this general objective several specific goals were proposed:

1. To collate the published information on feasible preventive mechanisms to minimize the risk of new introductions of *F. circinatum* into disease-free areas (**Article I**).

2. To understand the molecular mechanisms involved in *F. circinatum* pathogenicity through the study of its mycoviruses and transcriptional responses (**Article II & III**).

3. To identify defense mechanisms of the hosts by studying their coding and non-coding transcriptome during *F. circinatum* infection (**Article III & IV**).

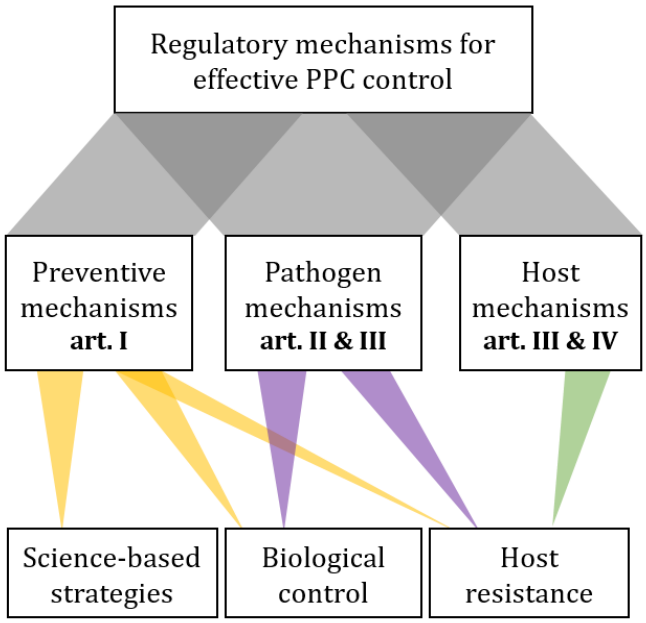

Figure 2.1: Conceptual model of the thesis.

Chapter 3: Materials and Methods

A brief description of materials and methods is given below. For detailed information, please refer to the original articles (Roman numerals).

1. MATERIALS

1.1. *Fusarium* isolates

- Spanish *F. circinatum* isogenic isolates Fc072v (**II & III**) and Fc072 (**II & IV**), obtained in the Forest Entomology and Pathology Laboratory (University of Valladolid).

1.2. Plant material

- Six-month-old seedlings of *P. radiata* (Provenance: Galicia, Spain) (**IV**).
- One-year old seedlings of *P. radiata* (Provenance: Galicia, Spain) and *P. pinea* (Provenance: Meseta Norte, Spain) (**III**).

2. METHODS

Characterization of *Fusarium circinatum* isolates

Cultured isolates

- Solid medium for colony maintenance (Figure 3.1): PDA medium (3.90% w/v potato dextrose agar, Scharlab S.L., Barcelona, Spain) (**II, III & IV**).
- Liquid medium for mycelium formation (**II**) and sporulation (**III & IV**): PDB medium (2.40% w/v potato dextrose broth, Scharlab S.L., Barcelona, Spain).

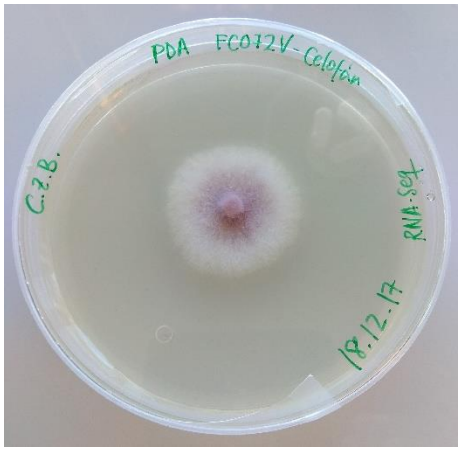

Figure 3.1. *Fusarium circinatum* Fc072v colony growing on PDA medium.

Molecular biology

a) RNA extraction (**II, III & IV**)

- Spectrum™ Plant Total RNA Kit (Sigma Aldrich, St. Louis, MO, USA) following the manufacturer's protocols.
- Genomic DNA removal by on-column DNase Digestion (DNASE10-1SET, Sigma-Aldrich, St. Louis, MO, USA).
- Concentration and purity of the RNA measurement by spectrophotometer.

b) Complementary DNA (cDNA) synthesis

- Reverse transcription reactions were performed in order to detect mycoviruses by polymerase chain reaction (PCR).
- Extracted RNA was used as template for cDNA synthesis with PrimeScript™ Reverse Transcriptase (TAKARA) using random hexamers (Thermo Scientific™ Random Hexamer Primer).

c) Polymerase chain reaction (PCR)

- Amplification of the synthesized cDNA for mycoviruses FcMV1 and FcMV2-1 detection was performed using specific primers: FMC1F1 (5'-CGTGGATTAAAACCCACAAA-3′) /FMC1Rev1 (5′-TGGTAATCTACCATAGCAATTAYTC-3') and FMC3F1 (5'-GAYAGAACTTTTACTCAAGATCC -3')/FMC3Rev1 (5'-ATTCATCTYTTGGCAAATTCATA-3').

d) Electrophoresis

- The fragments generated with the primer sets were scored using electrophoresis on agarose (1% TAE) gels and visualization was carried out using 0.1 µl ml^{-1} GelRed dye (Biotium) and a UV transilluminator.
- Extracted RNA was checked on agarose (1% TAE) gels for integrity measurement of the molecules (**II, III & IV**).

Next generation sequencing (NGS)

- Illumina Stranded RNA library construction and sequencing by Illumina HiSeq 4000 platform for the generation of 75-bp raw paired-end reads (**II**).
- Strand-specific RNA-Seq library construction with TruSeq Stranded mRNA LT sample preparation Kit and sequencing by Illumina NovaSeq 6000 platform for the generation of 150-bp raw paired-end reads (**III & IV**).

Pathogenicity analyses

Sporal suspensions (**III & IV**)

- Preparation of spore suspensions and adjustment of concentration (10^6 conidia mL^{-1}) with a haemocytometer.

Treatment of plant material (**III & IV**)

- Seedlings acclimation and handling.

Inoculation trial

- Stem inoculation technique from Martín-García *et al.,* 2017 (Figure 3.2) (**III & IV**).
- Seedling mortality was recorded daily (**IV**) or twice a week (**III**).

Figure 3.2. Stem inoculation method using 10 µL of sporal suspension and an inverted 'U' wound.

Physiological analyses (III)

Water potential measurements

- Midday stem water potential (Ψ_{md}, MPa) was measured for every seedling using a Scholander-type pressure chamber (PMS Instrument Co., Albany, OR, USA; Figure 3.3).

Needle gas exchange-related parameters measurements

- The apical shoot net CO_2 assimilation rate (A), transpiration rate (E), stomatal conductance (gs) and sub-stomatal CO_2 concentration (Ci) were measured using an infra-red gas exchange analyzer (LCpro-SD, ADC BioScientific Limited, Hoddesdon, U.K.) with a conifer-type chamber (Figure 3.4).

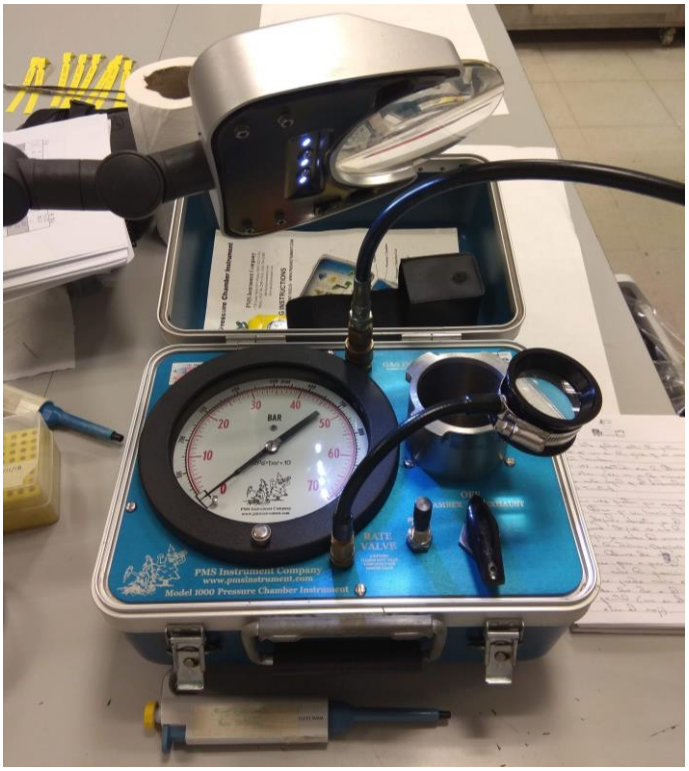

Figure 3.3. Scholander-type pressure chamber.

Figure 3.4. Infra-red gas exchange analyzer with a conifer-type chamber measuring needle gas exchange-related parameters of *Pinus radiata* (left) and *P. pinea* (right) seedings.

Bioinformatics analyses

Pre-processing of raw data

- Disposal of poor-quality terminal nucleotides using CLC Genomics Workbench 6.0.1. (**II**).
- Quality control of sequenced reads using FastQC v.0.11.9. (**III & IV**)
- Trimming of raw reads for Illumina adaptor sequences and low-quality base-calls using Trimmomatic v.0.38. (**III & IV**)

Genome mapping

- Reads were mapped to the *F. circinatum* FSP 34 annotated genome (provided by FABI-UP) with CLC Genomics Workbench 6.0.1. (**II**).
- Reads were mapped to the *Pinus taeda* genome (downloaded from Treegenes database) with HiSat2 v.2.0.0 with the default settings (**III & IV**) and adding parameters for further transcript assembly (**IV**).
- Reads were mapped to the *F. circinatum* Fc072v genome (downloaded from NCBI) with HiSat2 v.2.0.0 with the default settings (**III & IV**).

Transcripts assembly

- StringTie v.2.1.4 was used to assemble the transcripts from each sample and merging all assemblies into an experiment-level reference assembly for the pathogen (**III)** and the *P. radiata* data (**IV**).
- Gffcompare v.0.12.1 and *P. taeda* GTF file were used for classifying pine-assembled transcripts in different class codes according to their nature/origin (**IV**).

Annotation of transcripts

- Annotation of *F. circinatum* FSP 34 transcripts, prediction of protein domains through InterProScan 5, Gene Ontology (GO) and Enzyme Code (EC) mapping were performed using the BLAST2GO program (**II**).
- The pipeline of EnTAP v.0.9.2 for open reading frame prediction, similarity search, and orthologous groups (SMART/Pfam), gene ontology (GO) terms and KEGG pathways assignment was used for *F. circinatum* Fc072v (**III**) and *P. radiata* assembled transcripts (**IV**) annotation.
- The GTF file for the *P. taeda* genome sequence (downloaded from Treegenes database) was used for pine transcripts annotation (**III**).

Expression quantification

- Expression levels were estimated and normalized into RPKM values with CLC Genomics Workbench 6.0.1. (**II**).
- Expression quantification in pine for gene regions specified in the corresponding GTF file for the *P. taeda* genome sequence (downloaded from Treegenes database) (**III**).
- The estimation of abundances of the transcripts assembled was performed with StringTie v.2.1. by mapping the reads to the experiment-level reference generated for the pathogen (**III**) and the *P. radiata* data (**IV**).

Long non-coding RNA (lncRNA) identification (**IV**)

- The pine-assembled transcripts were subjected to the coding potential predictor FEELnc v.0.2 tool.
- Similarity of pine lncRNAs to other plant transcripts recorded in non-coding RNA databases was performed using the blastn algorithm (E-value $<10^{-5}$) of the BLAST v.2.9.0 software suite.

Statistical analyses

Survival analysis (**III & IV**)

- Survival analysis was performed with the "Survival" package based on the nonparametric estimator Kaplan–Meier using the software R v.3.6.2.

Physiological parameter analysis (**III**)

- Shapiro–Wilk's and Bartlett's tests to test for data normality and homoscedasticity ($p \leq 0.05$).
- When data followed ANOVA's assumptions: Two-way analysis of variance (ANOVA).
- When data violated ANOVA's assumptions: heteroscedastic two-way ANOVAs using the generalized Welch procedure and a 0.1 trimmed mean transformation.

Differential expression analysis

- Comparisons of RPKM values were performed using Baggerley's Z test (**II**).
- The expression quantification of pine and fungal data (**III**) was analyzed using edgeR v.1.3.959 package using the software R v.3.6.2.
- The expression quantification of pine transcripts (**IV**) was analyzed using DESeq2 v.1.24.1 package using the software R v.3.6.2.
- The identification of differential expression genes was determined using the threshold of 2-fold change (**II**), $\log_2$ (|Fold-change|) ≥ 1.5 (**III**) or $\log_2$ (|Fold-change|) ≥ 1 (**IV**) at a false discovery rate of (FDR) lower than 0.05 (**II, III & IV**).

Functional analysis

- GO enrichment was performed using Fisher's exact test (FET) in the BLAST2GO program (FDR lower than 0.05) (**II**).
- GO and KEGG enrichment analysis were implemented by GOSeq v.1.38.0 based on the Wallenius non-central hyper-geometric distribution (FDR lower than 0.05) (**III & IV**).

Co-expression analysis of transcripts (**IV**)

- A weighted gene co-expression network analysis approach implemented in the R-based Co-Expression Modules Identification Tool (CEMiTool) package v.1.8.3 was conducted in R software.
- Calculation of the correlation coefficients by Spearman's method.
- The pairwise comparisons were evaluated using Wald tests.

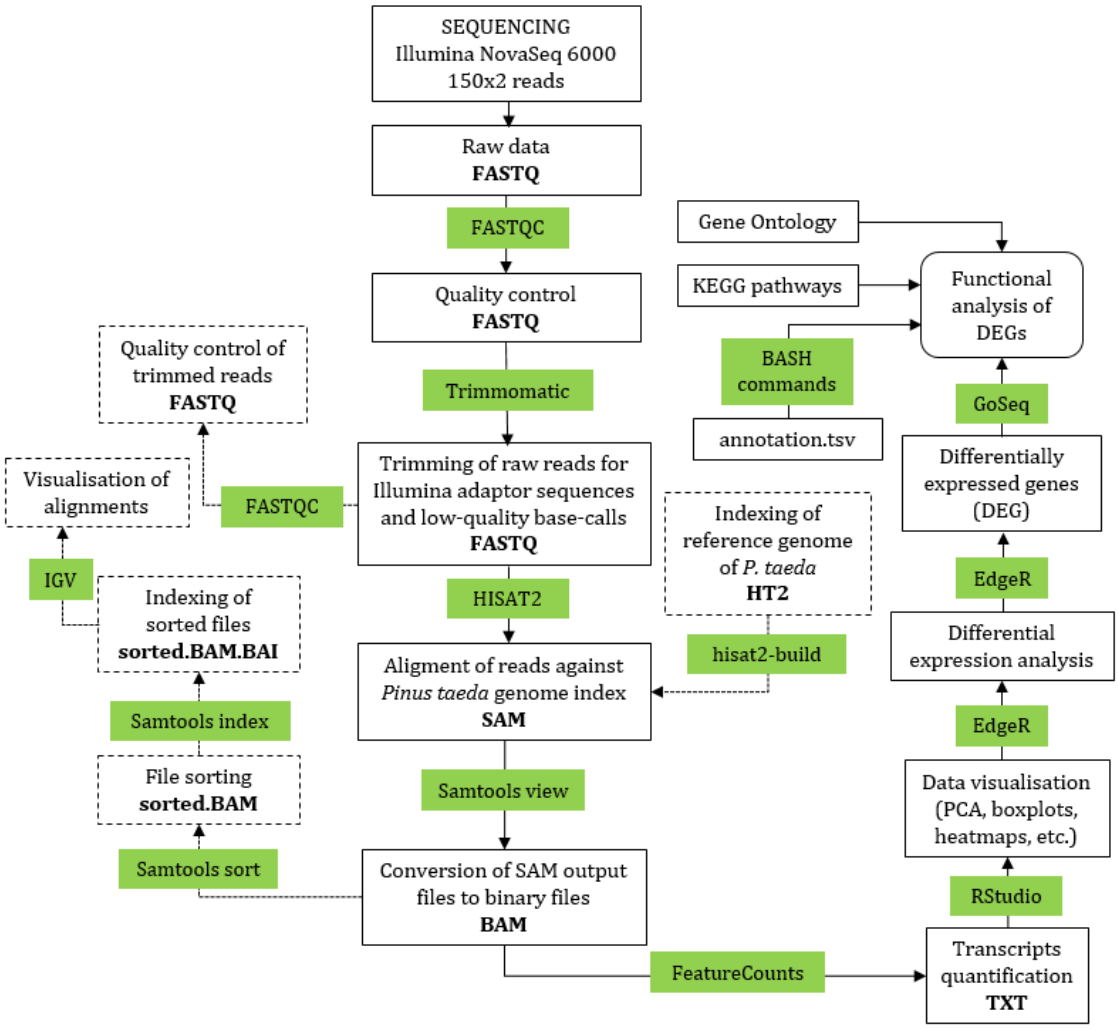

Figure 3.5. Workflow of the raw RNA-Seq reads processing for pine differential expression and functional analysis. Bold refers to file formats. Green boxes refer to the software used.

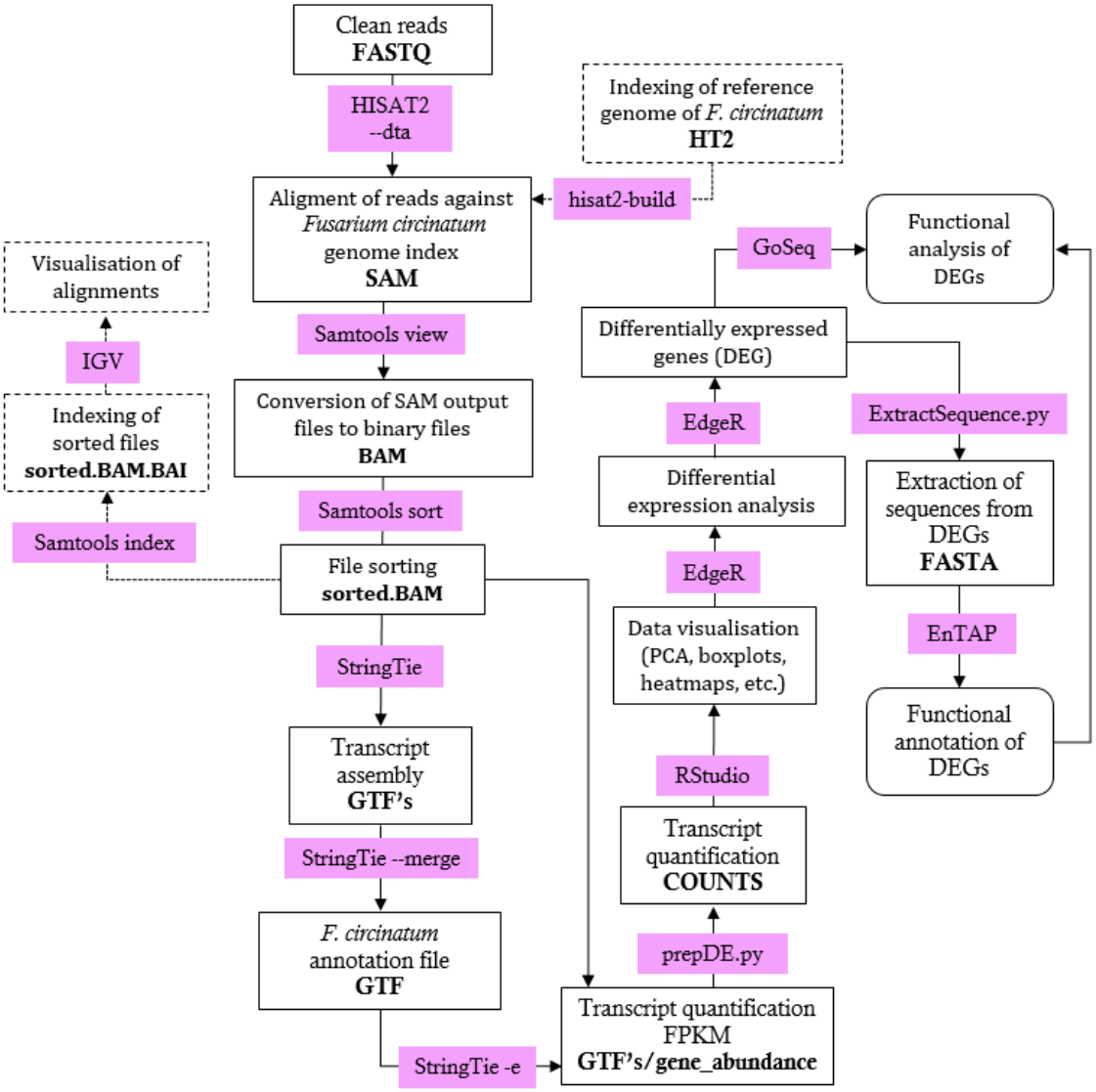

Figure 3.6. Workflow of the processing of clean RNA-Seq reads for *Fusarium circinatum* differential expression and functional analysis. Bold refers to file formats. Violet boxes refer to the software used.

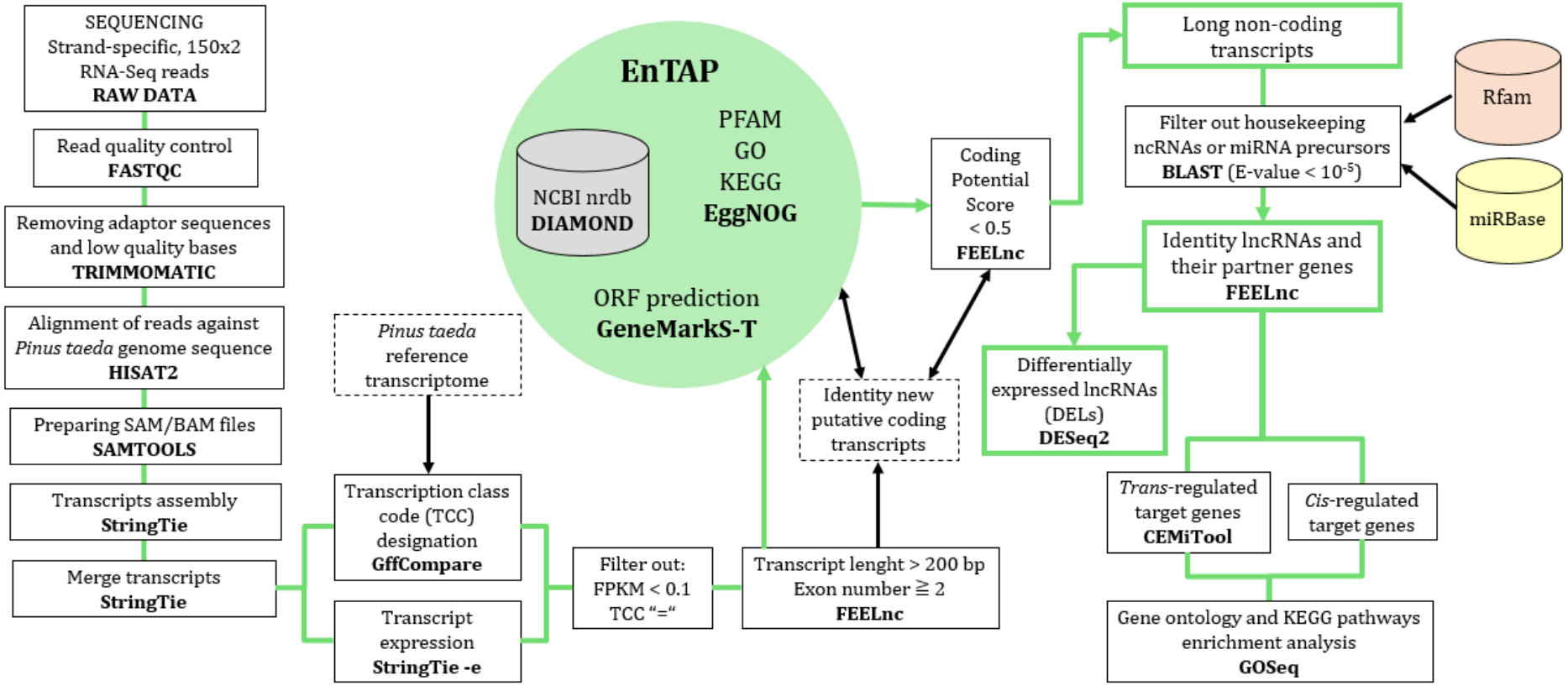

Figure 3.7. Simplified workflow for the identification of pine lncRNAs and those that respond to *Fusarium circinatum* infection. Bold refers to the software used.

Chapter 4: Original article I

Pine Pitch Canker (PPC): Pathways of pathogen spread and preventive measures

This chapter has been published as:

Zamora-Ballesteros, C.[1,2], Diez, J.J.[1,2], Martín-García, J.[1,2,3], Witzell, J.[4], Solla, A.[5], Ahumada, R.[6], Capretti, P.[7], Cleary, M.[4], Drenkhan, R.[8], Dvorák, M.[9], Elvira-Recuenco, M.[10], Fernández-Fernández, M.[2,11], Ghelardini, L.[7], Gonthier, P.[12], Hernández-Escribano, L.[10], Ioos, R.[13], Markovskaja, S.[14], Martínez-Álvarez, P.[2], Muñoz-Adalia, E.J.[15], Nowakowska, J.A.[16], Oszako, T.[17], Raposo, R.[10], Santini, A.[18] and Hantula, J.[19].
2019. Pine pitch canker (PPC): Pathways of pathogen spread and preventive measures. *Forests* **10**, 1158. https://doi.org/10.3390/f10121158.

[1]Department of Vegetal Production and Forest Resources, University of Valladolid, Av Madrid 44, 34004 Palencia, Spain.
[2]Sustainable Forest Management Research Institute, University of Valladolid—INIA, 28040 Madrid, Spain.
[3]Department of Biology and CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal.
[4]Southern Swedish Forest Research Centre, Swedish University of Agricultural Sciences, P.O.Box 49, 23053 Alnarp, Sweden.
[5]Faculty of Forestry, Institute for Dehesa Research (INDEHESA), University of Extremadura, Avenida Virgen del Puerto 2, 10600 Plasencia, Spain.
[6]Bioforest S.A., Km. 12, Camino a Coronel, 403 0000 Concepcion, Chile.
[7]Department of Agriculture, Food, Environment and Forestry (DAGRI), Università degli Studi di Firenze, Piazzale delle Cascine 18, 50144 Firenze, Italy.
[8]Institute of Forestry and Rural Engineering, Estonian University of Life Sciences, Fr. R. Kreutzwaldi 5, 51006 Tartu, Estonia.
[9]Depertment of Forest Protection and Wildlife Management, Faculty of Forestry and Wood Technology, Mendel University in Brno, Zemedelská 3, 61300 Brno, Cesko.
[10]Forest Research Centre (CIFOR), National Institute for Agricultural and Food Research and Technology (INIA), Ctra. La Coruña Km. 7.5, 28040 Madrid, Spain.
[11]Department of Agroforestry Sciences, University of Valladolid, Av Madrid 44, 34004 Palencia, Spain.
[12]Department of Agriculture, Forest and Food Sciences, University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco, Italy.
[13]ANSES Plant Health Laboratory, Unit of mycology, Domaine de Pixérécourt, Bât. E., 54220 Malzéville, France.
[14]Institute of Botany, Nature Research Centre, Laboratory of Mycology, Žaliuju¸ Ežeru¸ Str. 49, LT-08406 Vilnius, Lithuania.
[15]Centre Tecnològic Forestal de Catalunya, Crta. de St. Llorenç de Morunys Km. 2, 25280 Solsona, Spain.
[16]Faculty of Biology and Environmental Sciences, Cardinal Stefan Wyszynski University in Warsaw, Wóycickiego 1/3 Street, 01-938 Warsaw, Poland.
[17]Forest Protection Department, Institute of Forest Research, Braci Lesnej 3, 05-090 Sekocin Stary, Poland.
[18]Institute for Sustainable Plant Protection-C.N.R., Via Madonna del Piano 10, 50019 Sesto fiorentino, Italy.
[19]Department of Natural Resources, Natural Resources Institute Finland (Luke), Latokartanonkaari 9, 00790 Helsinki, Finland.

Chapter 5: Original article II

Residual effects caused by a past mycovirus infection in *Fusarium circinatum*

This chapter has been published as:

Zamora-Ballesteros, C.[1,2], Wingfield, B.D.[3], Wingfield, M.J.[3], Martín-García, J.[1,2], Diez, J.J.[1,2] 2021. Residual effects caused by a past mycovirus infection in *Fusarium circinatum*. *Forests* **12**, 11. https://dx.doi.org/10.3390/f12010011.

[1]Sustainable Forest Management Research Institute, University of Valladolid—INIA, 34004 Palencia, Spain.
[2]Department of Vegetal Production and Forest Resources, University of Valladolid, 34004 Palencia, Spain.
[3]Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria 0002, South Africa.

Chapter 6: Original article III

Dual RNA-Sequencing analysis of resistant (*Pinus pinea*) and susceptible (*Pinus radiata*) hosts during *Fusarium circinatum* challenge

This chapter has been published as:

Zamora-Ballesteros, C.[1,2], Pinto, G.[3], Amaral, J.[3], Valledor, L.[4], Alves, A.[3], Diez, J.J.[1,2], Martín-García, J.[1,2] 2021. **Dual RNA-Sequencing analysis of resistant (*Pinus pinea*) and susceptible (*Pinus radiata*) hosts during *Fusarium circinatum* challenge**. *International Journal of Molecular Sciences* 22, 5231.
https://doi.org/10.3390/ijms22105231

[1]Sustainable Forest Management Research Institute, University of Valladolid—INIA, 34004 Palencia, Spain.
[2]Department of Vegetal Production and Forest Resources, University of Valladolid, 34004 Palencia, Spain.
[3]Centre for Environmental and Marine Studies, CESAM, Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal.
[4]Department of Organisms and Systems Biology, University of Oviedo, 33071 Oviedo, Spain.

Chapter 7: Original article IV

Genome-wide identification and characterization of *Fusarium circinatum*-responsive lncRNAs in *Pinus radiata*

This chapter has been published as:

Zamora-Ballesteros, C.[1,2], **Martín-García, J.**[1,2], **Suárez-Vega, A.**[3], **Diez, J.J.**[1,2] 2022. Genome-wide identification and characterization of *Fusarium circinatum*-responsive lncRNAs in *Pinus radiata*. *BMC Genomics* **23**, 194.
https://doi.org/10.1186/s12864-022-08408-9

[1]Sustainable Forest Management Research Institute, University of Valladolid—INIA, 34004 Palencia, Spain.
[2]Department of Vegetal Production and Forest Resources, University of Valladolid, 34004 Palencia, Spain.
[3]Department of Animal Production, Faculty of Veterinary Medicine, University of León, Campus de Vegazana s/n, León, 24071, Spain.

Chapter 8: General discussion

Forests and woodland ecosystems are facing an alarming increase in the number of invasive pathogens, whose establishment is being accelerated by climate change (Anderson *et al.*, 2004). Consequently, the focus of forest pathogen management is shifting toward reduction of both the occurrence of new invasions and the impact of established diseases using environmentally friendly control methods. In this thesis, four scientific studies about PPC, a devastating disease of conifers caused by the invasive pathogen *Fusarium circinatum*, were carried out. The aim of these studies was to identify pathways of pathogen spread and proposing preventive measures to avoid its introduction into disease-free areas, as well as to unravel the molecular interactions of both mycovirus-pathogen and pathogen-pine using transcriptomics technologies.

F. circinatum is spread by multiple pathways, both naturally, which is mainly associated with local dissemination, and human-mediated, which involves global dispersal (Figure 8.1). The natural spread of the PPC pathogen is limited due to the short dispersal distances of the spores and the fairly short flight distances of disseminating insects. Most important, however, is the long-distance dispersion as a result of globalization, particularly of plant trade and movement of inconspicuously infected living plants, wood, bark, soil and seeds, which represents the most of introductions of the pathogen into disease-free regions (EFSA, 2010). The establishment of the disease in the field is of great concern since no viable intervention step aiming at the eradication or control of *F. circinatum* is available. In fact, eradication could be only possible with an early detection of the pathogen, especially if its presence is limited to nurseries or urban greeneries, as has been the case in France and Italy (Vainio *et al.*, 2019). Therefore, continued vigilance, monitoring and reliable diagnosis methods of PPC pathogen are essential to prevent its local establishment, spread and movement into disease-free areas. Field and laboratory protocols used for the identification and diagnostic of *F. circinatum* are described in detail in the **Annex II**.

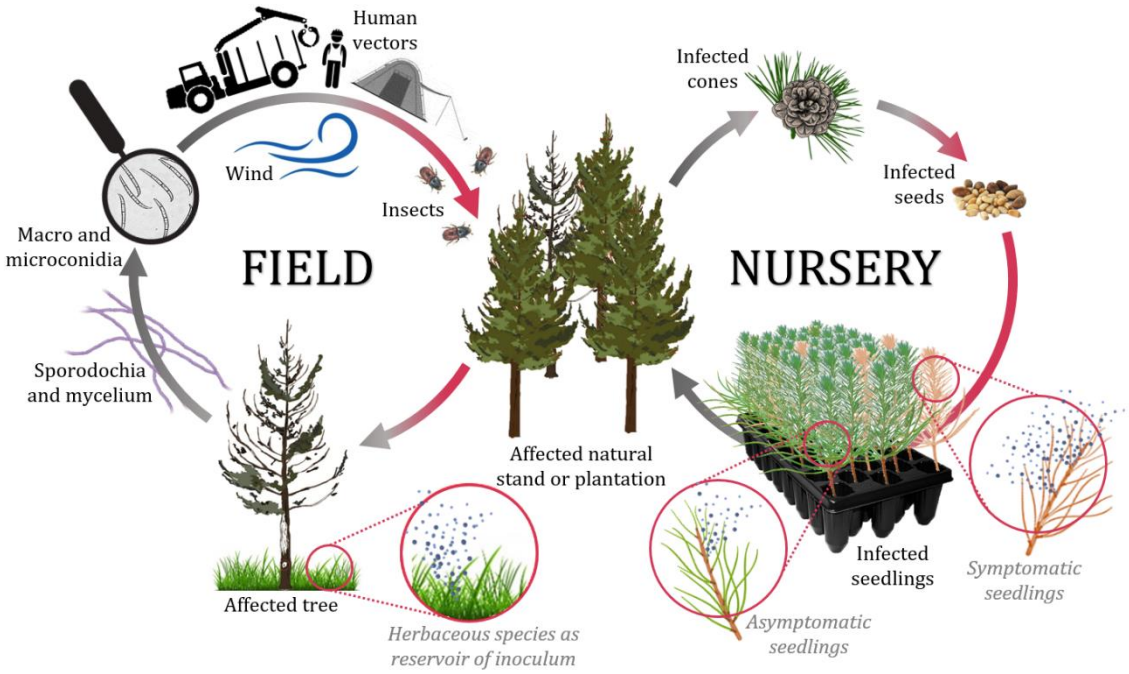

Figure 8.1. Dissemination pathways and potential sources of inoculum of *F. circinatum*.

Though it is an integral part of pathogen management, visual inspection for disease is not fool-proof, as the pathogen may exist at a stage that is not visually identifiable. Several studies have described the existence of a latent stage of *F. circinatum*, being capable of living inside the host remaining symptomless even for over a year (Elvira-Recuenco *et al.*, 2015; Swett *et al.*, 2016; Swett and Gordon, 2017; Martín-García *et al.*, 2018). The possible existence of asymptomatic seedlings together with the fact that *F. circinatum* produces similar symptoms to those caused by other pathogenic fungi (Vainio *et al.*, 2019), make molecular methods the most rapid and reliable tool for detection. Therefore, the development and implementation of robust diagnostic protocols is fundamental for the early and accurate detection of *F. circinatum* (Ioos *et al.*, 2019).

F. circinatum is a serious problem in forest nurseries, not only because it causes pre- and post-emergence damping-off through infection of seeds and roots, but also because infected nursery plants represent a source of infection for trees in the field (Drenkhan *et al.*, 2020), especially due to the possibility of infected plants remaining symptomless. Hence, regular monitoring should be carried out on symptomatic and asymptomatic seedlings for early detection of the pathogen. Moreover, sanitation practices, such as burning infected seedlings, chlorination or ozone treatment of irrigation water, clean trays and containers by immersion in hot water or steam, are critical to avoid new infestations and spreading of the disease to the field. Here in field, silvicultural methods such as pruning and characteristics in the planting site, including shallow soils, that could generate wounds or induce stress to the trees, should be avoided. Mechanical control such as the elimination of coarse woody debris colonized by insects before their emergence or the avoidance of storage of freshly cut logs have shown effective results in the management of insect vectors of PPC (Göthlin *et al.*, 2000; Wermelinger, 2004; Walmsley and Godbold, 2010; Torres-Vila *et al.*, 2015). In addition, high sanitary standards and common sense should be applied in forestry operations.

Direct chemical control of forest fungal pathogens has been widely applied in nurseries (Prospero *et al.*, 2021), but in the framework of an integrated pest management (IPM) strategy, the search for an effective biological control agent (BCA) becomes an imperative in these sites where the environment may be more controlled. In this sense, the use of *Trichoderma* species as a preventive formulation are likely the most promising BCA against *F. circinatum* in nurseries (Martín-García *et al.*, 2019). The potential of microbes as BCAs is based on their ability to produce toxins, parasitize or compete with the pathogen for the nutritional niche inside plants and even the stimulation of the defensive metabolism of the tree host (Gao *et al.*, 2010; Blumenstein *et al.*, 2015). The use of the microbiome associated with the healthy status of trees to suppress pathogenic fungi is gaining considerable interest as a field measure. In a recent study, although rhizobiomes of the *F. circinatum*-susceptible *P. radiata* and the resistant *P. pinea* species did not show significant changes after the stem inoculation of the pathogen, differences in the non-inoculated plants were found. In particular, the resistant species hosted higher abundance of bacterial taxa associated to disease protection (Leitão *et al.*, 2021). Therefore, further investigation about possible direct and indirect effects of microbiome on *F. circinatum* would be needed to assess the ecological importance of these biological populations for the disease development, and to evaluate possibilities to support pine resistance by microbiome manipulation.

As noted above, the seed movement has been one of the most important pathways of introduction of *F. circinatum* into new countries (Carey *et al.*, 2005; Coutinho *et al.*, 2007; Berbegal *et al.*, 2013) and forest nurseries (Storer *et al.*, 1998; Wingfield *et al.*, 2008). The

prevention of seed-borne infections is highly important, thus implementing thermotherapy, based on the lethal temperature of the PPC pathogen, could easily be implemented as an environmentally sound and affordable standard in commercial nurseries (Martín-García *et al.*, 2019). However, since there is no measure to eliminate internal infection of seeds (Storer *et al.*, 1998) and the ability of *F. circinatum* to asymptomatically infect seeds of non-host species (*Hypochaeris radicata* L.) (Hernández-Escribano *et al.*, 2018), restrictions on seed imports mainly from areas where the pathogen is known to occur should be considered. Likewise, avoidance of any movement of logs, bark, soil, litter, and wood chips from infested areas would minimize the risk of introducing *F. circinatum* propagules to areas free of disease (EFSA, 2010). Overall, science-based legislation considering new findings on the PPC disease such as the association between the pathogen and grasses and other non-*Pinus* plants should be a priority.

Overall, in the **article I** was reviewed the multiple pathways of spread of *F. circinatum* that make the disease a challenge to prevent. Optimizing the use of the most powerful tools for early detection and diagnosis of *F. circinatum* in seeds, plants, and vector insects are urgently required. The recent discovery of the endophytic colonization of non-coniferous species by *F. circinatum* illustrates the importance of biological and ecological knowledge for the design of effective intervention strategies. In this regard, further studies covering the role of insect vectors or microbiome in the spreading processes of the pathogen are needed. To ensure that the new, science-based strategies to suppress PPC comply with existing practices, regulations, and policies (Vettraino *et al.*, 2018), it is important that these strategies are developed through collaborations between phytosanitary authorities and researchers. Opinion-building actions, such as The Montesclaros Declaration (IUFRO, 2011), advocating the crucial target groups about the risks and measures to mitigate them are also needed to suppress the further spread of *F. circinatum* in nurseries and forests. The establishment of global collaborative networks focused on integrating management approaches and available knowledge is the only means by which we can realistically deal with tree pathogens (Wingfield *et al.*, 2015). In this regard, under the COST Action FP1406 "PINESTRENGTH" a special issue has been produced, including the **article I** of this thesis, of updated information on the biology, ecology, and spread pathways of *F. circinatum*. This Action represents one of the few examples where groups of forest scientists have joined to tackle a large-scale major threat.

The use of mycoviruses as BCAs in the control of *F. circinatum* is being explored. Spanish isolates of the fungus are commonly infected by three mitoviruses (FcMV1, FcMV2-1 and FcMV2-2); however, their effect on the host is still unclear. Mixed infections of single host fungi are frequent and can show a virus/virus interplay such as synergism, neutral with no effect on each other, or antagonism (Chiba and Suzuki, 2015). Traditionally, obtaining different combinations of virus infection in addition to the virus-free isolate on an identical genetic background (isogenic isolates) has been considered as the optimal approach to explore mitovirus-*F. circinatum* interactions. This would rely on obtaining these strains by curing or introduction of these mycoviruses. The lack of simple methods for artificial inoculation of mycoviruses has led many researchers to explore various approaches both physical (growing cultures from hyphal-tips, monosporic cultures, protoplast isolation) and chemical (exposure to antivirals) to eliminate mycoviruses (Kwon *et al.*, 2012; Cao *et al.*, 2019; Tran *et al.*, 2019; Espino-Vázquez *et al.*, 2020). In most cases, these methods were ineffective. Consistently, preliminary results of mycovirus curing assays of infected strains of *F. circinatum* using different approaches, such as thermal treatments or chemotherapy with antivirals (kanamycin, cycloheximide, cAMP

mixed with rifampicin and ribavirin), showed no success after 5 weeks of treatment (unpublished data). However, even when the virus is successfully cured from a virus-infected fungal cell, impairments caused by the treatment make the cured fungal cell inappropriate for reliable investigations (Song *et al.*, 2020). Similarly, the spontaneous loss of a mycovirus could have an impact on the transcriptomic machinery of the host fungus. The study performed in the **article II** is, as far as we know, the first time that a residual effect on the gene expression of a fungus caused by a past infection of a putative mycovirus has been reported. In particular, 12 known genes were identified as differentially expressed (DE) in *F. circinatum* after the spontaneous loss of the mitovirus FcMV1 using a RNA-Seq-based genome-wide expression analysis. Although the value concurs with the number of DE genes reported in response to the coinfection of four mycoviruses (FgV1, FgMV2, FgMV3, and FgMV4) in *Fusarium graminearum* (Lee *et al.*, 2014), other studies found a much larger number of DE genes as a result of mycovirus infections. For example, a total of 683 and 848 genes were DE in *Heterobasidion annosum* and *Phytophthora infectans* by the infection of the partitivirus HetPV13-an1 and PiRV-2, respectively (Vainio *et al.*, 2015; Cai *et al.*, 2019). Likewise, F. graminearum hypovirus 1 (FgHV1) caused a significant alteration in a total of 378 genes (Wang *et al.*, 2016). This could suggest that the residual effect of a past mycovirus infection is limited to a few genes and functions in comparison with those affected in the presence of the mycovirus.

Despite the reduced number of DE genes, several essential functions such as the methionine pathway were presumably upregulated after the loss of FcMV1. Allen *et al*. (2003) found that strains of *Cryphonectria parasitica* infected by the hypovirus CHV1 showed transcript accumulation for genes involved in the methionine pathway. Similar results were also observed in a study of yeast–totivirus interaction, where a fungal response to the viral infection was suggested (Mcbride *et al.*, 2013). Due to its central role in metabolism, it could be predicted that the increment in the expression of genes involved in the methionine pathway would be affecting many metabolic and physiologic processes, such as protein synthesis and membrane integrity (Allen *et al.*, 2003). Moreover, the induction of genes involved in the organization and formation of structures that serve as tracks for intracellular transport in the process of cytokinesis, suggests that the fungal cultures that had lost the virus have a higher cellular development than the virus-free isolate. In this sense, it is hypothesized that the oxidative burst represented by the upregulated genes involved in the response and regulation of oxidative stress, could be related to the development of the colony, inducing hyphae and macroconidium formation. This would be consistent with a preliminary study of FcMV1 infection, where the presence of the mitovirus caused a slight increase in *F. circinatum* virulence (Muñoz-Adalia *et al.*, 2016). Therefore, the use of isolates that have undergone mycovirus loss or have been subjected to invasive treatments to eliminate them is not recommended for a proper exploration of mycovirus-fungus interactions. This makes it imperative to find a method for securing an artificial virus-infected *F. circinatum* strain.

The results of the **article II** have also demonstrated the use of transcriptomics as an accurate method for mycovirus detection. This was reflected in the unexpected detection of FcMV2-1 in all the sequenced libraries, which was previously undetected using the conventional PCR method and its specific primers (Vainio *et al.*, 2015). Similar findings also occurred in a study with *H. annosum*, where cryptic mitovirus infections were not detected by dsRNA extraction but by RNA deep sequencing and RNA-Seq analysis (Vainio *et al.*, 2015, 2018). If the number of copies of the target molecule is very scarce, the use of real-time PCR or NGS technologies is more sensitive and, therefore, recommended (Barba *et al.*, 2014; Thekke Veetil *et al.*, 2016; Zhang and Vrient, 2020). In fact, deep sequencing of

virus-derived small RNA (resulting from antiviral RNA silencing) and fungal metatranscriptomes have allowed to identify the virome of various pathogenic fungi (Marzano *et al.*, 2016), including nine RNA viruses in ten strains of four different *Heterobasidion* spp. (Vainio *et al.*, 2015), 14 mycoviruses in four isolates of *Entoleuca* sp. (Velasco *et al.*, 2019) and a large variety of mycovirus species in three isolates of *Botrytis* spp. (Donaire and Ayllón, 2017). Additionally, RNA-Seq technique has been used for the efficient discovery of novel mycoviruses, including ten mycoviruses in five *Sclerotinia sclerotiorum* isolates (Khalifa *et al.*, 2016) and 57 viruses in 84 isolates of the same fungal species (Mu *et al.*, 2018), as well as 17 viruses in a *Fusarium poae* isolate (Osaki *et al.*, 2016), ten mycoviruses in *F. sacchari* and *F. andiyazi* strains (Yao *et al.*, 2020) and a large number of mycoviruses in *Rhizoctonia solani* isolates (Picarelli *et al.*, 2019). Therefore, virome sequencing can be used to characterize potential mycoviruses for biocontrol of fungal diseases in plants.

As noted above, the study of biological control of plant diseases is gaining interest as an important part of an integrated management approach. Since new legislative provisions give priority to non-chemical methods of plant protection, the environmentally sensitive methods and long-term solutions are clearly needed. In this regard, the most effective and eco-friendly approach to disease prevention involves breeding plants for resistance (Dodds and Rathjen, 2010). There is a large difference in resistance to *F. circinatum* among its multiple hosts, which provides a useful opportunity for the management of the PPC disease. Furthermore, the rapid growth of "omics" technologies and the availability of genomic resources for forest trees allow the exploration of different molecular responses in hosts with different degrees of susceptibility. Candidate genes associated with tree resistance can be inferred using functional genomics, e.g. transcriptomics, if these genes are differentially expressed under infection with the disease-causing pathogen. The **article III** aimed to understand the molecular processes that underlie the resistance of *P. pinea* and the susceptibility of *P. radiata* against *F. circinatum* using a dual RNA-Seq pipeline (Figure 8.2). Thus, the sensitivity of high-throughput sequencing allowed the simultaneous detection of both pine and fungus transcripts at the time of infection, although fungal RNA contributed to a low percentage of reads (~1.36%). This study was carried out at an early stage of the infection (4 dpi), so the relative fungal to pine biomass was not expected to be very high according to Martín-Rodrigues *et al.*, (2013) results.

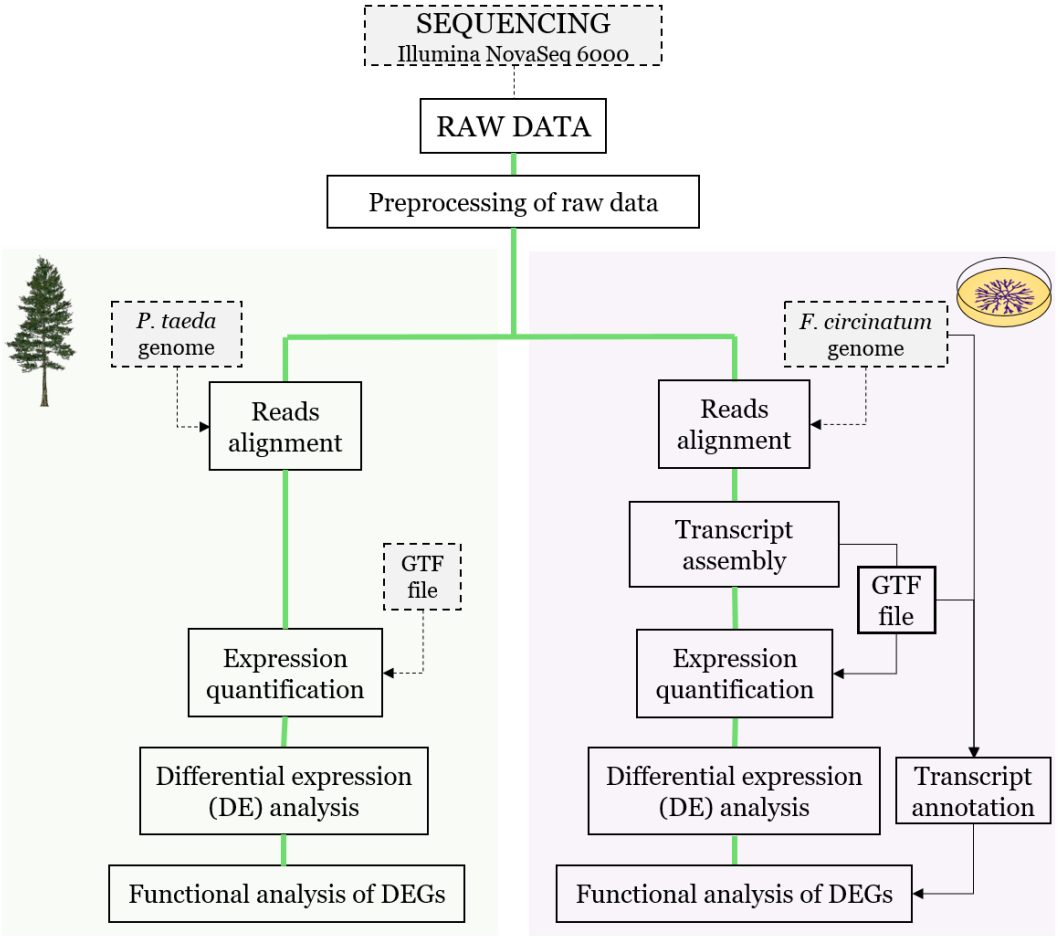

Figure 8.2. Simplified pipeline of the dual RNA-Seq experiment of **article III**. GTF (General Transfer Format) refers to the annotated file of a genome. The dashed lines enclose the files that have not been generated during this bioinformatics pipeline.

The early stage of infection is critical since the disease occurs when the pathogen is not detected by the plant (or is detected late) and, therefore, defensive responses have not yet been induced. Here the number of differentially expressed genes (DEGs) in *P. pinea* (1,822) was vastly higher than in *P. radiata* (371), a significant difference in transcriptional reprogramming by the pathogen infection that could help to find the resistance mechanisms in *P. pinea*. In previous studies, a marked trend has been observed in the increase in DEGs as the disease was progressing (Adomas *et al.*, 2007; Carrasco *et al.*, 2017; Visser *et al.*, 2019; Hernandez-Escribano *et al.*, 2020). Assuming that the number of DEGs is correlated with the phase of the host response to the infection, it could be hypothesized that *P. radiata* presents a delayed induction of defense activation, which could facilitate the entrance and spread of the pathogen inside the plant. By contrast, a degree of overlap existed between the resistant and susceptible host with regard to the enriched GO terms by the defense response mechanisms (Figure 8.3). This suggests a similar transcriptional change by *F. circinatum* infection in both species, but a lower number of genes involved in the response of the susceptible species. A closer look at the DEGs involved in pathogen perception in *P. pinea* revealed a large number of PRR containing domains such as LRR, lysine motifs (LysM), and lectin domain, as well as several R genes that were mainly up-regulated. On the contrary, the near absence of PRR induction in *P. radiata* and the lack of up-regulation of MAPK and CDPK transcripts could explain the weak downstream defense signaling.

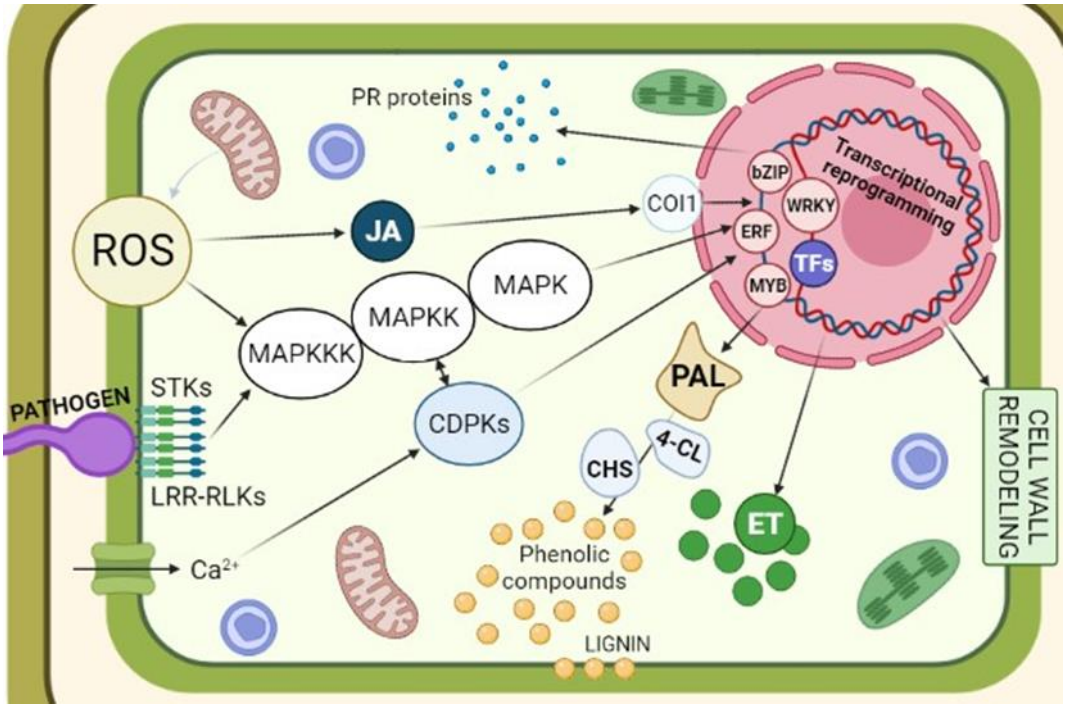

Figure 8.3. Model illustrating the main molecular mechanisms triggered in *Pinus* spp. infected with *F. circinatum*. The perception of the fungus by pine induce many defense mechanisms, signaling cascades, and stress response. LRR-RLKs: leucine-rich repeat receptor-like kinases; STKs: receptor-like serine/threonine-protein kinases; MAPKs: mitogen-activated protein kinases; CDPKs: Ca^{2+}-dependent protein kinases; ROS: reactive oxygen species; PR proteins: pathogenesis-related proteins; JA: jasmonic acid; COI1: coronatine insensitive 1; ET: ethylene; PAL: phenylalanine ammonia-lyase; 4-CL: 4-coumarate; CHS: chalcone synthase; TFs: Transcription factors.

The phytohormones jasmonic acid (JA) and ethylene (ET) seem to play an important role in the defense of pine trees. The PPC pathogen infection could have activated the JMT genes that catalyze the formation of methyl jasmonate (MeJA) from JA in both species. The pre-treatment of MeJA as an elicitor to induce resistance in young *P. pinaster* and *P. patula* seedlings was unsuccessful in protection against *F. circinatum* (Vivas *et al.*, 2012; Fitza *et al.*, 2013). In this regard, concentration levels and application strategy remain to be optimized, as some positive responses could be observed after MeJa application and, according to our results, this component could play an important role in the induction of response defenses in pine, such as terpene synthases and lipoxygenases (LOX) biosynthesis. However, it should be noted that the exogenous application of MeJA has been shown to increase the density of resin ducts in *P. pinaster* (Vivas *et al.*, 2012). Although these structures are considered a defense mechanism of *P. radiata* in advanced stages of PPC (Martín-Rodrigues *et al.*, 2013), their increase could facilitate vertical colonization of the stem by *F. circinatum* resulting in susceptibility (Amaral *et al.*, 2019). In fact, JA levels significantly decreased under PPC pathogen infection in the resistant *P. pinea* (Amaral *et al.*, 2019). In parallel, the down-regulation of coronatine insensitive 1 (COI1) together with the high induction of jasmonate ZIM-domain (JAZ) genes in *P. pinea* could denote a suppression of JA signaling at 4 dpi, as observed in *P. pinaster* infected with *F. circinatum* at 5 and 10 dpi (Hernández-Escribano *et al.*, 2020). Although Hernández-Escribano *et al.* (2020) hypothesized that *F. circinatum* may target and block JA signaling by COI1 suppression, the down-regulation of this gene could be a defense mechanism of *P. pinaster* and *P. pinea* to avoid pathogen spread. On the other hand, genes involved in the ET pathway were induced in both species. Notably, the responsive to antagonist 1 (RAN1)

gene, whose lack of expression leads to the constitutive expression of ET response (Hirayama *et al.*, 1999), was only induced in *P. pinea*, indicating a coordinated role of ET in the defense of this species.

Remarkably, the gene encoding phenylalanine ammonia-lyase (PAL), the key regulatory enzyme in altering the biosynthesis and accumulation of secondary metabolites and lignin, was not present in the DEGs of *P. radiata* at 4 dpi, as previously reported in the susceptible species *P. patula* at one dpi (Visser *et al.*, 2015). In contrast, PAL and downstream genes encoding for proteins of the flavonoid pathway were actively and widely induced by the pathogen in *P. pinea*. Interestingly, symptomatic *P. radiata* and *P. pinea* (10 dpi) showed an inverse pattern; where the change in PAL transcript abundance by *F. circinatum* infection was much lower in *P. pinea* than in *P. radiata* (Amaral *et al.*, 2019). Therefore, the prompt induction of the gene encoding PAL could be related to a solid defense response and, consequently, increased resistance to the PPC pathogen. Recent studies have reported the important role of flavonoid pathway components in the resistance of *Picea abies* to *Heterobasidion* infection (Nemesio-Gorriz *et al.*, 2016; Nemesio-Gorriz *et al.*, 2017; Kovalchuk *et al.*, 2019), being one of the main induced pathways in asymptomatic trees. Moreover, the moderate resistant *P. pinaster* challenged with *F. circinatum* showed that the induction of flavonoid biosynthesis was maintained over time (until 10 dpi; Hernández-Escribano *et al.*, 2020). Pathogenesis-related (PR) proteins including PR1, PR2, PR3, PR5, PR9, PR10, and PR14, which show strong antifungal and other antimicrobial properties, were also widely up-regulated in both pine species. However, a gene that encodes for β-1,3-endoglucanase (PR2) protein that greatly enhances the antifungal properties of chitinases (PR3) (Collinge *et al.*, 1993), was only induced in *P. pinea*, suggesting a less effective response of *P. radiata* at an early stage of the disease. On the other hand, some peroxidases (PR9) were hypothesized to be effector target of *F. circinatum* as previously reported in several *Phytophthora* species infecting *Carica papaya* (Moy *et al.*, 2004; Restrepo *et al.*, 2005; Porter *et al.*, 2009). Some of these PR9 were down-regulated in *P. pinea* in accordance to the results of interactions of *P. pinaster-F. circinatum* at 10 dpi (Hernández-Escribano *et al.*, 2020) and *Eucalyptus nitens-Phytophthora cinnamomi* at 5 dpi (Meyer *et al.*, 2016).

The results of the **article III** also revealed an important transcriptional reprogramming toward the reinforcement and lignification of the cell walls of *P. pinea*. Inoculation tests of *P. pinea* seedlings of the same age challenged by *F. circinatum* showed that the smaller plants were highly susceptible to the pathogen, while more developed and therefore more lignified plants showed resistance, suggesting that the level of lignification is an important mechanism of resistance against PPC (J. Martín-García, personal communication). A strong regulation of cell wall modification was shown in *P. pinea* through inhibition of genes associated with wall loosening. Genes encoding for XET enzymes, which are involved in the metabolism of xyloglucan, were detected as other potential *F. circinatum* target genes as previously reported in apple fruit infected by *Penicillium expansum* (Muñoz-Bertomeu and Lorences, 2014). The activity of the pectinesterases and their inhibitors (PMEI), implicated in the resistance of several plant species (Lionetti *et al.*, 2012), appeared to be perfectly coordinated in the resistant species. Additionally, the lignin biosynthesis pathway was also induced in *P. pinea*. Unlike *P. radiata*, these results suggest a quick *P. pinea* response in the reinforcement of cell walls by lignification. This strong reaction may be behind the activation of several genes encoding for cell wall-degrading enzymes (CWDE) such as laccases and glycoside hydrolases in *F. circinatum* when infecting the resistant species. Interestingly, *F. circinatum* showed different behavior depending on the infected pine tree. Hence, when the pathogen was infecting *P. radiata*, a noticeable

enrichment of DEGs in nutrient transport, especially of nitrogen sources including ammonium and amino acids, was observed in the fungus. Likewise, genes encoding proteins with roles in nutrient transport were highly up-regulated at the early stages of *F. oxysporum* colonization of susceptible seedlings of *Medicago truncatula* (Thatcher *et al.*, 2016). It has been demonstrated that the nitrogen availability in fungi modulates its growth, differentiation, and the biosynthesis of many secondary metabolites (Tudzynski, 2014); accordingly, pathogens will cause less disease in plants where nitrogen is limiting (Snoeijers *et al.*, 2000). Moreover, a general accumulation of amino acids, conceivably associated with plant stress response and/or pathogen hijacking of host metabolism, was reported in the susceptible *P. radiata* upon *F. circinatum* inoculation (Amaral *et al.*, 2019). Therefore, the enrichment of genes related to the uptake of nitrogen by *F. circinatum* infecting *P. radiata* could provide the pathogen a competitive advantage in the plant-pathogen interaction.

Additionally, the transcriptomic data was combined with a physiological approach in order to further support the results obtained at the transcriptional level, considering the post-transcriptional regulation. However, no significant changes in the physiological analysis were found upon pathogen infection. This may be because the timing of sampling was set too early to observe physiological changes, which are triggered by molecular responses. In fact, in similar studies, most significant changes in physiological parameters of *P. pinea*, *P. pinaster* and *P. radiata* seedlings infected by *F. circinatum* occurred once they started to develop disease symptoms, at 64, 17 and 10 dpi respectively (Amaral *et al.*, 2019, 2020).

The development of NGS technologies has revealed that the transcriptome is more complex and extensive than previously appreciated. Transcription, which involves mRNA processing, such as splicing and polyadenylation, transport and degradation, is regulated by RNA-binding proteins and non-coding RNAs (Buccitelli and Selbach, 2020). Among these ncRNAs, lncRNAs are gaining general attention as gene regulatory factors due to their participatory roles in important molecular processes including stress responses in plants (Waseem *et al.*, 2021). To my knowledge, this thesis includes the first analysis of lncRNAs responsive to biotic stress in conifers. The **article IV** presents a comprehensive genome-wide analysis of *P. radiata* lncRNAs and identifies those lncRNAs involved in defense reactions against *F. circinatum* at an early stage of the disease. For that, a strand-specific RNA-Seq approach with a high coverage sequencing (up to 84 million reads per sample) allowed the identification of 13,312 lncRNA transcripts in *P. radiata*. This methodological strategy was able to cope with the low levels of lncRNAs expression and the detection of lncRNAs transcribed from the opposite DNA strand of coding or non-coding genes, known as long non-coding natural antisense transcripts (lncNATs). *P. radiata* lncRNAs were shorter in terms of overall length, contained fewer exons being also shorter in length, and showed lower expression and GC content than the mRNAs, genomic features consistent with those previously characterized in other organisms (Cabili *et al.*, 2011).

A total of 164 *F. circinatum*-responsive lncRNAs were identified, which were mainly composed of intergenic lncRNAs. This was consistent with previous analyses where the number of lncRNAs in response to a biotic stress was comparable, and intergenic lncRNAs were also the most abundant responsive transcripts (Joshi *et al.*, 2016; Li *et al.*, 2017; Wang *et al.*, 2017; Fan *et al.*, 2018). The pattern appears to follow the same trend in conifer trees. Several differentially expressed lncRNAs of *P. radiata* inoculated by *F. circinatum* seem to function as decoys by sequestering RNA-binding proteins (RBP),

miRNAs or chromatin-modifying complexes, a conserved mechanism of action of the lncRNAs (Wang and Chang, 2011). Therefore, the reprogramming exerted by the infection of *F. circinatum* on pine transcription affects not only the protein-coding genes, but also the non-coding part of the genome.

As pointed out in **article III**, *F. circinatum* infection causes drastic transcriptomic reprogramming by inducing genes involved in signal perception and transduction, biosynthesis of defense hormone and secondary metabolites, and cell wall reinforcement and lignification. The functional analysis also indicated an association of the differentially expressed lncRNAs with these functional groups, including biological processes such as cell wall modification and signaling of the abscisic acid (ABA), ET and cytokinin hormones. Several studies have pointed to lncRNAs as participants in the complex network of hormone regulation (Li *et al.*, 2017; Wang *et al.*, 2017; Zhang *et al.*, 2018; Yu *et al.*, 2020; Feng *et al.*, 2021). Genes involved in phytohormone pathways have already been reported to play an important role in the defense against *F. circinatum* infection, and the results of **article IV** showed an implication of lncRNAs in these pathways. In particular, lncRNAPiRa.85000.6 potentially targets an ET receptor (*ETR2*) gene involved in the signal transduction pathway of ET, and lncRNAPiRa.47042.1 could influence the expression of a PP2C gene that negatively regulates ABA responses. Interestingly, both gene targets seem to be involved in the susceptibility of *P. radiata*. *ETR2* has been found to be induced by *F. circinatum* infection in the moderate resistant specie *P. pinaster* at 5 and 10 dpi (Hernández-Escribano *et al.*, 2020), but not in *P. radiata* at 2, 6 or 12 dpi (Carrasco *et al.*, 2017). These results were supported in *P. radiata* infected by *F. circinatum* at 4 dpi by **article III** and **IV**. On the other hand, the accumulation of PP2C protein in *P. radiata* has found to be positively correlated with ABA biosynthesis (Amaral *et al.*, 2020, 2021), network in which the lncRNA identified in the **article IV** could have a role. Therefore, when studying the role of phytohormones in the pine-*F. circinatum* interaction, in addition to gene transcription, the regulatory role of lncRNAs must be addressed.

Besides phytohormones, plant-signaling molecules such as protein kinases or reactive oxygen species (ROS) are essential for an adequate defense response. As reported in rice, where a lncNAT (LAIR) induced the expression of a receptor kinase (Wang *et al.*, 2018), two genes with predicted functions in receptor-like kinase were predicted to be *cis*-regulated by *P. radiata* lncRNAs, one of them being a lncNAT. A proteomics study of PPC interactions revealed an accumulation of chloroplastic redox proteins glutathione S-transferases (GSTs) in *P. radiata* (Amaral *et al.*, 2021), which concurs with our transcriptomics results that showed an up-regulation of GST-encoded genes after *F. circinatum* inoculation. In addition, lncRNAPiRa.64704.1 was predicted to *cis*-regulate a gene encoding GST. This finding together with the possible interaction between lncRNAPiRa.19024.1 with a non-symbiotic hemoglobin 1, seem to indicate that lncRNAs could be also involved in the cell detoxification after an oxidative burst provoked by a fungal infection.

As remarked in **article III**, the reinforcement and lignification of the cell wall is a highly important defensive system of pines. The pectinesterase activity, which appeared to be fine-tuned in *P. pinea* as opposed to *P. radiata* (**article III**), seems to be potentially targeted by two nearby lncRNAs in *P. radiata* (**article IV**). Moreover, the pectinesterase activity was also enriched in the *trans*-regulation analysis of lncRNAs and mRNAs. The transcriptional regulation of the enzymes involved in pectinesterase activity could be related to the susceptibility of *P. radiata* and would be worth further investigation. Other genes involved in cell wall modification, such as a gene encoding for 4CL3, were also

predicted to be *cis*-regulated by lncRNAs. The potential function of these transcripts in wood formation, including lignin biosynthesis, has been previously observed in different plant species such as *Populus* (Chen *et al.*, 2015; Shi *et al.*, 2017), cotton (Wang *et al.*, 2015) or *Paulownia tomentosa* (Wang *et al.*, 2017), and confirmed by our results.

Overall, our results evidenced the necessity for collaboration between phytosanitary authorities and researchers in order to develop science-based strategies to suppress PPC. Current legislation does not meet the necessary requirements to stop new introductions of *F. circinatum*, since it does not consider new findings on the disease, including the presence of the fungus in alternative hosts to pines or the endophytic lifestyle of the pathogen. The use of biological control demands a deep knowledge of the ecological behavior of the organisms involved and those of the environment, which requires detailed investigations in the laboratory and greenhouse. For this reason, it is still rarely used for pathogens of forest trees. In the case of the mycoviruses, although the development of NGS is allowing a rapid and straightforward detection of the virome of any sample, their manipulation in the laboratory results labor-intensive. The optimization of transfection experiments to introduce mycoviruses to natively virus-free *F. circinatum* strains are still needed in order to obtain mycovirus-free lines for further studies. Genetic resistance potentially provides an invaluable tool within an integrated management framework. The use of biotechnological tools in breeding programs, including genetic engineering, requires prior genomic and transcriptomic knowledge to identify genes of interest. Highly expressed genes in *P. pinea* associated with disease resistance have been identified in this thesis, which may be candidates to be considered in breeding programs. In addition, evidence has been provided for the implication of numerous lncRNAs in the defense of *P. radiata*. The experimental validation by using knockouts, RNA interference (if possible) or overexpression of these lncRNAs would be needed to support our hypotheses.

References

Adomas, A., Heller, G., Li, G., et al. (2007) Transcript profiling of a conifer pathosystem: response of *Pinus sylvestris* root tissues to pathogen (*Heterobasidion annosum*) invasion. *Tree Physiol.* **27**, 1441–1458.

Allen, T.D., Dawe, A.L. and Nuss, D.L. (2003) Use of cDNA Microarrays To Monitor Transcriptional Responses of the Chestnut Blight Fungus *Cryphonectria parasitica* to Infection by Virulence-Attenuating Hypoviruses. *Eukaryot. Cell* **2**, 1253–1265.

Amaral, J., Correia, B., António, C., Rodrigues, A.M., Gómez-Cadenas, A., Valledor, L., Hancock, R.D., Alves, A. and Pinto, G. (2019) Pinus susceptibility to pitch Canker triggers specific physiological responses in symptomatic plants: An integrated approach. *Front. Plant Sci.* **10**, 509.

Amaral, J., Correia, B., Escandón, M., *et al*. (2020) Temporal physiological response of pine to *Fusarium circinatum* infection is dependent on host susceptibility level: the role of ABA catabolism. *Tree Physiol.* **tpaa143**.

Amaral, J., Pinto, G., Flores-Pacheco, J.A., Díez, J.J., Cerqueira, A., Monteiro, P., Gómez-Cadenas, A., Alves, A. and Martín-García, J. (2019) Effect of *Trichoderma viride* pre-inoculation in pine species with different levels of susceptibility to *Fusarium circinatum*: physiological and hormonal responses. *Plant Pathol.* **68**, 1645–1653.

Amaral, J., Lamelas, L., Valledor, L., Castillejo, M.Á., Alves, A. and Pinto, G. (2021) Comparative proteomics of *Pinus–Fusarium circinatum* interactions reveal metabolic clues to biotic stress resistance. *Physiol. Plant.* **173**, 1–13.

Anderson, P.K., Cunningham, A.A., Patel, N.G., Morales, F.J., Epstein, P.R. and Daszak, P. (2004) Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends Ecol. Evol.* **19**, 535–544.

Barba, M., Czosnek, H. and Hadidi, A. (2014) Historical Perspective, Development and Applications of Next-Generation Sequencing in Plant Virology. *Viruses* **6**, 106–136.

Berbegal, M., Pérez-Sierra, A., Armengol, J. and Grünwald, N.J. (2013) Evidence for multiple introductions and clonality in Spanish populations of *Fusarium circinatum*. *Phytopathology* **103**, 851–861.

Blumenstein, K., Albrectsen, B.R., Martín, J.A., Hultberg, M., Sieber, T.N., Helander, M. and Witzell, J. (2015) Nutritional niche overlap potentiates the use of endophytes in biocontrol of a tree disease. *BioControl* **60**, 655–667.

Buccitelli, C. and Selbach, M. (2020) mRNAs, proteins and the emerging principles of gene expression control. *Nat. Rev. Genet.* **21**, 630–644.

Cabili, M., Trapnell, C., Goff, L., Koziol, M., Tazon-Vega, B., Regev, A. and Rinn, J.L. (2011) Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes Dev.* **25**, 1915–1927.

Cai, G., Fry, W.E. and Hillman, B.I. (2019) PiRV-2 stimulates sporulation in *Phytophthora infestans*. *Virus Res.* **271**.

Carey, W.A., Oak, S.W. and Enebak, S.A. (2005) Pitch canker ratings of longleaf pine clones correlate with *Fusarium circinatum* infestation of seeds and seedling mortality in containers. *For. Pathol.* **35**, 205–212.

Carrasco, A., Wegrzyn, J.L., Durán, R., Fernández, M., Donoso, A., Rodriguez, V., Neale, D. and Valenzuela, S. (2017) Expression profiling in *Pinus radiata* infected with *Fusarium circinatum*. *Tree Genet. Genomes* **13**.

Chen, J., Quan, M. and Zhang, D. (2015) Genome-wide identification of novel long non-coding RNAs in *Populus tomentosa* tension wood, opposite wood and normal wood xylem by RNA-seq. *Planta* **241**, 125–143.

Chiba, S. and Suzuki, N. (2015) Highly activated RNA silencing via strong induction of dicer by one virus can interfere with the replication of an unrelated virus. *Proc. Natl. Acad. Sci.* **112**, E4911–E4918.

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U. and Vad, K. (1993) Plant chitinases. *Plant J.* **3**, 31–40.

Coutinho, T.A., Steenkamp, E.T., Mongwaketsi, K., Wilmot, M. and Wingfield, M.J. (2007) First outbreak of pitch canker in a South African pine plantation. *Australas. PLANT Pathol.* **36**, 256–261.

Dodds, P.N. and Rathjen, J.P. (2010) Plant immunity: towards an integrated view of plant–pathogen interactions. *Nature* **11**, 539–548.

Donaire, L. and Ayllón, M.A. (2017) Deep sequencing of mycovirus-derived small RNAs from *Botrytis* species. *Mol. Plant Pathol.* **18**, 1127–1137.

Drenkhan, R., Ganley, B., Martín-García, J., *et al*. (2020) Global Geographic Distribution and Host Range of *Fusarium circinatum*, the Causal Agent of Pine Pitch Canker. *Forests* **11,** 724.

EFSA (2010) Risk assessment of *Gibberella circinata* for the EU territory and identification and evaluation of risk management options. *EFSA J.* **8**, 1620.

Elvira-Recuenco, M., Iturritxa, E. and Raposo, R. (2015) Impact of seed transmission on the infection and development of pitch canker disease in *Pinus radiata*. *Forests* **6**, 3353–3368.

Fan, G., Cao, Y. and Wang, Z. (2018) Regulation of long noncoding RNAs responsive to phytoplasma infection in *Paulownia tomentosa*. *Int. J. Genomics* **2018**, 16.

Feng, S., Fang, H., Liu, X., Dong, Y., Wang, Q. and Yang, K.Q. (2021) Genome-wide identification and characterization of long non-coding RNAs conferring resistance to *Colletotrichum gloeosporioides* in walnut (*Juglans regia*). *BMC Genomics* **22**, 15.

Fitza, K.N.E., Payn, K.G., Steenkamp, E.T., Myburg, A.A. and Naidoo, S. (2013) Chitosan application improves resistance to *Fusarium circinatum* in *Pinus patula*. *South African J. Bot.* **85**, 70–78.

Gao, F.K., Dai, C.C. and Liu, X.Z. (2010) Mechanisms of fungal endophytes in plant protection against pathogens. *African J. Microbiol. Res.* **4**, 1346–1351.

Göthlin, E., Schroeder, L.M. and Lindelöw, A. (2000) Attacks by *Ips typographus* and *Pityogenes chalcographus* on Windthrown Spruces (*Picea abies*) During the Two Years Following a Storm Felling. *Scand. J. For. Res.* **15**, 542–549.

Hernández-Escribano, L., Iturritxa, E., Elvira-Recuenco, M., Berbegal, M., Campos, J.A., Renobales, G., Garcia, I. and Raposo, R. (2018) Herbaceous plants in the understory of a pitch canker-affected *Pinus radiata* plantation are endophytically infected with *Fusarium circinatum*. *Fungal Ecol.* **32**, 65–71.

Hernández-Escribano, L., Visser, E.A., Iturritxa, E., Raposo, R. and Naidoo, S. (2020) The transcriptome of *Pinus pinaster* under *Fusarium circinatum* challenge. *BMC Genomics* **21**, 1–18.

Hirayama, T., Kieber, J.J., Hirayama, N., *et al*. (1999) RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in *Arabidopsis*. *Cell* **97**, 383–393.

Ioos, R., Aloi, F., Piškur, B., *et al*. (2019) Transferability of PCR-based diagnostic protocols: An international collaborative case study assessing protocols targeting the quarantine pine pathogen *Fusarium circinatum*. *Sci. Rep.* **9**, 8195.

IUFRO (2011) The Montesclaros Declaration. In IUFRO The Global Network for Forest Science and Cooperation., Cantabria, Spain.

Joshi, R.K., Megha, S., Basu, U., Rahman, M.H. and Kav, N.N.V. (2016) Genome Wide Identification and Functional Prediction of Long Non-Coding RNAs Responsive to *Sclerotinia sclerotiorum*

Infection in *Brassica napus*. *PLoS One* **11**, e0158784.

Khalifa, M.E., Varsani, A., Ganley, A.R.D. and Pearson, M.N. (2016) Comparison of Illumina *de novo* assembled and Sanger sequenced viral genomes: A case study for RNA viruses recovered from the plant pathogenic fungus *Sclerotinia sclerotiorum*. *Virus Res.* **219**, 51–57.

Kovalchuk, A., Zeng, Z., Ghimire, R.P., *et al*. (2019) Dual RNA-seq analysis provides new insights into interactions between Norway spruce and necrotrophic pathogen *Heterobasidion annosum* s.l. *BMC Plant Biol.* **19**, 2.

Lee, K.M., Cho, W.K., Yu, J., Son, M., Choi, H., Min, K., Lee, Y.W. and Kim, K.H. (2014) A comparison of transcriptional patterns and mycological phenotypes following infection of *Fusarium graminearum* by four mycoviruses. *PLoS One* **9**.

Leitão, F., Pinto, G., Amaral, J., Monteiro, P. and Henriques, I. (2021) New insights into the role of constitutive bacterial rhizobiome and phenolic compounds in two *Pinus* spp. with contrasting susceptibility to pine pitch canker. *Tree Physiol.* **tpab119.**

Li, W., Li, C., Li, S. and Peng, M. (2017) Long noncoding RNAs that respond to *Fusarium oxysporum* infection in "Cavendish" banana (*Musa acuminata*). *Sci. Rep.* **7**, 1–13.

Lionetti, V., Cervone, F. and Bellincampi, D. (2012) Methyl esterification of pectin plays a role during plant-pathogen interactions and affects plant resistance to diseases. *J. Plant Physiol.* **169**, 1623–1630.

Martín-García, J., Lukacevicova, A., Flores-Pacheco, J.A., Diez, J.J. and Dvorak, M. (2018) Evaluation of the Susceptibility of Several Czech Conifer Provenances to *Fusarium circinatum*. *Forests* **9**, 72.

Martín-García, J., Zas, R., Solla, A., *et al*. (2019) Environmentally-friendly methods for controlling pine pitch canker. *Plant Pathol.* **68**, 843–860.

Martín-Rodrigues, N., Espinel, S., Sánchez-Zabala, J., Ortíz, A., González-Murua, C. and Duñabeitia, M.K. (2013) Spatial and temporal dynamics of the colonization of *Pinus radiata* by *Fusarium circinatum*, of conidiophora development in the pith and of traumatic resin duct formation. *New Phytol.* **198**, 1215–1227.

Marzano, S.-Y.L., Nelson, B.D., Ajayi-Oyetunde, O., Bradley, C.A., Hughes, T.J., Hartman, G.L., Eastburn, D.M. and Domier, L.L. (2016) Identification of diverse mycoviruses through metatranscriptomics characterization of the viromes of five major fungal plant pathogens. *J. Virol.* **90**, 6846.

Mcbride, R.C., Boucher, N., Park, D.S., Turner, P.E., Townsend, J.P. and Townsend, J. (2013) Yeast response to LA virus indicates coadapted global gene expression during mycoviral infection. *FEMS Yeast Res* **13**, 162–179.

Meyer, F.E., Shuey, L.S., Naidoo, Sitha, Mamni, T., Berger, D.K., Myburg, A.A., Berg, N. van den and Naidoo, Sanushka (2016) Dual RNA-sequencing of *Eucalyptus nitens* during *Phytophthora cinnamomi* challenge reveals pathogen and host factors influencing compatibility. *Front. Plant Sci.* **7**, 1–15.

Moy, P., Qutob, D., Chapman, B.P., Atkinson, I. and Gijzen, M. (2004) Patterns of gene expression upon infection of soybean plants by *Phytophthora sojae*. *Mol. Plant-Microbe Interact.* **17**, 1051–1062.

Mu, F., Xie, J., Cheng, S., *et al*. (2018) Virome characterization of a collection of *Sclerotinia sclerotiorum* from Australia. *Front. Microbiol.* **8**, 2540.

Muñoz-Adalia, E.J., Flores-Pacheco, J.A., Martínez-Álvarez, P., Martín-García, J., Fernández, M. and Diez, J.J. (2016) Effect of mycoviruses on the virulence of *Fusarium circinatum* and laccase activity. *Physiol. Mol. Plant Pathol.* **94**, 8–15.

Muñoz-Bertomeu, J. and Lorences, E.P. (2014) Changes in xyloglucan endotransglucosylase/hydrolase (XTHs) expression and XET activity during apple fruit

infection by *Penicillium expansum* Link. A. *Eur. J. Plant Pathol.* **138**, 273–282.

Nemesio-Gorriz, M., Blair, P.B., Dalman, K., Hammerbacher, A., Arnerup, J., Stenlid, J., Mukhtar, S.M. and Elfstrand, M. (2017) Identification of Norway Spruce MYB-bHLH-WDR Transcription Factor Complex Members Linked to Regulation of the Flavonoid Pathway. *Front. Plant Sci.* **8**, 305.

Nemesio-Gorriz, M., Hammerbacher, A., Ihrmark, K., Källman, T., Olson, Å., Lascoux, M., Stenlid, J., Gershenzon, J. and Elfstrand, M. (2016) Different Alleles of a Gene Encoding Leucoanthocyanidin Reductase (PaLAR3) Influence Resistance against the Fungus *Heterobasidion parviporum* in *Picea abies*. *Plant Physiol.* **171**, 2671–2681.

Osaki, H., Sasaki, A., Nomiyama, K. and Tomioka, K. (2016) Multiple virus infection in a single strain of *Fusarium poae* shown by deep sequencing. *Virus Genes* **52**, 835–847.

Picarelli, M.A.S.C., Forgia, M., Rivas, E.B., Nerva, L., Chiapello, M., Turina, M. and Colariccio, A. (2019) Extreme diversity of mycoviruses present in isolates of *Rhizoctonia solani* AG2-2 LP from *Zoysia japonica* from Brazil. *Front. Cell. Infect. Microbiol.* **9**.

Pintos, C., González, B., Mansilla, J.P., Aguín, O. and Salinero, M.C. (2005) *Fusarium circinatum* Nirenberg & O´Donell: Teleomorfo *Gibberella circinata*. *Foresta* **31**, 9–11.

Porter, B.W., Zhu, Y.J. and Christopher, D.A. (2009) *Carica papaya* genes regulated by *Phytophthora palmivora*: A model system for genomic studies of compatible *Phytophthora*-plant interactions. *Trop. Plant Biol.* **2**, 84–97.

Prospero, S., Botella, L., Santini, A. and Robin, C. (2021) Biological control of emerging forest diseases: How can we move from dreams to reality? *For. Ecol. Manage.* **496**, 119377.

Restrepo, S., Myers, K.L., Pozo, O. Del, Martin, G.B., Hart, A.L., Buell, C.R., Fry, W.E. and Smart, C.D. (2005) Gene profiling of a compatible interaction between *Phytophthora infestans* and *Solanum tuberosum* suggests a role for carbonic anhydrase. *Mol. Plant-Microbe Interact.* **18**, 913–922.

Shi, W., Quan, M., Du, Q. and Zhang, D. (2017) The Interactions between the Long Non-coding RNA NERDL and Its Target Gene Affect Wood Formation in *Populus tomentosa*. *Front. Plant Sci.* **8**, 1035.

Snoeijers, S.S., Pérez-García, A., Joosten, M.H.A.J. and Wit, P.J.G.M. (2000) The effect of nitrogen on disease development and gene expression in bacterial and fungal plant pathogens. *Eur. J. Plant Pathol.* **106**, 493–506.

Song, H.-Y., Kim, N., Kim, D.-H. and Kim, J.-M. (2020) The PoV mycovirus affects extracellular enzyme expression and fruiting body yield in the oyster mushroom, *Pleurotus ostreatus*. *Sci. Rep.* **10**, 1094.

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

Swett, C.L. and Gordon, T.R. (2017) Exposure to a pine pathogen enhances growth and disease resistance in *Pinus radiata* seedlings. *For. Pathol.* **47**.

Swett, C.L., Kirkpatrick, S.C. and Gordon, T.R. (2016) Evidence for a Hemibiotrophic Association of the Pitch Canker Pathogen *Fusarium circinatum* with *Pinus radiata*. *Plant Dis.* **100**, 79–84.

Thatcher, L.F., Williams, A.H., Garg, G., Buck, S.A.G. and Singh, K.B. (2016) Transcriptome analysis of the fungal pathogen *Fusarium oxysporum* f. sp. *medicaginis* during colonisation of resistant and susceptible *Medicago truncatula* hosts identifies differential pathogenicity profiles and novel candidate effectors. *BMC Genomics* **17**, 1–19.

Thekke Veetil, T., Ho, T., Moyer, C., Whitaker, V.M. and Tzanetakis, I.E. (2016) Detection of strawberry necrotic shock virus using conventional and TaqMan® quantitative RT-PCR. *J. Virol. Methods* **235**, 176–181.

Torres-Vila, L.M., Zugasti, C., De-Juan, J.M., *et al*. (2015) Mark-recapture of *Monochamus galloprovincialis* with semiochemical-baited traps: population density, attraction distance, flight behaviour and mass trapping efficiency. *Forestry* **88**, 224–236.

Tudzynski, B. (2014) Nitrogen regulation of fungal secondary metabolism in fungi. *Front. Microbiol.* **5**, 656.

Vainio, E.J., Bezos, D., Bragança, H., *et al*. (2019) Sampling and detection strategies for the pine pitch canker (PPC) disease pathogen *Fusarium circinatum* in Europe. *Forests* **10**, 1–27.

Vainio, E.J., Jurvansuu, J., Hyder, R., *et al*. (2018) Heterobasidion Partitivirus 13 Mediates Severe Growth Debilitation and Major Alterations in the Gene Expression of a Fungal Forest Pathogen. *J. Virol.* **92**, 1–15.

Vainio, E.J, Jurvansuu, J., Streng, J., Rajamä, M.-L., Hantula, J. and Valkonen, J.P.T. (2015) Diagnosis and discovery of fungal viruses using deep sequencing of small RNAs. *J. Gen. Virol.* **96**, 714–725.

Vainio, E.J., Martínez-Álvarez, P., Bezos, D., Hantula, J. and Diez, J.J. (2015) *Fusarium circinatum* isolates from northern Spain are commonly infected by three distinct mitoviruses. *Arch. Virol.* **160**, 2093–2098.

Velasco, L., Arjona-Girona, I., Cretazzo, E. and López-Herrera, C. (2019) Viromes in Xylariaceae fungi infecting avocado in Spain. *Virology* **532**, 11–21.

Vettraino, A.M., Potting, R. and Raposo, R. (2018) EU legislation on forest plant health: An overview with a focus on *Fusarium circinatum*. *Forests* **9**, 1–26.

Visser, E.A., Wegrzyn, J.L., Steenkamp, E.T., Myburg, A.A. and Naidoo, S. (2019) Dual RNA-Seq analysis of the pine-*Fusarium circinatum* interaction in resistant (*Pinus tecunumanii*) and susceptible (*Pinus patula*) hosts. *Microorganisms* **7**, 7–9.

Visser, E.A., Wegrzyn, J.L., Steenkmap, E.T., Myburg, A.A. and Naidoo, S. (2015) Combined *de novo* and genome guided assembly and annotation of the *Pinus patula* juvenile shoot transcriptome. *BMC Genomics* **16**, 1057.

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

Walmsley, J.D. and Godbold, D.L. (2010) Stump Harvesting for Bioenergy - A Review of the Environmental Impacts. *Forestry* **83**, 17–38.

Wang, K.C. and Chang, H.Y. (2011) Molecular Mechanisms of Long Noncoding RNAs. *Mol. Cell* **43**, 904–914.

Wang, M., Yuan, D., Tu, L., *et al*. (2015) Long noncoding RNAs and their proposed functions in fibre development of cotton (*Gossypium* spp.). *New Phytol.* **207**, 1181–1197.

Wang, S., Zhang, J., Li, P., Qiu, D. and Guo, L. (2016) Transcriptome-Based Discovery of *Fusarium graminearum* Stress Responses to FgHV1 Infection. *Int. J. Mol. Sci.* **17**, 1922.

Wang, Y., Luo, X., Sun, F., Hu, J., Zha, X., Su, W. and Yang, J. (2018) Overexpressing lncRNA LAIR increases grain yield and regulates neighbouring gene cluster expression in rice. *Nat. Commun.* **9**, 1–9.

Wang, Zupeng, Liu, Y., Li, L., Li, D., Zhang, Q., Guo, Y., Wang, S., Zhong, C. and Huang, H. (2017) Whole transcriptome sequencing of *Pseudomonas syringae* pv. *actinidiae*-infected kiwifruit plants reveals species-specific interaction between long non-coding RNA and coding genes. *Sci. Rep.* **7**, 1–15.

Wang, Z., Zhai, X., Cao, Y., Dong, Y. and Fan, G. (2017) Long non-coding RNAs responsive to Witches' Broom disease in *Paulownia tomentosa*. *Forests* **8**, 348.

Waseem, M., Liu, Y. and Xia, R. (2021) Long Non-Coding RNAs, the Dark Matter: An Emerging

Regulatory Component in Plants. *Int. J. Mol. Sci.* **22**, 86.

Wermelinger, B. (2004) Ecology and management of the spruce bark beetle *Ips typographus* - a review of recent research. *For. Ecol. Manag.* **202**, 67–82.

Wingfield, M.J., Brockerhoff, E.G., Wingfield, B.D. and Slippers, B. (2015) Planted forest health: The need for a global strategy. *Science* **349**, 832–836.

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

Yao, Z., Zou, C., Peng, N., Zhu, Y., Bao, Y., Zhou, Q., Wu, Q., Chen, B. and Zhang, M. (2020) Virome identification and characterization of *Fusarium sacchari* and *F. andiyazi*: Causative agents of Pokkah Boeng disease in sugarcane. *Front. Microbiol.* **11**, 240.

Yu, Y., Zhou, Y.F., Feng, Y.Z., He, H., Lian, J.P., Yang, Y.W., Lei, M.Q., Zhang, Y.C. and Chen, Y.Q. (2020) Transcriptional landscape of pathogen-responsive lncRNAs in rice unveils the role of ALEX1 in jasmonate pathway and disease resistance. *Plant Biotechnol. J.* **18**, 679–690.

Zhang, L., Wang, M., Li, N., *et al*. (2018) Long noncoding RNAs involve in resistance to *Verticillium dahliae*, a fungal disease in cotton. *Plant Biotechnol. J.* **16**, 1172–1185.

Zhang, S. and Vrient, A. (2020) Rapid detection of plant viruses and viroids. In Applied Plant Virology., pp. 101–109. Elsevier.

Chapter 9: Conclusions

1) Since established PPC disease becomes extremely difficult to eradicate, preventive mechanisms to avoid its introduction into disease-free areas become essential. In this regard, new methods for detection and diagnosis of *F. circinatum* at a very early stage in seeds, plants, and vector insects are urgently needed. The multiple pathways of spread make *F. circinatum* challenging to prevent, especially with the recent discovery of its endophytic colonization of non-reported host species. This illustrates the importance of the biological and ecological knowledge for the design of effective intervention strategies. To ensure that the new, science-based strategies to suppress PPC comply with existing practices, regulations, and policies, it is important that these strategies had been developed through collaborations between phytosanitary authorities and researchers. Furthermore, opinion-building actions, such as The Montesclaros Declaration, as well as interdisciplinary research networks (e.g. COST Action FP1406), increasing knowledge of the disease and advocating the crucial target groups about the risks and measures to mitigate them, are also needed.

2) The possibility of combating a forest disease with the interaction mechanisms of the pathogen lies in the search for a putative hypovirulence-inducing mycovirus. However, the study, manipulation and conservation of mycoviruses within their hosts require meticulous endeavors in the laboratory. As a result, there is insufficient information to help fully elucidate the effects of viral infection on the fungal fitness. RNA-Seq technology was used to analyze the residual effect of the loss of the FcMV1 mycovirus on the *F. circinatum* transcriptome. The fungal isolate that had lost the mycovirus experienced a slight acceleration of metabolism through the increment in the expression of genes involved in the methionine pathway, which regulates many essential metabolic and physiologic processes. This was also reflected in the up-regulation of genes associated with cellular development as well as oxidative burst that could be related to the development of the colony, inducing hyphae and macroconidium formation. The residual effect provoked by the loss of a mycovirus in the host transcriptome reflects the difficulty in obtaining unaltered isolates for further studies. Future research focused on the generation of isogenic lines of *F. circinatum* with and without mycoviruses by transfection methods is required in order to better understand the feasibility of virocontrol of this forest pathogen.

3) Knowing the pathogenicity mechanisms used by *F. circinatum* during its infection to *Pinus* spp. species with different degrees of susceptibility greatly helps in the understanding of PPC disease resistance. The comparative transcriptomic response showed a greater focus of *F. circinatum* on cell wall and lignin degradation when infecting the resistant species *P. pinea* at an early stage of the disease. This may be associated with a higher lignin content in this species and, in turn, a stronger barrier against pathogen penetration. On the other hand, the pathogen had an active uptake of nutrients (such as nitrogen) of the susceptible species *P. radiata* during its infection, which could provide *F. circinatum* with a competitive advantage in the plant-pathogen interaction.

4) Genetic resistance is one of the most promising approaches for PPC management. The improvement of breeding programs for PCC-resistant pine commercialization requires a deep understanding of the regulatory mechanisms involved in the defense of the hosts. The comparative transcriptomic response showed the lack of a substantial response in the susceptible species (*P. radiata*) contrasted with an advanced

transcriptional reprogramming for defense in the resistant one (*P. pinea*) at an early stage of the disease. The weak response of *P. radiata* could be related to the impaired perception of the fungal infection since genes involved in early defense responses, including calcium flux, recognition by R proteins, or the activation of mitogen-activated protein kinases (MAPKs), were not expressed in this species. The failure during the initial infection stage that in turn is involved in signaling intermediate and late responses has presumably led to a weaker activation of a diverse array of defense pathways, including lignification, phytohormone biosynthesis, and the production of non-enzymatic antioxidants such as ascorbate and flavonoids. These findings allowed a better understanding of the tree defensive mechanisms and set the foundation for future studies for validating the association of these candidate genes with PPC resistance traits.

5) The discovery of new mechanisms regulating plant defence, such as long non-coding RNAs, implies their incorporation in decision-making during the generation of PCC-resistant material. The computational analysis using RNA-Seq data from the interaction of *P. radiata-F. circinatum* allowed to identify 13,312 lncRNAs in the pine. Compared to the protein-coding RNAs, the lncRNAs were shorter, with fewer exons and lower expression levels. A total of 164 lncRNAs were reported as responsive to *F. circinatum* infection. Functional analysis of genes that either overlap with or are neighbours of these pathogen-responsive lncRNAs suggested involvement of important defense processes including signal transduction and cell wall reinforcement. These results present a comprehensive map of lncRNAs in *P. radiata* under *F. circinatum* infection and provide a starting point to understand their regulatory mechanisms and functions in conifer defense responses to biotic stress. In turn, a thorough understanding of the mechanism of gene regulation will contribute to the improvement of breeding programs for resistant pine commercialization.

Chapter 10: Conclusiones

1) Dado que la enfermedad del PPC una vez establecida en el monte es extremadamente difícil de erradicar, los mecanismos preventivos para evitar su introducción en zonas libres de la enfermedad se vuelven esenciales. En este sentido, se necesitan urgentemente nuevos métodos que permitan una rápida detección y diagnóstico de *F. circinatum* en semillas, plantas e insectos vectores. Sus múltiples vías de propagación hacen de *F. circinatum* un verdadero reto a la hora de combatirlo, especialmente con el reciente descubrimiento de su colonización endofítica no solo en especies de pinos, sino también en otras coníferas y herbáceas. Esto pone de manifiesto la importancia del conocimiento biológico y ecológico para el diseño de estrategias de intervención eficaces. Con el fin de garantizar que las nuevas estrategias de control de PPC basadas en el conocimiento científico se ajusten a las prácticas, reglamentos y políticas existentes, es importante que estas estrategias se hayan desarrollado mediante la colaboración entre las autoridades fitosanitarias y los investigadores. Además, también son necesarias acciones de creación de opinión, como la Declaración de Montesclaros, así como redes de investigación interdisciplinarias (por ejemplo, la Acción COST FP1406), que aumenten el conocimiento de la enfermedad y sensibilicen a los agentes implicados sobre los riesgos y las medidas para mitigarlos.

2) La posibilidad de combatir una enfermedad forestal con los mecanismos de interacción del patógeno reside en la búsqueda de un micovirus propio que induzca hipovirulencia. Sin embargo, el estudio, la manipulación y la conservación de los micovirus dentro de sus hospedadores requieren un esfuerzo meticuloso en el laboratorio. Como resultado, no hay suficiente información para ayudar a dilucidar completamente los efectos de la infección viral en los hongos. La tecnología RNA-Seq fue utilizada para analizar el efecto residual de la pérdida del micovirus FcMV1 en el transcriptoma de *F. circinatum*. El aislado fúngico que había perdido el micovirus experimentó una ligera aceleración del metabolismo a través de un incremento en la expresión de genes implicados en la vía de la metionina, la cual regula muchos procesos metabólicos y fisiológicos esenciales. Esto también se reflejó en la sobreexpresión de genes asociados al desarrollo celular, así como a la explosión oxidativa que podría estar relacionada con el desarrollo de la colonia, induciendo la formación de hifas y macroconidios. El efecto residual que provoca la pérdida de un micovirus en el transcriptoma del hospedador refleja la dificultad de obtener aislados inalterados para estudios posteriores. Se requieren futuras investigaciones centradas en la generación de líneas isogénicas de *F. circinatum* con y sin micovirus por métodos de transfección para comprender mejor la viabilidad del virocontrol de esta patología forestal.

3) Conocer los mecanismos de patogenicidad utilizados por *F. circinatum* durante su infección en especies de *Pinus* spp. con diferentes grados de susceptibilidad ayuda en gran medida a la comprensión de la resistencia a la enfermedad del PPC. La respuesta transcriptómica comparativa mostró una mayor actividad de *F. circinatum* en degradar la pared celular y la lignina cuando infecta a la especie resistente *P. pinea* en una etapa temprana de la enfermedad. Esto puede estar asociado a un mayor contenido de lignina en esta especie y, a su vez, a una mayor barrera contra la penetración del patógeno. Por otro lado, el patógeno tuvo una absorción activa de los nutrientes (como el nitrógeno) de la especie susceptible *P. radiata* durante su infección, lo que podría proporcionar a *F. circinatum* una ventaja competitiva en la interacción planta-patógeno.

4) La resistencia genética es una de las estrategias más prometedoras para el manejo del PCC. La optimización de los programas de mejora genética para la comercialización de pinos resistentes al PCC requiere un profundo conocimiento de los mecanismos reguladores implicados en la defensa de los hospedadores. La respuesta transcriptómica comparativa mostró la ausencia de una respuesta sustancial en la especie susceptible (*P. radiata*) en contraste con una amplia reprogramación transcripcional para la defensa en la resistente (*P. pinea*) en una fase inicial de la enfermedad. La débil respuesta del *P. radiata* podría estar relacionada con la falta de percepción de la infección fúngica, ya que los genes implicados en las primeras respuestas de defensa, incluyendo el flujo de calcio, el reconocimiento por parte de las proteínas R, o la activación de las proteínas quinasas activadas por mitógenos (MAPKs), no se expresaron en esta especie. El fallo durante la etapa inicial de la infección, que a su vez está implicado en la señalización de las respuestas posteriores, llevó posiblemente a una activación más débil de un conjunto diverso de vías de defensa, incluyendo la lignificación, la biosíntesis de fitohormonas y la producción de antioxidantes no enzimáticos como el ascorbato y los flavonoides. Nuestros hallazgos permitieron una mejor comprensión de los mecanismos defensivos del árbol y sentaron las bases para futuros estudios de validación de la asociación de estos genes candidatos con los caracteres de resistencia al PPC.

5) El descubrimiento de nuevos mecanismos que regulan la defensa de las plantas, como los ARN no codificantes de cadena larga (ARNlnc), implica su incorporación en la toma de decisiones durante la generación de material resistente al PPC. El análisis computacional utilizando datos de RNA-Seq de la interacción *P. radiata-F. circinatum* permitió identificar 13.312 ARNlnc en el pino. En comparación con los ARN que codifican proteínas, los ARNlnc eran más cortos, con menos exones y niveles de expresión más bajos. Un total de 164 ARNlnc se asociaron a la infección por *F. circinatum*. El análisis funcional de los genes que se solapan o son vecinos de estos ARNlnc de respuesta al patógeno predijo su participación en importantes procesos de defensa, incluyendo la transducción de señales y el refuerzo de la pared celular. Estos resultados presentan un mapa completo de los ARNlnc en *P. radiata* durante la infección de *F. circinatum* y proporcionan un punto de partida para entender sus mecanismos de regulación y funciones en las respuestas de defensa de las coníferas al estrés biótico. A su vez, un conocimiento profundo de los mecanismos de regulación de los genes contribuirá a la optimización de los programas de mejora genética para la comercialización de pinos resistentes.

Annexes

Annex I:

Chapter 27: Pine pitch canker: an introduction, an overview

Cristina Zamora-Ballesteros, Jorge Martín-García, M. Milagros Fernández-Fernández and Julio J. Diez

Zamora-Ballesteros, C., Martín-García, J., Fernández-Fernández, M.M. and Diez, J.J. (2022) Pine Pitch canker: an introduction, an overview. In: Asiegbu, F.O. and Kovalchuk, A. (Eds.) Forest Microbiology. Volume 2: Forest Tree Health. Academic Press Inc. (Elsevier), pp. 300. ISBN: 9780323850421

Abstract

Pine pitch canker (PPC) is a serious disease of *Pinus* spp. and *Pseudotsuga menziesii* globally. The infection of its causal agent, *Fusarium circinatum*, causes pitch or resin-soaked cankers on trunks and lateral branches of mature hosts. In nurseries, the main symptoms are damping-off and tip dieback of seedlings. However, the pathogen, with a hemibiotrophic nature, can remain endophytic in seedlings that do not show symptoms of infection. Since the first report in 1946 in North America, the presence of *F. circinatum* has been notified in 14 countries in America, Asia, Africa and Europe. Several factors have contributed to the spread of the disease to all these continents, the most important being globalization in terms of trade in reproductive plant material. Wind, raindrops and forest insects associated with pines contribute to the local dispersion of the pathogen. Therefore, the implementation of early diagnostic methods is crucial to prevent the PPC establishment in disease-free areas. Worryingly, some herbaceous plants growing under the canopy of PPC-affected stands may also act as a source of inoculum for *F. circinatum*, as recent studies have reported. Since the viability of the inoculum is highly moisture-dependent and needs warm temperatures to germinate, coast areas represent the most suitable climate for PPC development. In fact, the highest incidence occurs in Mediterranean and sub-tropical climates and temperate regions. Under future climate change scenarios, cooler latitudes with the presence of susceptible hosts would become suitable for the disease establishment. For this reason, the use of tolerant host conifers might reduce outbreaks of PPC in free-disease areas. The range of susceptibility to *F. circinatum* has been found to vary significantly among species and intraspecifically, posing as a potential disease management strategy. According to this, the development of massive sequencing technologies is allowing a deeper understanding of the plant-pathogen interaction of this pathosystem, promoting the improvement of breeding programs to obtain resistant pine reproductive material. Eco-friendly methods such as the use of endophytic fungi and bacteria with antagonistic effect to *F. circinatum*, plant essential oils, chitosan or phosphite have been also investigated for reducing the impact of the PPC. Moreover, thermotherapy to eliminate the pathogen from infected seeds is a simple and low-cost method to minimize the risk of introducing contaminated seed into nurseries in disease-free areas. However, the lack of effective intervention measures in the field, and the difficulties to avoid its transmission to the forest due to asymptomatic nursery plants, make PPC an unsolved problem for the coming years. Therefore, great efforts will be necessary to address the integrated management of this disease through the use of environmental-friendly methods in the near future.

Annex II:

Chapter 3: Field and laboratory procedures for *Fusarium circinatum* identification and diagnosis

Cristina Zamora-Ballesteros, Reinaldo Pire, Julio J. Diez

Zamora-Ballesteros, C., Pire, R., Diez, J.J. (2022) Field and laboratory procedures for Fusarium circinatum identification and diagnosis. In: Luchi N. (Eds.) Plant Pathology: Method and Protocols. Methods in Molecular Biology. Humana, New York, NY. (In Press).

Abstract

Fusarium circinatum is a serious invasive pathogen affecting conifers and causes the disease commonly known as pine pitch canker. Due to the outbreak in European countries, regulations stipulate that Member States must conduct annual official surveys for the fungus on their territory and report the results to the European Commission. Here, we describe the field and laboratory protocols used for the identification and diagnostic of the pathogen.