Universidade de Aveiro Departamento de Biologia
Ano 2016

Andreia Filipa Lages Cerqueira

Effects of phosphite in *Pinus radiata-Fusarium circinatum* interaction

Efeitos do fosfito na interação *Pinus radiata-Fusarium circinatum*

DECLARAÇÃO

Declaro que este relatório é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

Universidade de Aveiro Departamento de Biologia
Ano 2016

Andreia Filipa Lages Cerqueira

Effects of phosphite in *Pinus radiata-Fusarium circinatum* interaction

Efeitos do fosfito na interação *Pinus radiata-Fusarium circinatum*

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Glória Catarina Cintra da Costa Pinto, Professora auxiliar convidada do Departamento de Biologia da Universidade de Aveiro, e coorientação do Doutor Artur Jorge da Costa Peixoto Alves, Investigador principal do Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro da Comissão Diretiva do Programa Operacional Competitividade e Internacionalização (POCI-01-0145-FEDER-016785)

Apoio financeiro da Fundação para a Ciência e Tecnologia (FCT) no âmbito do projeto PTDC/AGR-FOR/2768/2014

"Don’t be afraid. There are exquisite things in store for you. This is merely the beginning."
Oscar Wilde, *The Picture of Dorian Gray*

o júri

presidente	Professora Doutora Maria de Lourdes Pereira Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro
arguente principal	Doutora Lia-Tânia Rosa Dinis Investigadora de Pós-Doutoramento do Departamento de Biologia e Ambiente da Universidade de Trás-os-Montes e Alto Douro
orientadora	Doutora Glória Catarina Cintra da Costa Pinto Professora Auxiliar convidada do Departamento de Biologia da Universidade de Aveiro

agradecimentos

Finda mais uma etapa da minha vida, quero agradecer a toda a gente que, de uma forma ou de outra, esteve lá para mim.

À Doutora Glória Pinto e ao Doutor Artur Alves, obrigada por me terem acolhido no vosso laboratório, pela generosidade e confiança depositadas em mim e por tudo o que me ensinaram.

À Barbara, obrigada por tudo o que me ensinaste e pela paciência em responder a todas as minhas questões. Ao Pedro e ao Helder, por me ajudarem sempre que precisei.

Aos amigos de sempre, que mesmo longe estão sempre por perto. Em particular, à Rita obrigada por me ouvires a falar de biologia horas e horas, mesmo sem gostar, e por me animares sempre que tudo parecia perdido. À Leandra, obrigada pelos 14 anos de amizade e por estares presente em todas as ocasiões, do menos bom ao bom.

Ao melhor grupo de amigas que podia pedir - à Bárbara, à Cláudia, à Filipa, à Gracinha, à Natacha e à Vanessa. Obrigada pelos almoços no CUA e pelas bifanas, pelos dias passados na biblioteca, pelos risos e ombro amigo, por ouvirem os meus desabafos e por terem sido a minha família longe de casa.

Às outras pessoas importantes que Aveiro me trouxe - ao João, por teres tornado 4 destes 5 anos melhores, por te preocupares e por me ensinares o que é lutar pela felicidade. À Ana Rita, por me compreenderes mesmo sem palavras e por teres sido a melhor companheira de quarto/casa/aventuras. À Sara, por partilhares comigo o amor às coisas belas da vida, como a música, os céus e Coura. Ao Alexandre, à Liane, à Diana e à Maria João por tornarem a minha passagem por Aveiro ainda mais especial.

E por fim, aos meus pais e irmão. Obrigada pelos sacríficos, ao longo desde 5 anos, e pelo amor, carinho e apoio ao longo de toda a minha vida. A vocês tudo devo e tudo o que faço é por vocês e para vocês.

palavras-chave *Fusarium circinatum*, *Pinus radiata*, fosfito, indução de resistência, fisiologia

resumo

O cancro resinoso, provocado pelo fungo *Fusarium circinatum*, é uma doença que afeta *Pinus* spp. e *Pseudotsuga menziesii* em todo o mundo e está sujeita a medidas de quarentena. Caracteriza-se pela formação de grandes cancros resinosos que rodeiam rebentos, ramos e troncos e levam à morte do hospedeiro. Até à data não existem meios para o controlo da doença e, com a crescente necessidade de reduzir o uso de fungicidas, outras abordagens devem ser estudadas. Um método para o controlo de doenças fitopatogénicas passa pela indução da resistência do hospedeiro, através do pré-tratamento de plantas com compostos químicos ou de origem biológica que estimulam as defesas. O fosfito (Phi) é um sal inorgânico que apresenta a capacidade de indução da resistência e uma potencial estratégia mais amiga do ambiente. Neste estudo, a utilização do fosfito de potássio (KPhi) na redução do desenvolvimento dos sintomas da doença do cancro resinoso, assim como os seus efeitos no crescimento do fungo, foram estudados em diferentes concentrações. Numa primeira fase, colónias de *F. circinatum* foram crescidas em PDA suplementado com Phi (0%, 1% e 4%) para avaliação do seu efeito no crescimento radial. Posteriormente foram estudados os efeitos da aplicação foliar de Phi (0%, 1% e 4%) em plântulas de *Pinus radiata*, inoculadas e não inoculadas. A taxa de sobrevivência e a performance fisiológica (potencial hídrico, trocas gasosas e performance fotoquímica, pigmentos, peroxidação lipídica, libertação de eletrólitos, prolina e carbohidratos) foram avaliados. Os resultados mostram que aplicação de Phi atrasa o desenvolvimento dos sintomas de doença numa forma dependente da concentração de Phi, em semelhança ao observado relativamente à inibição do crescimento do micélio *in vitro*. Alterações fisiológicas ao nível da prolina e carbohidratos, peroxidação lipídica e trocas gasosas foram observadas. A aplicação de Phi apresenta-se como uma potencial alternativa viável na gestão da doença do cancro resinoso.

keywords

Fusarium circinatum, *Pinus radiata*, phosphite, induction of resistance, physiology

abstract

The pitch canker, caused by the fungus *Fusarium circinatum*, is a disease under quarantine measures affecting *Pinus* spp. and *Pseudotsuga menziesii* worldwide. Characterized by the formation of large resinous cankers that girdle shoots, branches, and trunks, leads to the death of the host. To date, there are no means for the control of the pitch canker and, with the growing need to reduce the use of fungicides, another approaches must be studied. A method for the control of phytopathogenic diseases is the enhancement of host resistance, through pre-treatment of seedlings with chemicals or biologically derived compounds that stimulate defense responses. Phosphite (Phi) is an inorganic salt with the capability of inducing host resistance and presents an approach more environmentally friendly. In this study, the ability of potassium phosphite (KPhi) in delaying the pitch canker symptom development, as well as its effects in fungal growth, were studied at different concentrations. In a first phase, *F. circinatum* colonies were grown in PDA medium supplemented with Phi (0%, 1% and 4%) to evaluation in the radial growth of the fungus. Posteriorly, were studied the effects of foliar application of Phi (0%, 1% and 4%) in *Pinus radiata* seedlings, inoculated and non-inoculated. Survival and physiological performance (water potential, gas exchange and photochemical performance, pigments, lipid peroxidation, electrolyte leakage, proline and carbohydrates) were assessed. Results showed that Phi application delayed disease symptoms in a dose dependent manner similarly to what was observed in mycelial growth inhibition during *in vitro* assays. Physiological alterations in proline, carbohydrates, lipid peroxidation and gas exchange parameters were observed. Thus, Phi application presents a potential viable alternative to the management of the pitch canker disease.

Index

Part I

The genus *Pinus*: a brief description of *Pinus radiata*

Considered to be the most significant tree genus in the world, the genus *Pinus* represents an outstanding group of gymnosperms in the Plant Kingdom and is found in almost any terrestrial habitat (Richardson & Rundel 2000; Ioannou et al. 2014). It is composed of a total of 111 species that have the ability to spread into a remarkably wide range of environments and grow continually throughout the year (Ghildiyal et al. 2010; Gernandt et al. 2015). These plants grow naturally or are introduced in both hemispheres, being essentially distributed over the Northern hemisphere but also occurring in subtropical and tropical regions of Central America and Asia (Ioannou et al. 2014).

In Europe, pine forests cover an area of more than 50 million hectares. In Portugal, *Pinus* is the second most planted genus, representing approximately 29% of the Portuguese forest (ICNF 2013b), being *Pinus pinaster* Ait. the most planted *Pinus* species. The forest products represent an important economic asset, accounting for 2.5% of the national gross domestic product (ICNF 2013a). They are a source of timber, pulp and paper, food (particularly seeds), charcoal, resin, construction materials and other products (Richardson & Rundel 2000).

Belonging to the closed-cone pine group (subsection Attenuatae), Monterey pine (*Pinus radiata* D. Don) is the most relevant pine species in the world (Mead 2013). Its provenance is almost exclusively from Mexico to California (Farjon 2013), but has large estates established in Australia, Chile, New Zealand, South Africa, Spain and Uruguay, representing 4.2 million ha in plantations worldwide (Rogers 2004; Stone et al. 2012). In Portugal, *Pinus radiata* is part of the group "another resinous trees" that represents 2% of the total planted area (ICNF 2013b).

Due to its versatility and rapid growth, *P. radiata* has a huge success in commercial forestry. Similarly, as happen for any forest ecosystem, Monterey pine diseases can be troublesome to plantations which can result in damage of plantation resources, end-use and value (Mead 2013). More than 400 pests and pathogens have been recorded on *P. radiata*, including *Diplodia sapinea*, *Dothistroma septosporum*, and *Fusarium circinatum* (Dick et al. 2014; Iturritxa et al. 2015).

Fusarium circinatum, the causal agent of the pitch canker

Fusarium circinatum Nirenberg & O'Donnell (teleomorph *Gibberella circinata* Nirenberg & O'Donnell 1998) is an ascomycete fungus with a great virulence known to cause the pitch canker disease (Vivas et al. 2012; Fitza et al. 2013). Exclusively a pathogen of coniferous trees, *F. circinatum* has been reported on more than 60 species of pine (*Pinus*) and on Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), representing a significant threat to regions where these species occur naturally or are commercially grown (Wingfield et al. 2008; Berbegal et al. 2013). It was first reported in 1946 in North Carolina, United States (U.S.), and is now known to be present across the southeastern U.S. as well as in California (Iturritxa et al. 2011). Since then, the pitch canker has been described in Japan (Muramoto & Dwinell 1990), South Africa (Viljoen et al. 1994), Mexico (Britz et al. 2001), Chile (Wingfield et al. 2002), and Brazil (Pfenning et al. 2014). The European Union referenced *F. circinatum* for the first time in 2005, in the north of Spain, in nurseries of *Pinus radiata* (Landeras et al. 2005). Italy was the second country reporting the disease, being the first symptoms observed in adult trees of *Pinus halepensis* and *Pinus pinea* (Carlucci et al. 2007). In Portugal it was first reported in 2008 in a tree nursery on plant groups of *P. radiata* and *Pinus pinaster* (Bragança et al. 2009), and in France in *Pseudotsuga menziesii* (EPPO 2009) (**Figure 1**).

Figure 1: Occurrence points of *F. circinatum* in the European Union until the end of 2009. Obtained from EFSA Panel on Plant Health (2010).

Fusarium circinatum was subject to Commission Decision 2007/433/EC of 18 June 2007 recommended as quarantine pathogen and a National Priority Action plan coordinated by ICNF (2014).

Morphological identification

Macroscopically, colonies of *F. circinatum* have margin entire, white cottony aerial mycelium, with a purple or dark violet color in the center. In the reverse, presents a greyish-white to gray pigmentation (Nirenberg & O'Donnell 1998; Pérez-Sierra et al. 2007; de Paiva 2011) (**Figure 2**).

Fusarium circinatum is characterized by sterile coiled hyphae, sympodially branched conidiophores bearing polyphialides and conidiophores that are originated directly from the substrate hyphae. The conidiophores have 2 to 5 openings. Produces two types of conidia - macroconidia and microconidia. Macroconidia are typically 3-septate, slender, and cylindrical. Microconidia, usually unicellular, non-septate and oval or obovoid, are born in false heads on aerial polyphialides and abundant in the aerial mycelium. Presence of coiled sterile hyphae (circinate) is one of the distinctive features of this species from which derives the epithet "circinatum" (Nirenberg & O'Donnell 1998; Pérez-Sierra et al. 2007; EPPO 2009).

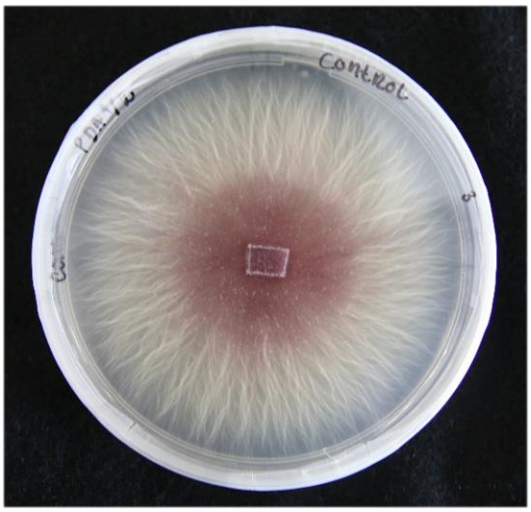

Figure 2: Macroscopic aspect of *Fusarium circinatum* in PDA medium.

Biology and epidemiology

The pitch canker is quite dynamic and every outbreak has a unique case history since symptom expression and disease severity are strongly correlated with the activity of biotic or abiotic agents (Gordon 2006; Wingfield et al. 2008).

Several environmental factors are known to contribute to pitch canker disease establishment and severity (Wingfield et al. 2008). Nutrient levels can affect susceptibility, especially high levels of nutrients, both in soil and foliage. The amount of water available (*i.e.* drought or waterlogging)

increases vulnerability (Iturritxa et al. 2011), as well as edaphic factors, in particular, shallow soils and plantations at high stand densities (Hammerbacher 2006).

Temperature affects the infection process, as the pitch canker fungus appears to prefer warmer climates (Inman et al. 2008). In addition, humidity may also affect the establishment of disease. If sufficient moisture is not available, the infection may not occur, even where temperatures are within the optimal range (Iturritxa et al. 2014).

Successful infections require wounds or openings on the tree as intact tissue is not vulnerable to invasion by the fungus (Iturritxa et al. 2011). Generally, pitch canker is associated with wounds created by insects (coleopterans), weather or mechanical damage (Sakamoto & Gordon 2006; Mitchell et al. 2011). Dispersal is easily linked to the presence of abundant airborne inoculum (Schweigkofler et al. 2004) and spores can be disseminated locally by the wind, rain, animals, insects or soil (de Paiva 2011). Over long distances, it can be disseminated by the movement of infected plant materials to new areas by man (EPPO 2005; Hammerbacher 2006). Inoculum can occur in any season of the year but highest spores frequency has been found to occur during the autumn-winter months (Wingfield et al. 2008; Donoso et al. 2015).

Despite the vast host range of *F. circinatum*, there is a differential susceptibility of resistance within species (Wingfield et al. 2008). For example, *Pinus brutia* and *P. pinaster* appear to have relatively high levels of resistance to the disease, while there are indications that *P. radiata* is the most susceptible of all pines (Gordon et al. 2001). However, the degree to which individual trees develop pitch canker varies widely. The tolerance of an individual may change over time as a result of induced resistance, associated with the activation of natural defense mechanisms of the plant, and influence of environmental stress and inoculum pressure (Martín-Rodrigues et al. 2013). Therefore, the relative resistance is controlled by genetic factors as well as environmental factors (Lopez-Zamora et al. 2007).

Infection process and symptoms

Fusarium circinatum can infect all the stages of tree development: seeds, seedlings, and mature trees (Pérez-Sierra et al. 2007). In 2013, Martín-Rodrigues et al. reported the colonization dynamics of this pathogen. Spatially, *F. circinatum* reveals a homogeneous colonization pattern, initially colonizing the cortex and phloem, and later invading the xylem and resin ducts, reaching the pith. Temporal progression involves three different stages: the exponential growth phase (first week following inoculation), where the pathogen biomass grows exponentially; transition phase (from 7 days post-inoculation to 21 days post-inoculation), where fungal biomass keeps growing

but slower; and stationary phase (after 21 days post-inoculation), where the grown rate is stabilized.

The most distinctive feature of the disease is the development of large resinous cankers that girdle the shoots, branches, and trunks of the hosts (Fitza et al. 2013). These cankers are characterized by the excessive pitch flow resulting in complete impregnation of the wood inside the canker (Martín-Rodrigues et al. 2013) (**Figure 3**). The first symptoms include wilting and chlorosis of needles, which turn red and fall off resulting in branch dieback (de Paiva 2011). Multiple branch infections cause canopy dieback.

On seedlings, *F. circinatum* leads to tip dieback, root and collar disease and damping off (Santana et al. 2015). In the reproductive structures, the pitch canker disease leads to mortality of female flowers and mature cones as well as reduced germination of seeds (Hammerbacher et al. 2009).

Figure 3: Symptoms of the pitch canker disease in a plantation in the North of Spain: (a) resinous canker and (b) tissues impregnated with resin.

Pathogen classification

Depending on their lifestyle, plant pathogens are normally classified as biotrophs, hemibiotrophs, and necrotrophs (Kangasjärvi et al. 2012). Biotrophs derive energy from living cells; hemibiotrophic pathogens present an initial period of biotrophy followed by a necrotrophic phase;

and necrotrophs invade the plant through wounded sites, inducing necrosis in the cells (Oliver & Ipcho 2004; Kangasjärvi et al. 2012).

The genus *Fusarium* employs a broad range of infection strategies (Ma et al. 2013). *Fusarium circinatum* has a lifestyle typical of necrotrophic pathogens not presenting specialized infection structures to derive nutrients from sacrificed cells (Martín-Rodrigues et al. 2013). However, recently Swett et al. (2016) reported evidence for a hemibiotrophic lifestyle of *F. circinatum* in *Pinus radiata*, showing that the pathogen can grow intercellularly in the root cortex without damage to surrounding the tissues.

Host-pathogen interaction

Plant responses to abiotic or biotic stresses are complex and involve numerous physiological, molecular, and cellular adaptations (Atkinson & Urwin 2012). A first layer of plant immune system is based on the sensitive perception of the pathogen-associated molecular patterns (PAMP; synonymously called MAMPs for microbe-associated molecular patterns) (Pastor et al. 2013), which activates PAMP-triggered immunity (PTI). Generally, PTI stops the infection before the microbe gains a hold in the plant (Chisholm et al. 2006). However, to contour PTI, pathogens secrete virulence effector molecules that deregulate the signaling pathways controlling plant innate immunity. With this, plants developed the effector-triggered immunity (ETI), which requires *R* (resistance) genes (Göhre et al. 2012). Generally, PTI and ETI give rise to similar responses, although ETI is stronger, inducing a hypersensitive response (HR) programmed cell death, production of salicylic acid (SA), and the systemic acquired resistance (SAR) (Dodds & Rathjen 2010; Hua 2013).

Systemic acquired resistance is part of the systemic plant immune responses, which also includes the induced systemic resistance (ISR) (Conrath et al. 2015). ISR is typically activated upon colonization of plant roots by growth-promoting bacteria and fungi. It is a jasmonic acid (JA) and ethylene (ET) dependent pathway and is not associated with the direct activation of *pathogenesis-related* (PR) genes (Walters & Heil 2007; Pieterse et al. 2009). SAR develops locally and/or systemically in response to necrotizing pathogen infection and requires SA (Conrath et al. 2015). Upon pathogen infection, SA travels through the vascular system to activate defense responses in distal tissues and coordinates the activation of *SAR* genes, some of which encode PR proteins (Conrath et al. 2002; Pieterse et al. 2009). The accumulation of SA mediates the development of

HR, which is a form of rapid host cell death and simultaneous localization of the pathogen (Dangl & Jones 2001).

Induced resistance (IR) represents a physiological state of enhancing defensive capacity elicited by abiotic and biotic stimuli, whereby the plant's innate defenses are potentiated against subsequent challenges (Vallad & Goodman 2004). IR can occur at the site of the initial attack (local defense) or be functional in distant parts of the plant or throughout the entire plant (systemic defense) (Eyles et al. 2010).

One key process in systemic immunity is defense priming. When primed, the plant is set in an "alert" state in which defenses are not actively expressed but in which the response to an attack occurs faster and/or stronger than unprimed plants (Jung et al. 2012). This phenomenon is established not only in local/distal tissue exposed to the biological agents but also to various chemicals that mimic biologically induced priming (Pastor et al. 2013; Conrath et al. 2015).

Plant physiological responses upon pathogenic attack

Plants put effort into growth and development. Infection by pathogens alters both primary and secondary metabolism of the host leading to changes in the plant growth and development (Fagard et al. 2014). Reprogramming host metabolism seems to be a general feature of plant-pathogen interactions and is a consequence of both defense and the requirement for pathogens to acquire nutrients, in particular carbon sources (*e.g.* through sugar accumulation) and nitrogen (N) sources (*e.g.* through amino acid accumulation), which in turn can affect defense activation (Bolton 2009; Pieterse et al. 2009; Fagard et al. 2014). The expense of fitness associated with a pathogenic attack is called fitness cost and is associated with the allocation of large quantities of resources to resistance instead to growth, reproduction, and yield (Heil & Baldwin 2002; Vos et al. 2013).

It is clear that, due to the high demand to activate plant defense pathways, the photosynthetic performance may be altered. During photosynthesis, two key events occur: light reactions, in which plants harvest light energy to generate ATP and reducing power in the form of NADPH, and dark reactions, in which CO_2 is fixed into carbohydrates (needed for various biological processes such as defense) by utilizing ATP and NADPH (Ashraf & Harris 2013). The proper assessment of the photosynthetic performance of plants under pathogen infection can provide crucial insight into the mechanisms underlying their interactions, with the potential for identifying novel strategies for crop protections (Tatagiba et al. 2015). Methods such as gas-exchange measurements, particularly when combined with chlorophyll (Chl) *a* fluorescence, can provide a detailed analysis of how an infected leaf may respond to a certain infection (Rolfe & Scholes 2010; Tatagiba et al. 2015).

Characteristic gas exchange parameters, such as CO_2 assimilation rate (A), transpiration (E), internal CO_2 concentration (Ci) and stomatal conductance (gs), are usually the most assessed parameters. Two hypotheses are proposed to explain the relationship between photosynthesis and defense response (Bolton 2009; Massad et al. 2012). Firstly, since photo-assimilates serve as source of carbon skeletons, energy, and reducing equivalents, defense induction requires an increased demand for photosynthesis (Göhre et al. 2012). On the other hand, the production of defense-related compounds (*e.g.* phytoalexins and phenolics) can become a priority and primary activities like photosynthesis are reduced until the pathogenic growth is terminated (Bolton 2009; Göhre et al. 2012).

Fluorescence is a very sensitive marker for the efficiency of photosynthesis since the energy of absorbed photons can either be used for photosynthetic electron transport or be dissipated as heat or fluorescence (Berger et al. 2007). This parameter responds to the changes in energy conversion at photosystem II (PSII) reaction centers and is sensitive to any limitations in the dark enzymatic steps of the complex process of photosynthesis (Berger et al. 2007). Changes of chlorophyll fluorescence measurements were verified, for example, in *Glycine max* infected by *Pseudomonas syringae* (Zou et al. 2005) and in *Triticum aestivum* infected by *Pyricularia aryaze* (Göhre et al. 2012). A typical response observed in host-pathogen interactions is an initial reduction in the quantum yield of PS II photochemistry (ϕPSII), an increase in the non-photochemical processes and a decline in maximum quantum yield of PSII photochemistry (Fv/Fm) measurements. At later stages of infection, all of these parameters decline as the photosynthetic apparatus is destroyed (Rolfe & Scholes 2010). Apart from photosynthesis, photorespiration varies during defense response. Plant respiration is known to be stimulated since this pathway is a major source of energy and reactive oxygen species (ROS) (Kangasjarvi et al. 2011; Sørhagen et al. 2013; Rojas et al. 2014).

In terms of primary metabolism, carbohydrate metabolism is among the most important variables to quantify. Carbohydrates support the production of plant phenolics and terpenes, many of which play a role in defense (Berger et al. 2007). Many plant responses to the attack of a pathogen are closely associated with the pathways regulating the level of sugars in the plant cell (Morkunas & Ratajczak 2014). Total soluble sugars, such as glucose and sucrose, also function as signaling molecules (Wind et al. 2010), affecting the expression of certain genes that enhance the expression of anthocyanin biosynthesis genes. Anthocyanins are one class of flavonoids responsible for the red, pink, purple and blue pigmentation in plants (Landi et al. 2015). Induced under stress conditions and infection by pathogens, anthocyanins serve as protectors against oxidative damage (Zhang et al. 2014).

Additionally, N metabolism is strongly affected by pathogen infection, probably as a result of both defense activation and attempted pathogen manipulation of host metabolism for nutritional purposes (Schultz et al. 2013; Fagard et al. 2014). Amino acids are sources of N and their metabolism can impact plant-pathogen interactions. Proline (Pro) is a proteinogenic amino acid essential for primary metabolism. Pro presents osmoprotective functions, and its accumulation is related with biotic and abiotic stresses (Szabados & Savouré 2009).

Furthermore, many tiers of activated defense involve the production of ROS (*e.g.* superoxide and hydrogen peroxide) and the perception of an invading pathogen leads to the increase of production of these metabolites (O'Brien et al. 2012). One of the most rapid defense reactions is commonly referent to as oxidative burst and is accompanied by changes in extracellular pH, ion fluxes and protein phosphorylation (Kangasjärvi et al. 2012; O'Brien et al. 2012). ROS have been implicated not only in direct antimicrobial roles but also in cellular signaling associated with the induction of defense gene expression, the hypersensitive response (HR), cell wall protein cross-linking, phytoalexin production, callose deposition and SAR. ROS can react with proteins, DNA, and membrane lipids to reduce photosynthesis, increase electrolyte leakage, and accelerate senescence and cell death (O'Brien et al. 2012). One of the most used indicators of oxidative stress is lipid peroxidation, which usually increases under stress conditions (Sharma et al. 2012).

Disease management

Control methods to reduce the effects of pitch canker have been unsuccessfully investigated (Conejero 2013), thus proactive prevention is the best way to prevent losses due to pitch canker (Dreaden & Smith 2010). Management programs consist in the proper nursery and silvicultural management, monitoring plantations, adequate quarantine measures (*e.g*. burn infected trees, removing logging slash and pruned infected branches), exportation bans and genetic selection of resistant species (Dreaden & Smith 2010; Vivas et al. 2012). In plantations, the best way to avoid outbreaks is the correct and appropriate utilization of forestry techniques. Environmental stress rises the incidence of disease, so plantation sites must be appropriate with a correct tree density and drainage (de Paiva 2011). The excessive use of fertilizer should be avoided because it can increase disease incidence and severity (Lopez-Zamora et al. 2007). In nurseries, one of the most important means of prevention is the utilization of seeds and seedlings free of pathogen (Wingfield et al. 2008).

Thus, no cost-effective strategy for the use of anti-fungal materials to manage pitch canker has yet been developed. An integrated disease management (IDM) approach can, however, reduce the economic impact of the disease (Hammerbacher 2006) as well as avoid the use of fungicides.

The role of phosphites

At present, effective management of fungal plant diseases is generally achieved by the use of fungicides. However, these present undesirable traits such as high and acute environmental and health toxicity, and their use is now limited (Satish et al. 1999; Bock et al. 2012). The requirement for more environmental-friendly methods to manage forest diseases justifies exploration of alternative approaches (Machinandiarena et al. 2012).

The use of resistance elicitors within an integrated disease management (IDM) strategy offers new opportunities for control of plant pathogens as well as enabling lower fungicide inputs (Lyon & Newton 1997). These elicitors are characterized as biocompatible chemical compounds that enhance disease resistance in plants (Machinandiarena et al. 2012; Fitza et al. 2013), and must have low cost, favorable safety profile, and low toxicity (Deliopoulos et al. 2010). Inorganic salts (*e.g.* chlorides, phosphates or bicarbonates) present these properties making them desirable for inclusion in IDM programs (Deliopoulos et al. 2010). Among them, phosphites have received particular attention. Phosphites are a group of inorganic salts that can be used in the control of pathogens. The term phosphite ($H_2PO_3^-$; Phi) is a generic name used for alkali metal salts of phosphorus acid (H_3PO_3) (Silva et al. 2011). It is a simple compound with high solubility and capability for association with other compounds (*e.g.* potassium, zinc, copper) (Daniel & Guest 2005; Lobato et al. 2008; Bock et al. 2012; Machinandiarena et al. 2012). With an ambimobile nature, Phi accommodates the potential for protection of plant tissues and organs distant from the point of application (Daniel & Guest 2006). They also are relatively inexpensive and present low toxicity to invertebrates, aquatic organisms, and animals at an effective application rate (Ali et al. 1999; Scott et al. 2013).

After Second World War, phosphites were proposed as fertilizers providing a source of phosphorus (P), which is a non-renewable resource and essential nutrient for the metabolic processes for all living organisms (López-Arredondo & Herrera-Estrellla 2012). However, the data available about Phi potential as P source are ambiguous, being dependent on the crop and type of application (Thao & Yamakawa 2009). Recently, phosphite compounds have been studied due to their capability for reducing infection by *Pythium* spp. in turfgrasses (Cook et al. 2009), by *Fusarium solani* and *Phytophthora infestans* in *Solanum tuberosum* (Lobato et al. 2008), or *Plasmopara*

viticola in *Vitis vinifera* (Pereira et al. 2012), among others. On woody species, phosphite reduced infection by *Phytophthora plurivora* in *Fagus sylvatica* (Dalio et al. 2014), by *Pseudomonas syringae* in *Aesculus hippocastanum* (Percival & Banks 2015) and by *Phytophthora cinnamomi* in *Banksia grandis* and *Eucalyptus marginata* (Scott et al. 2015).

The mode of action of Phi is complex and not fully understood (Silva et al. 2011; Bock et al. 2012). Possibly, it includes direct inhibition of pathogen growth and stimulation of host defenses through the synthesis of antimicrobials and ROS, cell wall changes, and induction of PR proteins (Machinandiarena et al. 2012; Gozzo & Faoro 2013). This complexity of mechanisms has limited the development of pathogen resistance to these substances (Landschoot & Cook 2005).

The application methods depend on the crop-pathogen combination (Deliopoulos et al. 2010). The most common techniques are foliar sprays and trunk injections. However, they require specialized equipment and training for proper application, which makes its use restrictive and laborious (Scott et al. 2015). Other approaches are root or soil drenches, seed treatments, through drip irrigation, and mixing in hydroponic nutrient solutions. More recently, soluble, and slow-release implants of Phi have been developed that can be inserted into the stems, without the need to mix chemicals or use injection equipment. Yet, it requires drilling into trees and may not be feasible for rapid widespread use in natural ecosystems (Deliopoulos et al. 2010; Scott et al. 2015).

Thesis main purposes

Phytopathogenic organisms compromise the sustainability of native forests and the productivity of plantations, posing a severe threat to these essential resources. While is difficult to provide a realistic estimate of the economic losses, plant diseases have been reported to cause damage to the forestry economy in many parts of the world.

Pitch canker caused by *Fusarium circinatum* is one of the most important diseases affecting *Pinus* species. It is frequently associated high levels of tree mortality and, to date, there are no proven methods for the disease control. The quarantine measures applied reveal to be expensive and it is necessary to find solutions not only economically viable but also environmentally friendly. The induction of resistance, through the pretreatment of plants with chemical or biological elicitors, presents a viable approach for the control of phytopathogenic diseases. Phosphite (Phi) salts are one example of inexpensive and innocuous elicitors that may also present a direct effect in the pathogen.

The main objectives of this work were (i) to study the effects of potassium phosphite (KPhi) in the control of *F. circinatum* in *Pinus radiata*; (ii) to understand the effects of Phi in the plant physiology; and (iii) to understand the effects of Phi in *F. circinatum* mycelial growth. In consequence, *in vitro* and *in vivo* assays were performed.

With the results from this study, I expect to contribute for the improvement of current disease management strategies, and gather knowledge about the interaction fungus-elicitor-host and its effects on the host physiology.

Part II

Phosphite delays the progression of *Fusarium circinatum* on *Pinus radiata*: a physiological approach

Cerqueira A[1], Alves A[1], Berenguer H[1], Correia B[1], Monteiro P[1], Casero J[2,3], Pinto G[1*]

[1] Department of Biology & CESAM – Centre for Environmental and Marine Studies, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[2] Sustainable Forest Management Research Institute, University of Valladolid - INIA, Avenida de Madrid 44, Palencia, Spain

[3] Department of Vegetal Production and Forest Resources, University of Valladolid, Avenida de Madrid 44, Palencia, Spain

* corresponding author: gpinto@ua.pt

Abstract

Fusarium circinatum is a necrotrophic fungus responsible for the pitch canker disease affecting *Pinus* spp. and *Pseudotsuga menziesii* all over the word. Characterized by the formation of large resinous cankers that girdle shoots, branches, and trunks, leading to the death of the host, the pitch canker is subject of management strategies and quarantine measures that represent significant economic losses. Thus, there is the need for alternative, inexpensive and environmentally friendly approaches for disease control. Induction resistance, through pretreatment of plants with chemicals or biologically derived compounds, presents an alternative for the control of phytopathogenic organisms. Phosphite (Phi) is an inorganic salt that not only presents effects in the pathogen, but also has the capability of inducing host resistance. In this study, we tested the efficiency of potassium phosphite (KPhi) on *Pinus radiata* plants, inoculated and non-inoculated, as well as its effects in the fungal growth. Initially, *F. circinatum* colonies were grown in PDA medium supplemented with Phi (0%, 1% and 4%) to evaluate its effects on the radial growth of the fungus. Posteriorly, *P. radiata* plants were sprayed with Phi (0%, 1% and 4%) 17 days prior to inoculation with *F. circinatum*. Survival and physiological performance (water potential, gas exchange and photochemical performance, pigments, lipid peroxidation, electrolyte leakage, proline and carbohydrates) were assessed. Results showed that Phi was effective in delaying the pitch canker symptoms in a dose dependent manner similarly to what was observed regarding mycelial growth

inhibition during *in vitro* assays, and the effects of *Fusarium* inoculation were appeased by Phi application. Therefore, these results suggest that the application of Phi salts offers a viable alternative to the management of the pitch canker disease.

Keywords

Pitch canker disease, *Pinus radiata*, phosphite, induced resistance, physiology

Introduction

In Europe, forests play important roles at natural and anthropic levels, representing 33% of the total land area (215 million ha) (Ayanz et al. 2015). Conifers account for 112.95 million of the European forest area, being mainly composed by *Pinus* spp. (de Rigo et al. 2016; Pividori et al. 2016). *Pinus* is the most ecologically and economically significant tree genus in the world (Richardson & Rundel 2000). In addition to its many effects in the ecosystems, pines represent an important source of timber, pulp and paper, seeds, charcoal, resin, and construction materials (Richardson & Rundel 2000; Ioannou et al. 2014).

Phytopathogenic organisms pose a serious threat to the conservation and the productivity of native forests and plantations (Iturritxa et al. 2015). The pitch canker caused by the necrotrophic fungus *Fusarium circinatum* Nirenberg & O'Donnell 1998 (teleomorph *Gibberella circinata*) is one of the most important pathogens affecting *Pinus* spp. and *Pseudotsuga menziesii* (Mirb.) Franco worldwide (Wingfield et al. 2008; Berbegal et al. 2013). Since it was first described in 1946 in the United States (Hepting & Roth 1946), *F. circinatum* has been reported in Japan (Muramoto & Dwinell 1990), South Africa (Viljoen et al. 1994), Mexico (Britz et al. 2001), Chile (Wingfield et al. 2002), Spain (Landeras et al. 2005), Italy (Carlucci et al. 2007), France (EPPO 2009), Portugal (Bragança et al. 2009) and Brazil (Pfenning et al. 2014). In Europe, *F. circinatum* appears on the list of pests (A2) recommended for regulation as quarentine pest (EPPO 2015).

Successful pathogens have the ability to subvert plant defense mechanisms either by avoiding their recognition or by reprogramming host metabolism, leading to changes in the host growth and development (Dalio et al. 2014; Fagard et al. 2014). Altered metabolism involves changes in the photosynthetic performance and carbohydrate metabolism, nitrogen metabolism and in the production of reactive oxygen species (ROS) and antioxidants (Berger et al. 2007; Bolton 2009; O'Brien et al. 2012). *F. circinatum* affects all stages of tree development, provoaking the development of large resinous cankers that impregnate the tissues and stop the moviment of water and nutrients (Iturritxa et al. 2011; Martín-Rodrigues et al. 2013). Major symptoms include wilting

and chlorosis of the needles, and damping of seedlings causing canopy dieback and tree mortality (Fitza et al. 2013; Santana et al. 2015). Despite the vast host range, pine species present differential resistance to *F. circinatum* (Wingfield et al. 2008). One of the most economically relevant pine species in the world, Monterey pine (*Pinus radiata* D. Don), appears to be the most susceptible species to *F. circinatum* infection (Gordon et al. 2001; Mead 2013).

To date, there are no means to control the pine pitch canker pathogen and current strategies represent elevated economic costs (Pérez-Sierra et al. 2007; Wingfield et al. 2008). Management programs include monitoring of plantations, quarantine measures (*e.g.* burn infected trees, removing logging slash and pruned infected branches), exportation bans and genetic selection of resistance species (Wingfield et al. 2008; Vivas et al. 2012). The induction of resistance is one alternative disease management approach that has been widely explored in agriculturally important crop plants (reviewed by Walters et al. 2013). Induced resistance (IR) represents a physiological state of enhanced defensive capacity stimulated by a biotic or abiotic elicitors, whereby the plant's innate defenses are potentiated against subsequent challenges (Vallad & Goodman 2004). Induced resistance prompts a highly coordinated and integrated response which involves anatomical modifications (*e.g.* lignification), and production of chemicals (*e.g.* the production of terpenoids) and proteins (*e.g.* pathogenesis related [PR] proteins) with antimicrobial activity (Fitza et al. 2013).

Phosphites ($H_2PO_3^-$; Phi), a group of alkali metal salts of phosphorus acid, are used as fertilizers, fungicides and present the capability of activating plant resistance (Silva et al. 2011; Liu et al. 2016) functioning as resistance elicitor. Despite its mode of action being complex and not fully understood (Silva et al. 2011; Bock et al. 2012), it certainly includes inhibition of pathogen growth and stimulation of host defense through synthesis of antimicrobials and ROS, cell wall changes, and induction of PR proteins (Machinandiarena et al. 2012; Gozzo & Faoro 2013). Phi is known for reducing infection by *Pythium* spp. (Cook et al. 2009), *Fusarium solani* and *Rhizoctonia solani* (Lobato et al. 2008), *Phytophthora* spp. (Gentile et al. 2009; Dalio et al. 2014; Scott et al. 2015), *Pseudomonas syringae* (Percival & Banks 2015), and *Plasmopara viticola* (Pereira et al. 2012). Despite these potentialities, there is not report for the use of Phi in the control of *F. circinatum* infection.

In this study, we investigated the mechanism of action of potassium phosphite (KPhi) on plant physiological performance of treated *Pinus radiata* plants in order to better understand its protective role in *Pinus-Fusarium circinatum* interaction. The effects of Phi in the *in vitro* pathogen

growth were evaluated. With study we expect to contribute to the improvement of current *F. circinatum* disease management programs as well as gather more knowledge on the interaction between fungus, elicitor, and the plant physiological performance.

Material and methods

In vitro assay

An isolate of *Fusarium circinatum* was obtained from the collection of the Forest Entomology and Pathology Laboratory at the University of Valladolid. Isolates were grown on half strength potato dextrose agar (PDA; Merck, Darmstadt, Germany) medium with addition of potassium phosphite (KPhi from now on identified as Phi; Trafos Sinergy, Nutrisapec, Portugal) at 0%, 1% and 4% prepared in distilled water (v/v; pH 5.6). Colonized agar plugs were inoculated at the center of each petri dish, followed by incubation at 20 ± 2°C. Measurements of the mycelia radial growth were performed every day until the control reached the edge of the petri dish (8 days).

In vivo assay

Plant material

Six-month old *Pinus radiata* (25 ± 1 cm height) plants were obtained from Cultiflor Nursery (Pombal, Leiria; 40°00'22.5"N, 8°48'20.0"W). The plants were placed in 1L plastic pots filled with 3:2 (w/w) peat:perlite and kept in an climate chamber (Fitoclima D1200, Aralab, Portugal) under a 16 h light/8 h darkness photoperiod at 25°C/15°C, respectively, and 60% relative humidity. Photon flux density (PFD) during the day was 500 µmol m^2 s^{-1}. The plants were acclimatized to these conditions for 2 months and well-watered every other day and fertilized (Frutifol, Nufarm, Portugal) every week.

Experimental procedure

After the acclimatization period three groups of 90 *P. radiata* plants each were treated as follows: (i) plants untreated with Phi (C); (ii) plants sprayed with Phi at 1% (Phi $_{1\%}$); (iii) plants sprayed with Phi at 4% (Phi $_{4\%}$).

The solutions of Phi (pH 5.6) at 1% (v/v) and 4% (v/v) were prepared from a commercial solution (Trafos Sinergy, Nutrisapec, Portugal). Foliar sprays of potassium phosphite were applied until run-off using a hand-held sprayer 17 days prior to inoculation to allow the absorption and uptake of Phi before plant inoculation. The untreated control group were sprayed with distillated water.

For inoculations, *F. circinatum* isolates were grown on half strength PDA medium at 20 ± 2°C for 7 days. Inoculation was performed as described by Cinelli et al. (2015). Seedlings were wounded with a sterile scalpel and an agar plug with mycelium grown was placed into each wound and sealed with Parafilm®. Sterile PDA plugs were used as a negative control. The above treatments (i, ii and iii) were then divided in two groups of inoculated with *Fusarium circinatum* and non-inoculated plants resulting in a total of six treatments: (i) plants with 0% Phi non-inoculated (C); (ii) plants with 0% Phi inoculated (F); (iii) plants with 1% Phi non-inoculated ($Phi_{1\%}$); (iv) plants with 1% Phi inoculated (F+$Phi_{1\%}$); (v) plants with 4% Phi non-inoculated ($Phi_{4\%}$); and (vi) plants with 4%Phi inoculated (F+$Phi_{4\%}$). The all experiment was conducted as described above for the acclimatization period. The plants were watered every other day until field capacity and were randomly moved every two days.

Sample collection

Samples were collect when 50% of the inoculated plants within each group displayed the initial typical disease symptoms (tip dieback). Also, to confirm Koch's postulates, cuts of the stem (above the inoculation point) were place in PDA medium and incubated at 20 ± 2°C for 7 days.

After the measurement of *in vivo* related parameters such as survival, growth, water potential, gas exchange, chlorophyll fluorescence and electrolyte leakage, needles were collected and stored at -80°C for biochemical analysis (quantification of pigments, proline, total soluble sugar content and lipid peroxidation). Only symptomatic plants were measured.

Growth, visual aspect evaluation and plant survival

To assess growth, the height of all plants was recorded for each treatment. This parameter was calculated taking in consideration the height (cm) of the plants in the end of the experiment and at the time of inoculation.

The visual aspect of plant was evaluated throughout the experiment by visual observation in order to assess plant health status (tip dieback and wilting of needles). Plant survival was monitor weekly and until all plants displayed symptoms.

Water potential

Water potential was measured above the point of inoculation with a Scholander-type pressure chamber (PMS Instrument Co., OR) in six independent biological replicates per each treatment.

Gas exchange parameters

Net CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (gs, molH_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and intercellular CO_2 concentration content (Ci, ppm) were measured with a gas-exchange system (LCpro-SD, ADC BioScientific Limited, Hertfordshire, UK) with a conifer type chamber. Inside the chamber, the following conditions were maintained during all the measurements: Ca (ambient CO_2 concentration): 410 µmol m^2 s^{-1}; air flux: 201 µmol s^{-1}; block temperature: 21.5°C. To find out the saturation light intensity A/PPFD (light response curves of CO_2 assimilation) curves were performed with the following photosynthetic photon flux density (PPFD): 2500, 2000, 1500, 1000, 750, 500, 250, 100, 50 and 0 µmol m^{-2} s^{-1}. Measurements at saturation light intensity were performed at 1000 µmol m^{-2} s^{-1}. Data were recorded when the measured parameters were stable (2–6 min).

Chlorophyll fluorescence

Chlorophyll fluorescence was estimated by measurement, at room temperature, of chlorophyll fluorescence kinetics on six plants using MINI-PAM photosynthesis yield analyzer (Walz Company, Effeltrich, Germany). Light adapted components of chlorophyll were measured: steady-state fluorescence (F), maximal fluorescence (F'm), variable fluorescence (F'v, equivalent to F'm − F) and quantum yield of PSII photochemistry (ϕPSII) equivalent to (F'm − F)/F'm.

For dark adapted components, the needles were kept in the dark using dark leaf clips for 20 min for minimum fluorescence (F0), maximum fluorescence (Fm), variable fluorescence (Fv, equivalent to Fm – F0) and maximum quantum yield of PSII photochemistry (Fv/Fm) measurements.

Electrolyte leakage

Electrolyte leakage (EL) was measured according to Escandón et al. (2016). Six biological replicates of 100 mg of needle pieces (1 cm long) were collected from each treatment, washed three times and immersed in 20 mL of Milli-Q water, and then incubated for 12 h at room temperature, under agitation, at 150 rpm on a Lab-Line Orbit Shaker. Maximum conductivity was measured after autoclaving for 20 min (1100 kP and 121°C), and total conductivity was measured after cooling at room temperature under agitation for 5 h. Leakage was calculated using the equation EL% = [(Cexp – Ci / Ct – Cii)] x 100, where C is water conductivity under control condition (Ci), control condition after autoclaving (Cii), experimental condition (Cexp) and after autoclaving (Ct).

Pigment analysis

For pigment content determination, six replicate samples (50 mg) from each treatment were used. Total chlorophyll and carotenoids were quantified according to Sims & Gamon (2002). Chlorophyll a (Chla), chlorophyll b (Chlb), and carotenoids (Car) were extracted with acetone/Tris (50 mM) buffer (80:20, v/v) at pH 7.8. After homogenization and centrifugation, supernatants were used to read absorbances at 663, 537, 647 and 470 nm and the content of each pigment was calculated using the equations proposed by Sims & Gamon (2002).

Anthocyanin (AA) content was determined according to Sanchez-Zabala et al. (2013). Pigments were extracted with acidified methanol (methanol:HCl, 99:1, v/v). Then, the homogenate was immersed in boiling water for 1.5 min and left for 24 h in the dark at 5°C. After centrifugation (10 000 g for 10 min at 5°C), absorbance was measured at 529 and 650 nm. Anthocyanin content was determined according the equation proposed by Sims & Gamon (2002). The average of anthocyanin content was calculated using an extinction coefficient of 30 000 mol^{-1} cm^{-1}.

Lipid peroxidation

Lipid peroxidation was estimated by measuring the amount of malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids. The tissues (50 mg) of six biological replicates from each treatment were homogenized in 1.25 mL of 5% trichloroacetic acid (TCA) and centrifuged at 10 000 g for 5 min. Afterwards, 1 mL of supernatant was mixed with 1 mL of 0.65% of thiobarbituric acid (TBA) in 20% TCA with 0.01% of butylated hydroxytoluene (BHT) and incubated at 95°C for 30 min. This was immediately cooled on ice and absorbance was read at 532 and 600 nm. Lipid peroxidation was calculated applying the formula by Heath and Packer (1968).

Proline content

Proline content was determined as described by Bates et al. (1973) with slight modifications. Plant tissue (100 mg) from six biological replicates were homogenized with 1.5 mL of sulphosalicylic acid (3%, w/v). Following centrifugation, 1 mL of supernatant was collected and 1 mL of ninhydrin acid and 1 mL of glacial acetic acid were added. After incubation at 100°C and cooling on ice, 2 mL of toluene were added to the solution and the absorbance was read at 520 nm. Free proline content was calculated using a standard curve.

Total soluble sugars

Total soluble sugars were extracted as described by Chow & Landhäusser (2004). Plant material (100 mg) from six biological replicates was homogenized with 4 mL of ethanol (80:20, v/v). Then, the samples were boiled for 15 min and the supernatant was collected after centrifugation (10 000g, 5 min). The resulting pellet was re-extracted twice more and the supernatants were combined. Total soluble sugars were quantified by the anthrone method (Irigoyen et al. 1992). Total soluble sugars content was calculated using a D-glucose standard curve.

Statistical analysis

The results are presented as mean with standard deviations of six independent replicates. All statistical procedures were performed using SigmaPlot for Windows (Systat Software for Windows v. 12.0 Systat Software Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was performed for non-inoculated and inoculated groups followed by post hoc multiple comparison using Holm-Sidak test was performed to estimate the significance of the results. Different letters indicate significant differences between conditions ($p < 0.05$), capital letters refer to inoculated plants group and lowercase letters refers to non-inoculated plant groups. When needed, a t-student test was performed to compare *Fusarium* effects within a given concentration of Phi.

Results

***In vitro* assay**

The effect of Phi on the in vitro growth of *F. circinatum* cultures at the end of the assay is shown in figure 1. The cultures grown in PDA supplemented with $Phi_{1\%}$ presented an average radial growth of 4.52 cm (b) when compared to control (a), while the cultures grown with $Phi_{4\%}$ presented an average radial growth of 1.05 cm (c).

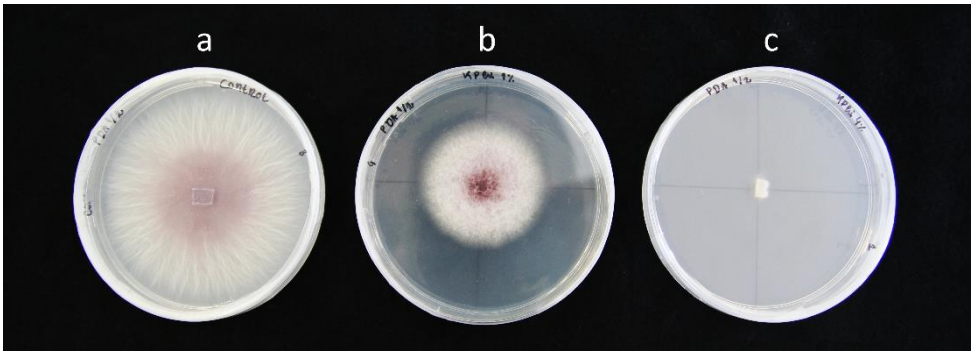

Figure 1: Aspect of the *F. circinatum* cultures after 8 days growing in PDA medium: control (a; Phi 0%), and supplemented with Phi 1% (b) and Phi 4% (c).

Growth, visual aspect and plant survival

Considering plants subject to pre-treatment with Phi, $Phi_{1\%}$ presented a significant increase in growth when compared to control, whereas $Phi_{4\%}$ plants showed a decreased rate (table 1). Inoculated plants presented a general decrease in growth when compared to control (table 1).

At morphological level, control plants and treated with $Phi_{1\%}$ showed no visual signals of damage in the needles (figure 2). On contrary, $Phi_{4\%}$ treatment induced extensive browning of the needles (figure 2). However, in the end of experiment signs of recovery were observed, particularly in the apical shoot.

Table 1: Height increment (in percentage) of *P. radiata* plants after *F. circinatum* inoculation and Phi treatment. Data are presented as mean ± SD. Different capital letters indicate differences between inoculated plants treatments and lowercase letters indicate differences between non-inoculated plants ($p < 0.05$). The asterisk (*) indicates differences between inoculated and non-inoculated plants within a given concentration of Phi ($p < 0.05$).

	C	**F**	**$Phi_{1\%}$**	**F+$Phi_{1\%}$**	**$Phi_{4\%}$**	**F+$Phi_{4\%}$**
Height	6.90 ± 1.71 a	4.70 ± 2.54 A *	11.70 ± 4.44 b	5.69 ± 2.71 A *	4.00 ± 2.65 a	0.00 ± 0.00 B *

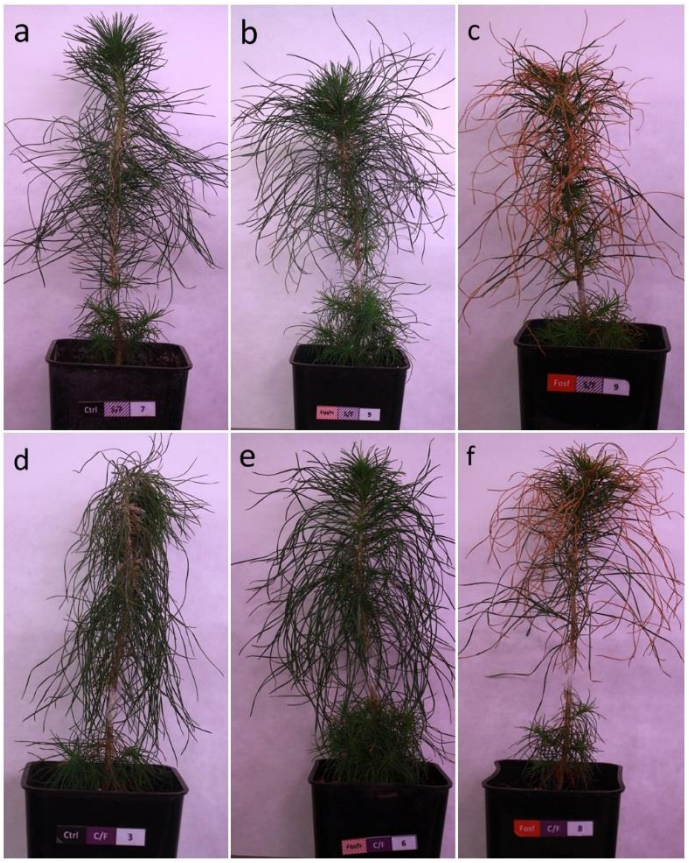

Figure 2: Morphological aspect of *P. radiata* plants at the end of the assay: (a) with 0% Phi non-inoculated; (b) with 1% Phi non-inoculated; (c) with 4% Phi non-inoculated; (d) with 0% Phi inoculated; (e) with 1% Phi inoculated; and (f) with 4% Phi inoculated.

Infected plants without Phi pre-treatment showed the first symptoms of disease 7 days post-inoculation (dpi) and all plants presented concomitant dieback, while all F+Phi$_{1\%}$ and F+Phi$_{4\%}$ plants revealed symptoms 30 and 37 dpi, respectively, and died more gradually (figure 3). Irrespective to Phi pre-treatment or not, no non-inoculated plants were lost.

F. circinatum was re-isolated from artificially inoculated plants and no pathogen was recovered from control plants, therefore respecting Koch's postulates.

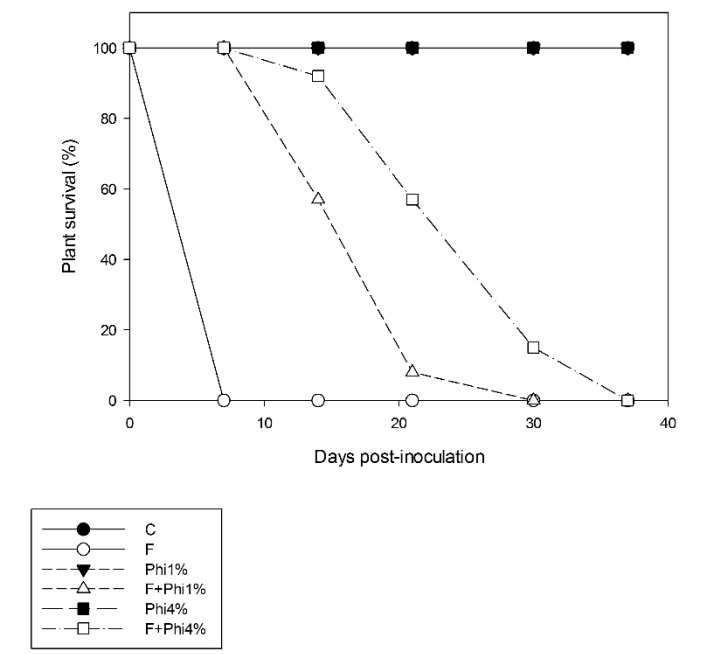

Figure 3: Time course of survival (in percentage) of *P. radiata* plants treated/untreated with Phi and inoculated/non-inoculated with *F. circinatum*. Day 0 (zero) corresponds to inoculation day.

Water potential

Considering water potential, only $Phi_{4\%}$ treatment showed significantly lower values when compared to control (figure 4). After inoculation, infected plants without Phi pre-treatment revealed a decrease in water potential in relation to control; however, this was not significant between inoculated and non-inoculated Phi pre-treated plants (figure 4).

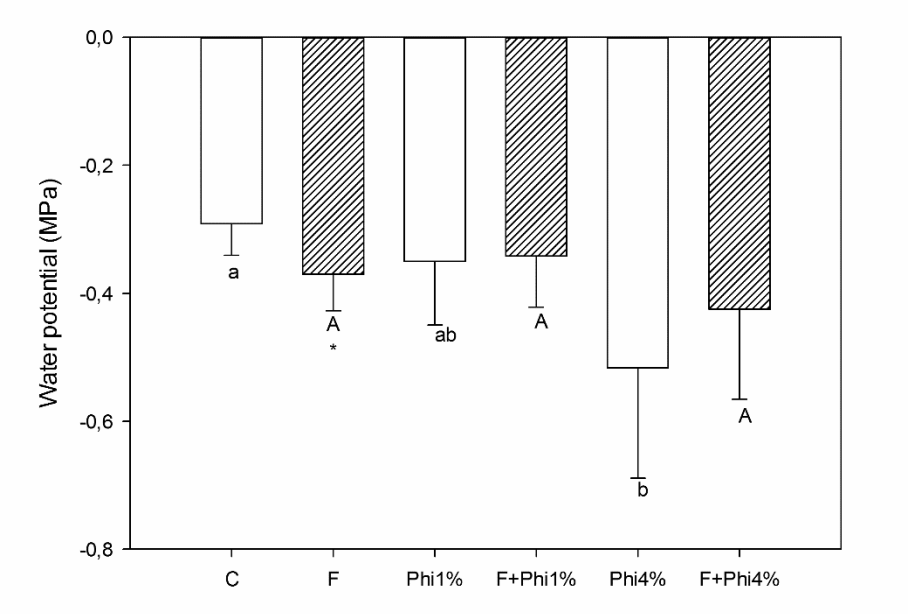

Figure 4: Water potential of *P. radiata* plants after *F. circinatum* inoculation and Phi treatment. Data are presented as mean ± SD. Different capital letters indicate differences between inoculated plants treatments and lowercase letters indicate differences between non-inoculated plants ($p < 0.05$). The asterisk (*) indicates differences between inoculated and non-inoculated plants within a given concentration of Phi ($p < 0.05$).

Gas exchange parameters

Pre-treatment with $Phi_{1\%}$ led to a significant increase of stomatal conductance (gs), transpiration rate (E) and intercellular CO_2 concentration (Ci) while the photosynthetic rate (A) kept unaffected when compared with control (figure 5). On the other hand, $Phi_{4\%}$ presented increases in E and Ci, gs kept unchanged and A significantly decreased in relation to control (figure 5).

After inoculation, infected plants without Phi pre-treatment revealed a sharp decrease in gas exchange in relation to control, with E and A decreasing to almost zero (figure 5a, b), in addition to gs (figure 5c), and only Ci exhibited a higher value (figure 5d). When pre-treated with $Phi_{1\%}$ and $Phi_{4\%}$, inoculated plants displayed significantly higher values when compared to non-treated inoculated plants. Comparing to two Phi concentrations, differences were found in E, A and Ci with $Phi_{1\%}$ revealing higher values (figure 5). The concurrent analysis of inoculation and Phi pre-treatment revealed a difference in E, with Phi pre-treated plants showing lower transpiration rates (figure 5b).

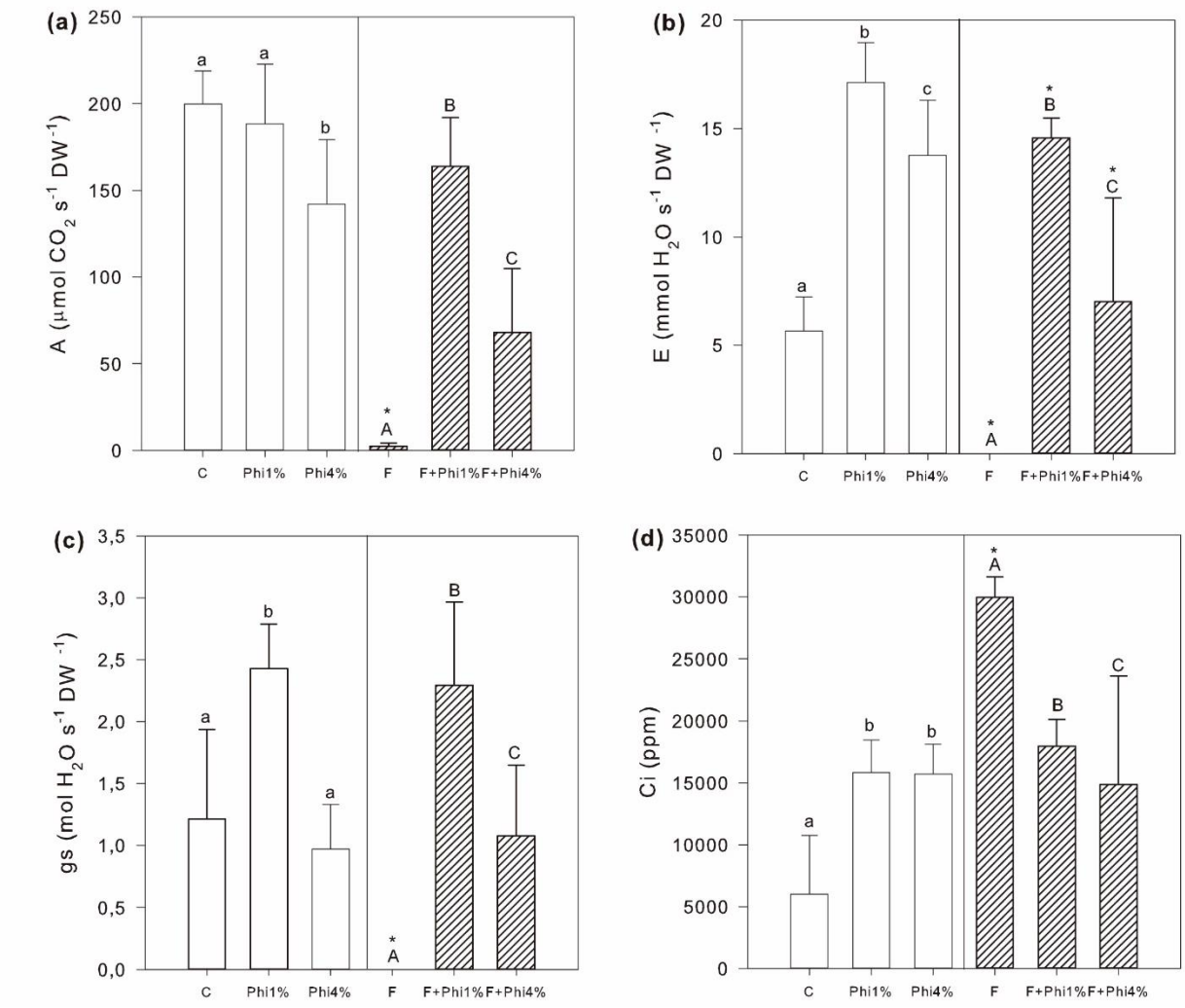

Figure 5: Gas exchange parameters: (a) net CO_2 assimilation rate (A), (b) transpiration rate (E), (c) stomatal conductance (gs) and (d) intercellular CO_2 concentration content (Ci) of *P. radiata* plants after *F. circinatum* inoculation and Phi treatment. Data are presented as mean ± SD. Different capital letters indicate differences between inoculated plants treatments and lowercase letters indicate differences between non-inoculated plants ($p < 0.05$). The asterisk (*) indicates differences between inoculated and non-inoculated plants within a given concentration of Phi ($p < 0.05$).

Chlorophyll fluorescence

Considering chlorophyll fluorescence, the maximal quantum yield of PSII (Fv/Fm) was significantly affected by the fungal inoculation; however, this effect disappeared in Phi pre-treated plants, although both inoculated and non-inoculated $Phi_{4\%}$ pre-treated plants showed a decreased in this parameter (figure 6a).

In relation to the effective photochemical quantum yield of PSII (ΦPSII), fungal inoculation was characterized by a significant reduction compared to non-inoculated plant and irrespective to Phi

pre-treatment. However, a global increase in ΦPSII was observed on $Phi_{1\%}$ pre-treatment in both inoculated and non-inoculated plants (figure 6b).

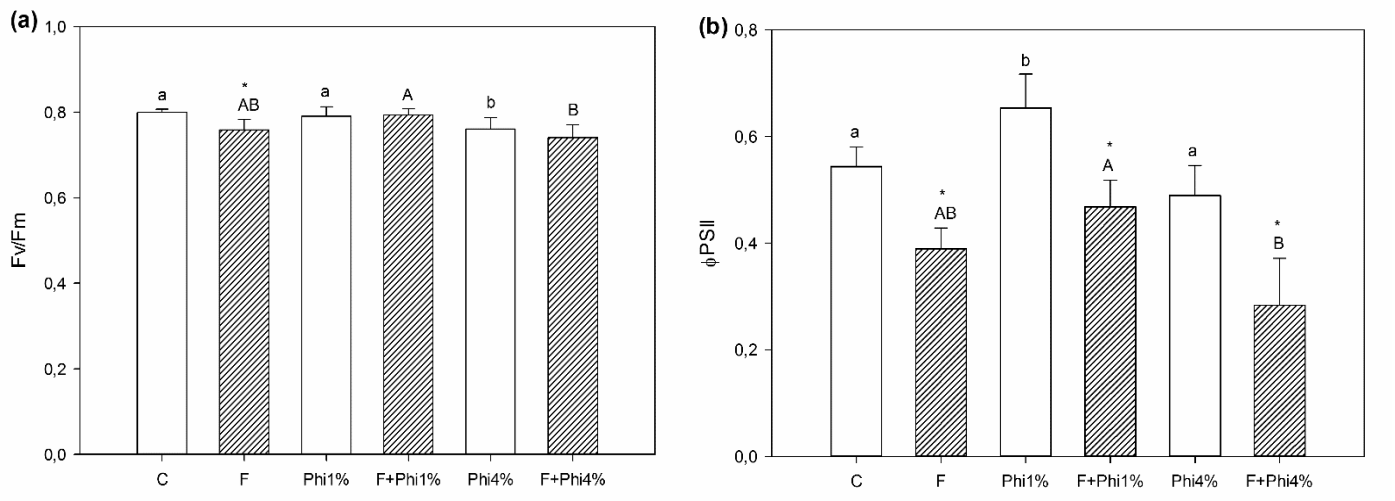

Figure 6: Maximal quantum yield of PSII (a; Fv/Fm) and effective quantum yield of PSII (b; ΦPSII) of *P. radiata* plants after *F. circinatum* inoculation and Phi treatment. Data are presented as mean ± SD. Different capital letters indicate differences between inoculated plants treatments and lowercase letters indicate differences between non-inoculated plants ($p < 0.05$). The asterisk (*) indicates differences between inoculated and non-inoculated plants within a given concentration of Phi ($p < 0.05$).

Electrolyte leakage

Application of Phi on non-inoculated did not affect electrolyte leakage (figure 7). On inoculated plants, the leakage increased in a dose-response manner with differences between all inoculated groups, but only $Phi_{4\%}$-treated inoculated plants presented a significant raise regarding to the corresponding non-inoculated group (figure 7).

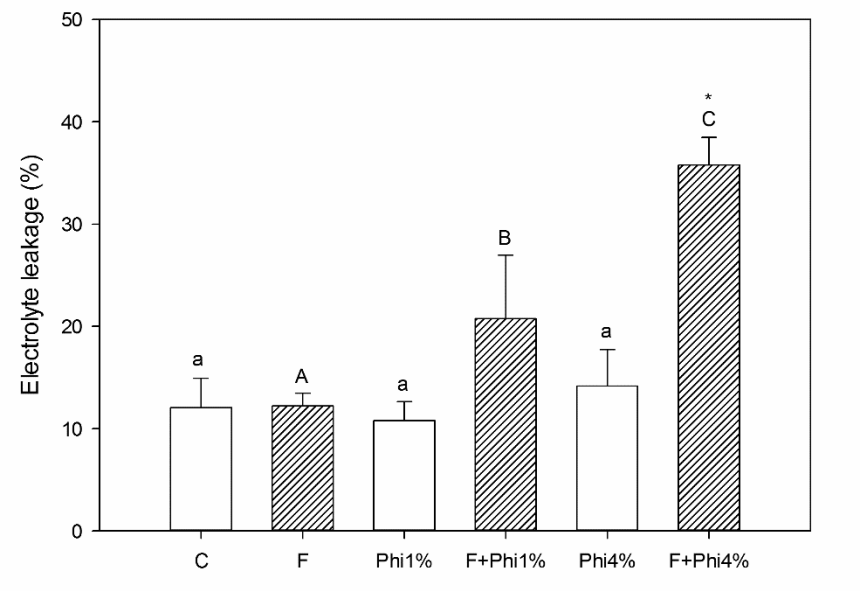

Figure 7: Electrolyte leakage of *P. radiata* plants after *F. circinatum* inoculation and Phi treatment. Data are presented as mean ± SD. Different capital letters indicate differences between inoculated plants treatments and lowercase letters indicate differences between non-inoculated plants ($p < 0.05$). The asterisk (*) indicates differences between inoculated and non-inoculated plants within a given concentration of Phi ($p < 0.05$).

Pigment content

Chlorophyll a (Chla), chlorophyll b (Chlb) and carotenoid (Car) contents displayed similar profiles (figure 8). The treatments with $Phi_{1\%}$ lead to a significant increase of Chla (figure 8a) and Car (figure 8c) and anthocyanins (figure 8d), while $Phi_{4\%}$ pre-treatment only significantly increased to a Car levels (figure 8c). Fungal inoculation had no significant effect on pigment content (figure 8).

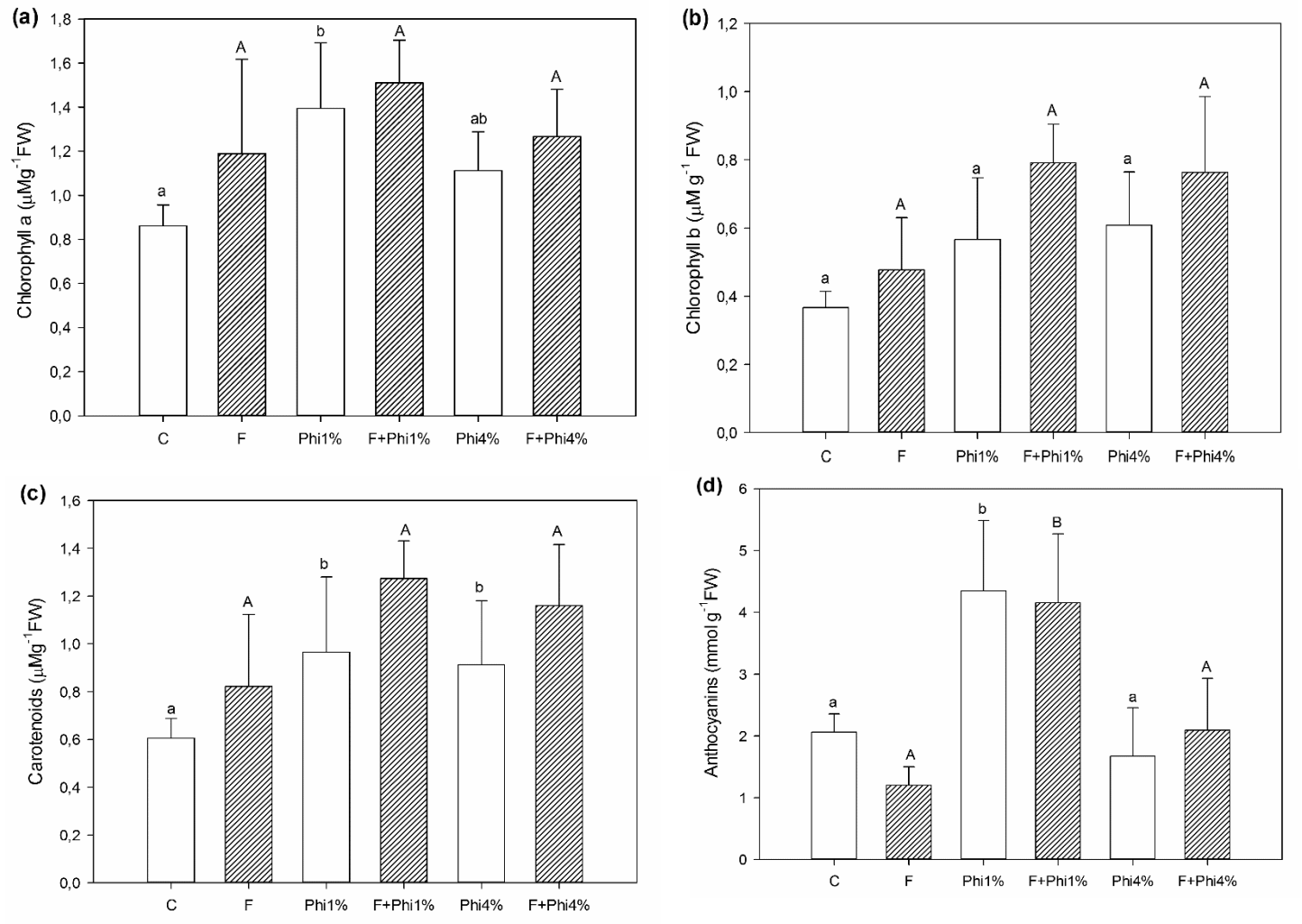

Figure 8: Chlorophyll a (a), chlorophyll b (b), carotenoids (c) and anthocyanins (d) content of *P. radiata* plants after *F. circinatum* inoculation and Phi treatment. Data are presented as mean ± SD. Different capital letters indicate differences between inoculated plants treatments and lowercase letters indicate differences between non-inoculated plants ($p < 0.05$). The asterisk (*) indicates differences between inoculated and non-inoculated plants within a given concentration of Phi ($p < 0.05$).

Lipid peroxidation

Lipid peroxidation, estimated in terms of malondialdehyde (MDA) content, showed a decreased, in both inoculated and non-inoculated plants, significant in $Phi_{4\%}$ (figure 9). After inoculation, all Phi pre-treated and non-treated plants also revealed a decrease in relation to the corresponding non-inoculated group.

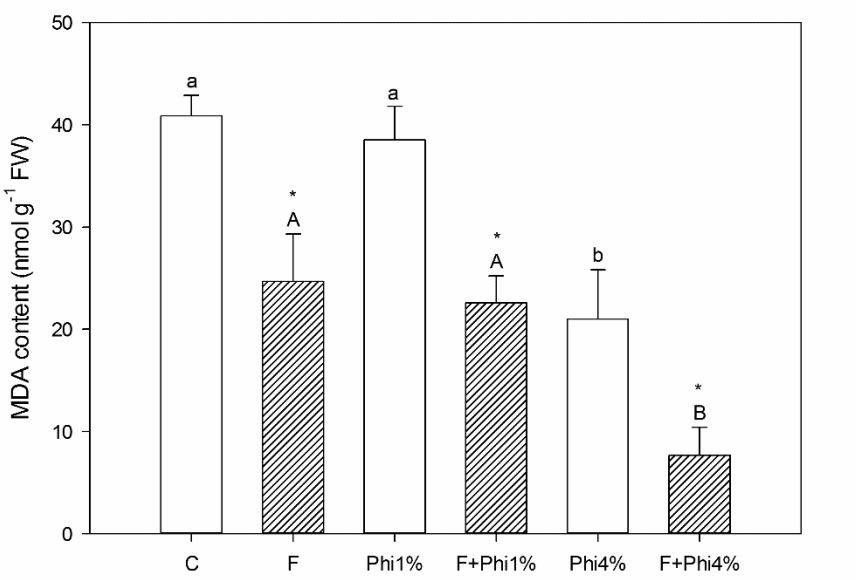

Figure 9: Malondialdehyde (MDA) content of *P. radiata* plants after *F. circinatum* inoculation and Phi treatment. Data are presented as mean ± SD. Different capital letters indicate differences between inoculated plants treatments and lowercase letters indicate differences between non-inoculated plants ($p < 0.05$). The asterisk (*) indicates differences between inoculated and non-inoculated plants within a given concentration of Phi ($p < 0.05$).

Proline content

Proline content was not present significantly altered by Phi pre-treatment in relation to control, but the inoculation led to an increase in this amino acid concentration (figure 10). Considering the inoculated plants, this increase was only significant on non-Phi pre-treated plants in relation to the corresponding non-inoculated group (figure 10).

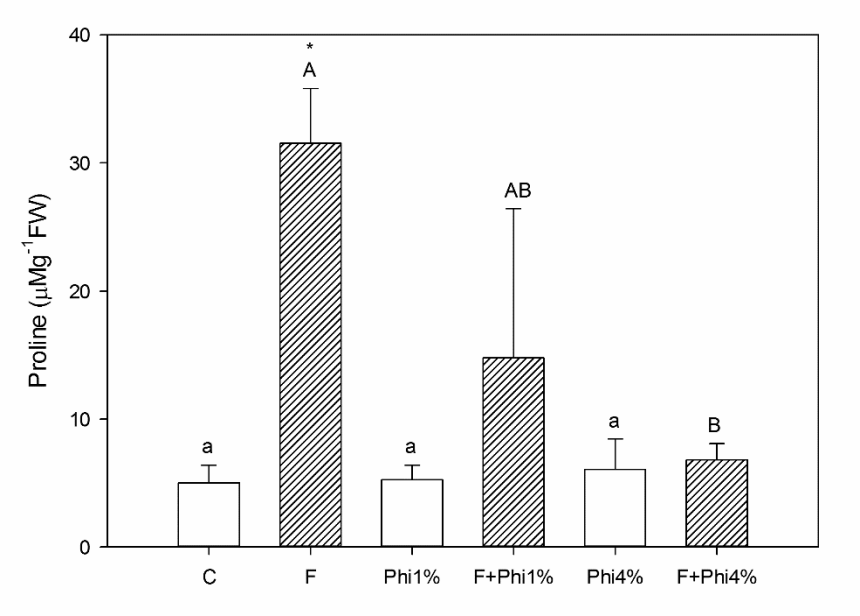

Figure 10: Proline content of *P. radiata* plants after *F. circinatum* inoculation and Phi treatment. Data are presented as mean ± SD. Different capital letters indicate differences between inoculated plants treatments and lowercase letters indicate differences between non-inoculated plants ($p < 0.05$). The asterisk (*) indicates differences between inoculated and non-inoculated plants within a given concentration of Phi ($p < 0.05$).

Total soluble sugars

Regarding total soluble sugars (TSS) content, Phi pre-treatment significantly decreased their content compared to non-treated plants. After inoculation, only $Phi_{1\%}$ showed an increment of TSS (figure 11).

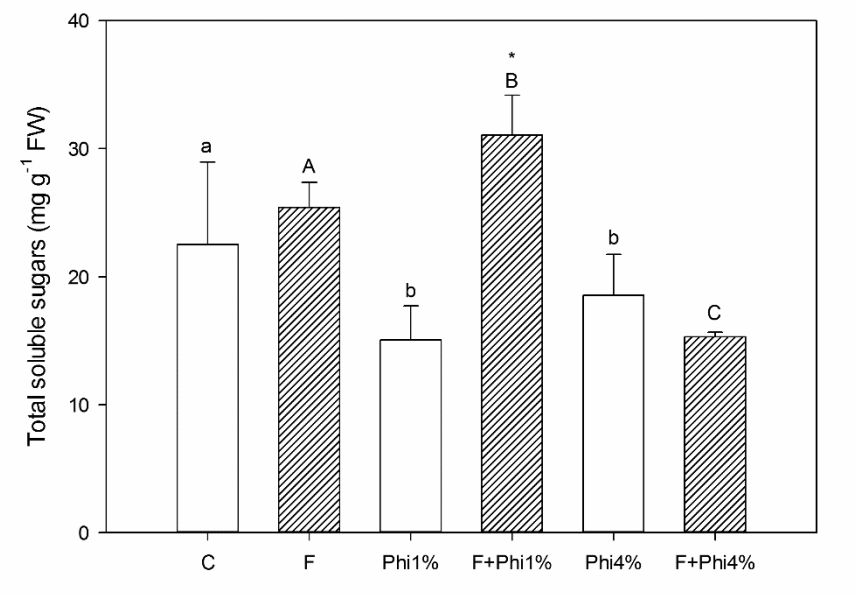

Figure 11: Total soluble sugars content of *P. radiata* plants after *F. circinatum* inoculation and Phi treatment. Data are presented as mean ± SD. Different capital letters indicate differences between inoculated plants treatments and lowercase letters indicate differences between non-inoculated plants ($p < 0.05$). The asterisk (*) indicates differences between inoculated and non-inoculated plants within a given concentration of Phi ($p < 0.05$).

Discussion

Phosphite provides an efficient disease protection in many different plant pathosystems and is well known to control oomycetes on a wide range of horticultural crops and native ecosystems (King et al. 2010). Several studies have shown that Phi presents direct effects on pathogen growth and triggers indirect responses through plant defense stimulation (Lobato et al. 2010; Bock et al. 2012; Machinandiarena et al. 2012; Burra et al. 2014; Dalio et al. 2014; Liu et al. 2016). Particularly to forestry species, the study of how plants respond to elicitors and pathogens, and the mechanisms behind phosphite-induced resistance is still in its infancy. Dalio et al. (2014) investigated the mechanisms of KPhi on physiology and gene regulation of treated *Fagus sylvatica* saplings to understand the protective role of this chemical in host-*Phytophtora plurivora* interaction. Here we contribute to fulfill this gap using *Pinus-Fusarium* as a model to decipher the underlying physiological processes mediating Phi and plant-pathogen interaction and to further assist new protection strategies for forestry disease management. We found that Phi inhibited *in vitro F.*

circinatum mycelial growth in a dose-response manner as also reported by Dalio et al. (2014) and by Liu et al. (2016) for *Phytophthora* spp.

Looking at plant responses level, a similar pattern was found *in vivo* and, Phi foliar applications delayed the development of pitch canker symptoms in a dose-response way. However, $Phi_{4\%}$ pre-treated plants presented severe side-effect damage on the needles, while $Phi_{1\%}$ treated ones presented vestigial or no damages. The Phi phytotoxicity is not consensual and may be mediated by other factors such as phosphate (Pi) fertilization status of the plant (Thao & Yamakawa 2009). Pilbeam et al. (2011) reported Phi phytotoxicity on *Eucalyptus marginata* plants in the range of 0.25 to 1%, describing irreparable damage to the plant crown. Nevertheless, and within in this range of Phi concentration (0.5%), Dalio et al. (2014) reported no phytotoxicity and no physiological disorders on *Fagus sylvatica* seedlings while reporting a remarkable efficiency in protecting beech against *P. plurivola*. This suggests that the tolerance to different Phi concentrations differs between species and within plant development stages (Lobato et al. 2008), and preliminary tests before use are necessary. Phosphite is generally considered to have low phytotoxicity (Guest & Grant 1991), but foliar phytotoxicity has been reported in selected horticultural and ornamental species. When Phi concentration reach the phytotoxic threshold ($Phi_{4\%}$) a growth reduction and photochemical damages were observed. At the sub-toxic concentration ($Phi_{1\%}$), a height increment was register in comparison with control plants. The later results are in agreement with Pilbeam et al. (2000), where the use of Phi at 0.5% to 2% lead to the promotion of growth in *Adenanthos barbiger*. Phi-treated plants showed a decrease of total soluble sugars probably to support the energy demand needed for growth and production of defense related compounds. At sub-toxic concentration, a higher content of chlorophyll *a* was accompanied by an increase of the quantum yield of photosystem II (Φ PSII) photochemical efficiency, suggesting an enhancement of the linear electron transporter chain.

The application of Phi induced positive effects on plant vitality (increased leaf chlorophyll content, leaf chlorophyll fluorescence [Fv/Fm]) in tobacco plants (Percival & Banks 2014). Carotenoids recognized as been involved in photosynthesis, antioxidation, and phytohormone biosynthesis (Lu & Li 2008) increased in both Phi treatments. Anthocyanins known as mediators of reactive oxygen species (ROS)-induced signalling cascades (Hatier & Gould 2008) increased with $Phi_{1\%}$ suggesting that a kind of defense alert was triggered resulting in the protection of the photosynthethic apparatus. The phytotoxic effects induced by $Phi_{4\%}$ (severe damages in the needles) led to a reduction of growth, a decrease in gas exchange parameters, due to the reduction of the

photosynthetic area, and to a decrease on photochemical performance. This damages may hide the action of this concentration as inducer of host defense responses; however, the decrease on lipid peroxidation, which indicates the enhancement of the antioxidant machinery and activation of the protection against an imposed stress, proves its action as inducer of host defense.

The susceptibility of the widely planted commercial species *P. radiata* to *F. circinatum* is well documented and has been of concern to pine forest industries worldwide (Ganley et al. 2009; Gordon et al. 2015). In recent years, research has focus its efforts in understanding the differential susceptibility between and within species (Iturritxa et al. 2013; Mitchell et al. 2013; Mitchell et al. 2014), the intricate mechanisms behind the *Pinus-Fusarium* interaction (Martín-Rodrigues et al. 2013; Donoso et al. 2015; Martín-Rodrigues et al. 2015) and the development of disease control (Vivas et al. 2012; Berbegal et al. 2015; Martínez-Álvarez et al. 2016; Muñoz-Adalia et al. 2016) with different degrees of success.

It was not surprising to find that 7 dpi all plants non Phi-treated and inoculated with *F. circinatum* showed wilting needles with crown dieback symptoms, a common morphological feature widely reported (Martínez-Álvarez et al. 2016; Muñoz-Adalia et al. 2016). Unfortunately, most of phytopathological studies do not take into consideration the plant physiological status which may elucidate the fine-tuned infection mechanisms. Is well known that pathogenic attack alters primary metabolism (Berger et al. 2004; Swarbrick et al. 2006; Doehlemann et al. 2008). Photosynthetic changes are one of the most common physiological disorders reported (Bonfig et al. 2006; Berger et al. 2007; Prokopová et al. 2010) and several studies led to the proposal that plants switch off photosynthesis and other assimilatory metabolism to initiate respiration and other processes required for defense (Scharte et al. 2005). This assumption is supported by our data, where both stomatal and not stomatal limitations led to the decrease of photosynthesis in inoculated (non-treated) plants. The decrease of photochemical efficiency (Fv/Fm and ΦPSII) as well the increase of Ci together with decrease in gs, A and E are most visible and immediate consequences of pathogen attack.

Another point worthy of attention is the higher levels of proline accumulation, a commonly used as a marker of plant stress response such as drought (Yamada et al. 2005; Man et al. 2011; Filippou et al. 2014), on untreated inoculated plant. *Fusarium circinatum* is known for colonizing the cortex and phloem and, in later stages, the xylem and resin ducts stopping the flux of water and nutrients (Martín-Rodrigues et al. 2013) which is in line with the decrease in water potential values compared with control healthy plants. Besides its role in osmotic regulation, proline can function as a

chaperone, protecting proteins and membranes from degradation (Gomes et al. 2010; Kaushal et al. 2011). There are also studies linking proline accumulation to ROS scavenging activity (Reddy et al. 2004; Ahmad et al. 2008) which may explain why membrane integrity measure in terms of lipid peroxidation and electrolyte leakage did not changed after the first symptoms signals.

The most notorious aspect of our work is the fact that Phi application delayed the onset of symptoms in a dose concentration manner similarly as happened with *in vitro* mycelia growth inhibition. There is still no conclusive explanation regarding the mode of action of Phi and its potential targets in plants. In this Phi-pathogen interaction most of the physiological parameters evaluated have similar patterns as reported when Phi was assessed alone. An exception was noticed for total TSS and EL. Soluble sugars support plant metabolic processes and under stress (*e.g.* defense) plant tissues accumulate these carbohydrates. There were no considerable differences on F, but the high levels of soluble sugars in F+$Phi_{1\%}$ indicate a possible plant defense strategy. Several reports concluded that Phi might act in a dual way (Dalio et al. 2014) both at host and fungi level. Phi is a systemic fungicide with an ambimobile nature translocated in both the xylem and the phloem (Hardy et al. 2001; Daniel & Guest 2006). Therefore, Phi circulating on these tissues may have had a direct action on the fungus, leading to a similar effect to what was observed *in vitro*. Gao et al. (2016) reported that the disruption of *Fusarium graminearum* cell membranes treated with thymol led to an increased electrolyte leakage of the growth medium in a dose-response manner. King et al. (2010) observed hyphal distortion and lysis of cell walls in parallel with a down-regulation of many genes encoding proteins involved in cell wall synthesis and cytoskeleton functioning on *P. cinnamomi*. It is important to note that electrolyte leakage measures all charged solutes in the external medium and is not necessarily correlated with membrane damage of the plant (Bajji et al. 2002; Rolny et al. 2011). The Phi present on the systemic vases may have caused a disruption on the fungal membrane and led to leakage of metabolites. The above results support that the higher leakage levels evident on inoculated plants treated with Phi could be originated by the leakage of metabolites by the fungus to the external medium, and not by the disruption of the plant cell wall. The fungistatic nature of the concentration used in our study was confirmed by evaluation the reversibility of inhibition of *F. circinatum* mycelial growth in PDA medium (data not shown) supporting earlier studies (Aguín et al. 2006; Lobato et al. 2010).

Our data proved that the amelioration of severity of pitch canker disease was due to a mixed action of Phi direct on fungus growth and indirectly by enhancing plant defenses. The mode of action of phosphite might, therefore, best be described as mixed, rather than direct or indirect.

Conclusion

The effects of Phi application on *Pinus radiata* are here reported for the first time, considering the physiological effects of this elicitor on either *P. radiata* alone or in the interaction *P. radiata-F. circinatum*. The parallel induction of the inhibition of mycelial growth on *in vitro* assays, and the *in vivo* survival rate together with the increasing concentration of applied Phi reveals a protective effect of this compound towards the infected plant. Despite the phytotoxic effect assessed by visual damages, the application of Phi at 4% this concentration led plant to enhance specific priming defenses, as shown by alterations in proline content, and total soluble sugars content, and lipid peroxidation. Below the phytotoxic concentration, Phi application ameliorated overall plant physiological performance, indicating a positive influence on the primary metabolism. On inoculated plants, the impact effects caused by *F. circinatum* infection on the photosystem was ameliorated by pre-treatment with Phi. Therefore, preventive applications of Phi present a viable alternative for the management of the pitch canker disease.

Although the ameliorating effect of $Phi_{4\%}$, the phytotoxic disorders make its application questionable plants of this age and further studies must be performed, in order to optimize Phi concentrations while minimizing phytotoxicity symptoms, must be performed. Also, it is necessary to investigate the effects of Phi on older plants, different *Pinus* spp. and with other routes of application. Finally, mechanisms of *F. circinatum* infection and the specificity of the host must be better understood in order to development better management strategies.

References

Ahmad, P., John, R., Sarwat, M., & Umar, S. (2008). Responses of proline, lipid peroxidation and antioxidative enzymes in two varieties of *Pisum sativum* L. under salt stress. *International Journal of Plant Production*, *2*(4), 353-366.

Aguín, O., Mansilla, J. P., & Sainz, M. J. (2006). *In vitro* selection of an effective fungicide against *Armillaria mellea* and control of white root rot of grapevine in the field. *Pest Management Science*, *62*(3), 223-228.

Ayanz, J. S. M., Torres, G. D., Gasparini, P., Ståhl, G., Parviainen, J., Lier, M., Asensio, I. A., Cienciala, E., Freudenschuss, A., Ferrera, P. G., Korhonen, K., Lanz, A., Nabuurs, G., Parviainen, J., Verkek, H. & Weiss, G. (2015). Maintenance and appropriate enhancement of forest resources and their contribution to global carbon cycles *in* FOREST EUROPE, 2015: State of Europe's Forests 2015.

Bajji, M., Kinet, J. M., & Lutts, S. (2002). The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. *Plant Growth Regulation*, *36*(1), 61-70.

Bates, L. S., Waldren, R. P., & Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil*, *39*(1), 205-207.

Berbegal, M., Pérez-Sierra, A., Armengol, J., & Grünwald, N. J. (2013). Evidence for multiple introductions and clonality in Spanish populations of *Fusarium circinatum*. *Phytopathology*, *103*(8), 851-861.

Berger, S., Papadopoulos, M., Schreiber, U., Kaiser, W., & Roitsch, T. (2004). Complex regulation of gene expression, photosynthesis and sugar levels by pathogen infection in tomato. *Physiologia Plantarum*, *122*(4), 419-428.

Berger, S., Sinha, A. K., & Roitsch, T. (2007). Plant physiology meets phytopathology: plant primary metabolism and plant–pathogen interactions. *Journal of Experimental Botany*, *58*(15-16), 4019-4026.

Bock, C. H., Brenneman, T. B., Hotchkiss, M. W., & Wood, B. W. (2012). Evaluation of a phosphite fungicide to control pecan scab in the southeastern USA. *Crop Protection*, *36*, 58-64.

Bolton, M.D., (2009). Primary metabolism and plant defense - fuel for the fire. *Molecular Plant-Microbe Interactions: MPMI*, 22(5), pp.487–497.

Bonfig, K. B., Schreiber, U., Gabler, A., Roitsch, T., & Berger, S. (2006). Infection with virulent and avirulent *P.* syringae strains differentially affects photosynthesis and sink metabolism in *Arabidopsis* leaves. *Planta*, *225*(1), 1-12.

Bragança, H., Diogo, E., Moniz, F., & Amaro, P. (2009). First report of pitch canker on pines caused by *Fusarium circinatum* in Portugal. *Plant Disease*,*93*(10), 1079-1079.

Britz, H., Couhnho, T. A., Gordon, T. R., & Wingfield, M. J. (2001). Characterisation of the pitch canker fungus, *Fusarium circinatum*, from Mexico. *South African Journal of Botany*, *67*(4), 609-614.

Burra, D. D., Berkowitz, O., Hedley, P. E., Morris, J., Resjö, S., Levander, F., Liljeroth, E., Andreasson, E. & Alexandersson, E. (2014). Phosphite-induced changes of the transcriptome and secretome in *Solanum tuberosum* leading to resistance against *Phytophthora* infestans. *BMC plant biology*, *14*(1), 1.

Carlucci, A., Colatruglio, L., & Frisullo, S. (2007). First report of pitch canker caused by *Fusarium circinatum* on *Pinus halepensis* and *P. pinea* in Apulia (Southern Italy). *Plant Disease*, *91*(12), 1683-1683.

Chow, P. S., & Landhäusser, S. M. (2004). A method for routine measurements of total sugar and starch content in woody plant tissues. *Tree Physiology*,*24*(10), 1129-1136.

Cinelli, T., Mondello, V., Marchi, G., Burruano, S., Alves, A., & Mugnai, L. (2015). First Report of *Diaporthe eres* Associated with Cane Blight of Grapevine (*Vitis vinifera*) in Italy. *Plant Disease*, PDIS-08.

Cook, P. J., Landschoot, P. J., & Schlossberg, M. J. (2009). Inhibition of *Pythium* spp. and suppression of *Pythium* Blight of turfgrasses with phosphonate fungicides. *Plant Disease*, *93*(8), 809-814.

Dalio, R. J., Fleischmann, F., Humez, M., & Osswald, W. (2014). Phosphite protects *Fagus sylvatica* seedlings towards *Phytophthora plurivora* via local toxicity, priming and facilitation of pathogen recognition. *PloS one*, *9*(1), e87860.

Daniel, R., & Guest, D. (2006). Defence responses induced by potassium phosphonate in *Phytophthora palmivora*-challenged *Arabidopsis thaliana*. *Physiological and Molecular Plant Pathology*, *67*(3), 194-201.

de Rigo, D., Bosco, C., San-Miguel-Ayanz, J., Houston Durrant, T., Barredo, J.I., Strona, G., Caudullo, G., Di Leo, M., & Boca, R. (2016). Forest resources in Europe: an integrated perspective on ecosystem services, disturbances and threats. pp 8-19 *in* San-Miguel-Ayanz, J., de Rigo, D., Caudullo, G., Houston Durrant, T., Mauri, A. (Eds.), 2016. European Atlas of Forest Tree Species. *Publication Office of the European Union*, Luxembourg.

Doehlemann, G., Wahl, R., Horst, R. J., Voll, L. M., Usadel, B., Poree, F., Stitt, M., Pons-Kühnemann, J., Sonnewald, U., Kahmann, R., & Kämper, J. (2008). Reprogramming a maize plant: transcriptional and metabolic changes induced by the fungal biotroph *Ustilago maydis*. *The Plant Journal*,*56*(2), 181-195.

Donoso, A., Rodriguez, V., Carrasco, A., Ahumada, R., Sanfuentes, E., & Valenzuela, S. (2015). Relative expression of seven candidate genes for pathogen resistance on *Pinus radiata* infected with *Fusarium circinatum*. *Physiological and Molecular Plant Pathology*, *92*, 42-50.

Escandón, M., Cañal, M. J., Pascual, J., Pinto, G., Correia, B., Amaral, J., & Meijón, M. (2016). Integrated physiological and hormonal profile of heat-induced thermotolerance in *Pinus radiata*. *Tree Physiology*, *36*(1), 63-77.

European and Mediterranean Plant Protection Organization. 2015. EPPO A1 and A2 List of Pests Recommended for Regulation as Quarentine Pests. Available at http://archives.eppo.int/EPPOStandards/PM1_GENERAL/pm1-02(24)_A1A2_2015.pdf.

Fagard, M., Launay, A., Clément, G., Courtial, J., Dellagi, A., Farjad, M., & Masclaux-Daubresse, C. (2014). Nitrogen metabolism meets phytopathology. *Journal of Experimental Botany*, *65*(19), 5643-5656.

Filippou, P., Bouchagier, P., Skotti, E., & Fotopoulos, V. (2014). Proline and reactive oxygen/nitrogen species metabolism is involved in the tolerant response of the invasive plant species *Ailanthus altissima* to drought and salinity. *Environmental and Experimental Botany*, *97*, 1-10.

Fitza, K. N., Payn, K. G., Steenkamp, E. T., Myburg, A. A., & Naidoo, S. (2013). Chitosan application improves resistance to *Fusarium circinatum* in *Pinus patula*. *South African Journal of Botany*, *85*, 70-78.

Ganley, R. J., Watt, M. S., Manning, L., & Iturritxa, E. (2009). A global climatic risk assessment of pitch canker disease. *Canadian Journal of Forest Research*, *39*(11), 2246-2256.

Gao, T., Zhou, H., Zhou, W., Hu, L., Chen, J., & Shi, Z. (2016). The Fungicidal Activity of Thymol against *Fusarium graminearum* via Inducing Lipid Peroxidation and Disrupting Ergosterol Biosynthesis. *Molecules*, *21*(6), 770.

Gentile, S., Valentino, D., & Tamietti, G. (2009). Control of ink disease by trunk injection of potassium phosphite. *Journal of Plant Pathology*, 565-571.

Gomes, F. P., Oliva, M. A., Mielke, M. S., Almeida, A. A. F., & Aquino, L. A. (2010). Osmotic adjustment, proline accumulation and cell membrane stability in leaves of *Cocos nucifera* submitted to drought stress. *Scientia Horticulturae*,*126*(3), 379-384.

Gordon, T. R., Storer, A. J., & Wood, D. L. (2001). The pitch canker epidemic in California. *Plant Disease*, *85*(11), 1128-1139.

Gordon, T. R., Swett, C. L., & Wingfield, M. J. (2015). Management of *Fusarium* diseases affecting conifers. *Crop Protection*, *73*, 28-39.

Gozzo, F., & Faoro, F. (2013). Systemic acquired resistance (50 years after discovery): moving from the lab to the field. *Journal of Agricultural and Food Chemistry*, *61*(51), 12473-12491.

Guest, D., & Grant, B. (1991). The complex action of phosphonates as antifungal agents. *Biological Reviews*, *66*(2), 159-187.

Hardy, G. S. J., Barrett, S., & Shearer, B. L. (2001). The future of phosphite as a fungicide to control the soilborne plant pathogen *Phytophthora cinnamomi* in natural ecosystems. *Australasian Plant Pathology*, *30*(2), 133-139.

Hatier, J. H. B., & Gould, K. S. (2008). Foliar anthocyanins as modulators of stress signals. *Journal of Theoretical Biology*, *253*(3), 625-627.

Heath, R. L., & Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics*, *125*(1), 189-198.

Hepting, G. H., & Roth, E. R. (1946). Pitch canker, a new disease of some southern pines. *Journal of Forestry*, *44*(10), 742-744.

Ioannou, E., Koutsaviti, A., Tzakou, O., & Roussis, V. (2014). The genus *Pinus*: a comparative study on the needle essential oil composition of 46 pine species. *Phytochemistry Reviews*, *13*(4), 741-768.

Irigoyen, J. J., Einerich, D. W., & Sánchez-Díaz, M. (1992). Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativd*) plants. *Physiologia Plantarum*, *84*(1), 55-60.

Iturritxa, E., Ganley, R. J., Wright, J., Heppe, E., Steenkamp, E. T., Gordon, T. R., & Wingfield, M. J. (2011). A genetically homogenous popu lation of *Fusarium circinatum* causes pitch canker of *Pinus radiata* in the Basque Country, Spain. *Fungal Biology*, *115*(3), 288-295.

Iturritxa, E., Ganley, R. J., Raposo, R., García-Serna, I., Mesanza, N., Kirkpatrick, S. C., & Gordon, T. R. (2013). Resistance levels of Spanish conifers against *Fusarium circinatum* and *Diplodia pinea*. *Forest Pathology*,*43*(6), 488-495.

Iturritxa, E., Mesanza, N., & Brenning, A. (2015). Spatial analysis of the risk of major forest diseases in Monterey pine plantations. *Plant Pathology*, *64*(4), 880-889.

Kaushal, N., Gupta, K., Bhandhari, K., Kumar, S., Thakur, P., & Nayyar, H. (2011). Proline induces heat tolerance in chickpea (*Cicer arietinum* L.) plants by protecting vital enzymes of carbon and antioxidative metabolism. *Physiology and Molecular Biology of Plants*, *17*(3), 203-213.

King, M., Reeve, W., Van der Hoek, M. B., Williams, N., McComb, J., O'Brien, P. A., & Hardy, G. E. S. J. (2010). Defining the phosphite-regulated transcriptome of the plant pathogen *Phytophthora cinnamomi*. *Molecular Genetics and Genomics*, *284*(6), 425-435.

Landeras, E., García, P., Fernández, Y., Braña, M., Fernández-Alonso, O., Méndez-Lodos, S. & Beltrán, R. (2005). Outbreak of pitch canker caused by *Fusarium circinatum* on *Pinus* spp. in northern Spain. *Plant Disease*, *89*(9), 1015-1015.

Liu, P., Li, B., Lin, M., Chen, G., Ding, X., Weng, Q., & Chen, Q. (2016). Phosphite-induced reactive oxygen species production and ethylene and ABA biosynthesis, mediate the control of *Phytophthora capsici* in pepper (*Capsicum annuum*). *Functional Plant Biology*, *43*(6), 563-574.

Lobato, M. C., Olivieri, F. P., Altamiranda, E. G., Wolski, E. A., Daleo, G. R., Caldiz, D. O., & Andreu, A. B. (2008). Phosphite compounds reduce disease severity in potato seed tubers and foliage. *European Journal of Plant Pathology*, *122*(3), 349-358.

Lobato, M. C., Olivieri, F. P., Daleo, G. R., & Andreu, A. B. (2010). Antimicrobial activity of phosphites against different potato pathogens. *Journal of Plant Diseases and Protection*, *117*(3), 102-109.

Lu, S., & Li, L. (2008). Carotenoid metabolism: biosynthesis, regulation, and beyond. *Journal of Integrative Plant Biology*, *50*(7), 778-785.

Machinandiarena, M. F., Lobato, M. C., Feldman, M. L., Daleo, G. R., & Andreu, A. B. (2012). Potassium phosphite primes defense responses in potato against *Phytophthora infestans*. *Journal of Plant Physiology*, *169*(14), 1417-1424.

Man, D., Bao, Y. X., Han, L. B., & Zhang, X. (2011). Drought tolerance associated with proline and hormone metabolism in two tall fescue cultivars. *HortScience*, *46*(7), 1027-1032.

Martínez-Álvarez, P., Fernández-González, R. A., Sanz-Ros, A. V., Pando, V., & Diez, J. J. (2016). Two fungal endophytes reduce the severity of pitch canker disease in *Pinus radiata* seedlings. *Biological Control*, *94*, 1-10.

Martín-Rodrigues, N., Espinel, S., Sanchez-Zabala, J., Ortíz, A., González-Murua, C., & 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 Phytologist*,*198*(4), 1215-1227.

Martín-Rodrigues, N., Sanchez-Zabala, J., Salcedo, I., Majada, J., González-Murua, C., & Duñabeitia, M. K. (2015). New insights into radiata pine seedling root infection by *Fusarium circinatum*. *Plant Pathology*, *64*(6), 1336-1348.

Mead, D.J. (2013). Sustainable management of *Pinus radiata* plantations. FAO Forestry Paper No. 170. Rome, FAO.

Mitchell, R. G., Wingfield, M. J., Hodge, G. R., Steenkamp, E. T., & Coutinho, T. A. (2013). The tolerance of *Pinus patula*× *Pinus tecunumanii*, and other pine hybrids, to *Fusarium circinatum* in greenhouse trials. *New Forests*, *44*(3), 443-456.

Mitchell, R. G., Wingfield, M. J., Steenkamp, E. T., Roux, J., Verryn, S., & Coutinho, T. A. (2014). Comparison of the tolerance of *Pinus patula* seedlings and established trees to infection by *Fusarium circinatum*. *Southern Forests: a Journal of Forest Science*, *76*(3), 151-159.

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

Muramoto, M., & Dwinell, L. D. (1990). Pitch canker of *Pinus luchuensis* in Japan. *Plant Disease*, *74*(7).

O'Brien, J. A., Daudi, A., Butt, V. S., & Bolwell, G. P. (2012). Reactive oxygen species and their role in plant defence and cell wall metabolism. *Planta*, *236*(3), 765-779.

Percival, G. C., & Banks, J. M. (2014). Evaluation of plant defence activators for the potential control of *Pseudomonas syringae* pv. aesculi. *Arboricultural Journal: The International Journal of Urban Forestry*, *36*(2), 76-88.

Percival, G. C., & Banks, J. M. (2015). Phosphite-induced suppression of *Pseudomonas* bleeding canker (*Pseudomonas syringae* pv. aesculi) of horse chestnut (*Aesculus hippocastanum* L.). *Arboricultural Journal: The International Journal of Urban Forestry*, *37*(1), 7-20.

Pereira, V. F., Resende, M. L. V. D., Ribeiro Júnior, P. M., Regina, M. D. A., Mota, R. V. D., & Vitorino, L. R. R. (2012). Potassium phosphite on the control of downy mildew of grapevine and physicochemical characteristics of Merlot grapes. *Pesquisa Agropecuária Brasileira*, *47*(11), 1581-1588.

Pérez-Sierra, A., Landeras, E., León, M., Berbegal, M., García-Jiménez, J., & Armengol, J. (2007). Characterization of *Fusarium circinatum* from *Pinus* spp. in northern Spain. *Mycological Research*, *111*(7), 832-839.

Pfenning, L. H., Costa, S. D. S., Melo, M. P. D., Costa, H., Ventura, J. A., Auer, C. G., & Santos, Á. F. D. (2014). First report and characterization of *Fusarium circinatum*, the causal agent of pitch canker in Brazil. *Tropical Plant Pathology*, *39*(3), 210-216.

Pilbeam, R. A., Colquhoun, I. J., Shearer, B., & Hardy, G. S. J. (2000). Phosphite concentration: its effect on phytotoxicity symptoms and colonisation by *Phytophthora cinnamomi* in three understorey species of *Eucalyptus marginata* forest. *Australasian Plant Pathology*, *29*(2), 86-95.

Pividori, M., Gianetti, F., Barbati, A., Chirici, G. (2016). European Forest Types: tree species matrix. pp. 34-35 *in* San-Miguel-Ayanz, J., de Rigo, D., Caudullo, G., Houston Durrant, T., Mauri, A. (Eds.), 2016. European Atlas of Forest Tree Species. *Publication Office of the European Union*, Luxembourg.

Prokopová, J., Špundová, M., Sedlářová, M., Husičková, A., Novotný, R., Doležal, K., ... & Lebeda, A. (2010). Photosynthetic responses of lettuce to downy mildew infection and cytokinin treatment. *Plant Physiology and Biochemistry*, *48*(8), 716-723.

Reddy, A. R., Chaitanya, K. V., & Vivekanandan, M. (2004). Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *Journal of Plant Physiology*, *161*(11), 1189-1202.

Richardson, D. M., and P. W. Rundel. (2000). Ecology and biogeography of *Pinus*: an introduction. pp. 3–46 *in* D. M. Richardson, ed. Ecology and biogeography of *Pinus*. Cambridge Univ. Press, Cambridge, U.K.

Rolny, N., Costa, L., Carrión, C., & Guiamet, J. J. (2011). Is the electrolyte leakage assay an unequivocal test of membrane deterioration during leaf senescence?. *Plant Physiology and Biochemistry*, *49*(10), 1220-1227.

Sanchez-Zabala, J., Majada, J., Martín-Rodrigues, N., Gonzalez-Murua, C., Ortega, U., Alonso-Graña, M., & Duñabeitia, M. K. (2013). Physiological aspects underlying the improved outplanting performance of *Pinus pinaster* Ait. seedlings associated with ectomycorrhizal inoculation. *Mycorrhiza*, *23*(8), 627-640.

Santana, Q. C., Coetzee, M. P. A., Wingfield, B. D., Wingfield, M. J., & Steenkamp, E. T. (2015). Nursery-linked plantation outbreaks and evidence for multiple introductions of the pitch canker pathogen *Fusarium circinatum* into South Africa. *Plant Pathology*, 65, 357–368.

Scharte, J., Schön, H., & Weis, E. (2005). Photosynthesis and carbohydrate metabolism in tobacco leaves during an incompatible interaction with *Phytophthora nicotianae*. *Plant, Cell & Environment*, *28*(11), 1421-1435.

Scott, P. M., Dell, B., Shearer, B. L., Barber, P. A., & Hardy, G. S. J. (2013). Phosphite and nutrient applications as explorative tools to identify possible factors associated with *Eucalyptus gomphocephala* decline in South-Western Australia. *Australasian Plant Pathology*, *42*(6), 701-711.

Silva, O. C., Santos, H. A. A., Dalla Pria, M., & May-De Mio, L. L. (2011). Potassium phosphite for control of downy mildew of soybean. *Crop Protection*,*30*(6), 598-604.

Sims, D. A., & Gamon, J. A. (2002). Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. *Remote Sensing of Environment*, *81*(2), 337-354.

Swarbrick, P. J., Schulze-Lefert, P., & Scholes, J. D. (2006). Metabolic consequences of susceptibility and resistance (race-specific and broad-spectrum) in barley leaves challenged with powdery mildew. *Plant, Cell & Environment*, *29*(6), 1061-1076.

Thao, H. T. B., & Yamakawa, T. (2009). Phosphite (phosphorous acid): fungicide, fertilizer or bio-stimulator?. *Soil Science and Plant Nutrition*, *55*(2), 228-234.

Vallad, G. E., & Goodman, R. M. (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop science*, *44*(6), 1920-1934.

Viljoen, A., Wingfield, M. J., & Marasas, W. F. O. (1994). First report of *Fusarium subglutinans* f. sp. pini on pine seedlings in South Africa. *Plant Disease*, *78*(3), 309-312.

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

Walters, D. R., Ratsep, J., & Havis, N. D. (2013). Controlling crop diseases using induced resistance: challenges for the future. *Journal of Experimental Botany*, *64*(5), 1263-1280.

Wingfield, M. J., Jacobs, A., Coutinho, T. A., Ahumada, R., & Wingfield, B. D. (2002). First report of the pitch canker fungus, *Fusarium circinatum*, on pines in Chile. *Plant Pathology*, *51*(3), 397-397.

Wingfield, M. J., Hammerbacher, A., Ganley, R. J., Steenkamp, E. T., Gordon, T. R., Wingfield, B. D., & Coutinho, T. A. (2008). Pitch canker caused by *Fusarium circinatum*—a growing threat to pine plantations and forests worldwide. *Australasian Plant Pathology*, *37*(4), 319-334.

Yamada, M., Morishita, H., Urano, K., Shiozaki, N., Yamaguchi-Shinozaki, K., Shinozaki, K., & Yoshiba, Y. (2005). Effects of free proline accumulation in petunias under drought stress. *Journal of Experimental Botany*, *56*(417), 1975-1981.

Part III

Future perspectives

Fusarium circinatum is the causal agent of the pitch canker disease, an emerging threat affecting *Pinus* spp. and *Pseudotsuga menziesii* worldwide. Frequently associated high levels of seedling and tree mortality, the pitch canker represents elevated economic losses and compromises the sustainability of native forests and the productivity of plantations. In Europe, *F. circinatum* is under strict quarantine programs that are not cost-effective. Thus, this study arises from the current need for finding control strategies that not only inexpensive, but also environmentally friendly.

Recent research for the control of the pitch canker disease has used biologically derived compounds, chemicals, and other biological agents. Some of these studies are based on the induction of plant resistance, an extensive process of transcriptional and metabolic reprogramming that leads to an enhancement of plant defensive capacity. Phosphite (Phi) salts are inexpensive and innocuous to the environment, presenting the capacity of inducing plant resistance and having a direct effect on the pathogen. The use of these salts has been deeply explored in agriculturally important crop plants, presenting the capacity of combating infection by an array of phytopathogens. Despite these potentialities, there are no reports for the use of Phi in the control of *F. circinatum* infection or for its application on *Pinus* spp.

The aims of this work were to evaluated the potentialities of potassium phosphite (KPhi) in the control of *F. circinatum* on *Pinus radiata*, and understand its separate effects in the plant and pathogen. For this, different concentrations of Phi (0%, 1% and 4%) were used in *in vitro* and *in vivo* assays, and it was evident a relation between the delay of symptoms, the inhibition of pathogen growth and the concentration used.

The application of Phi independent of inoculation presented differential effects. At $Phi_{1\%}$ no significant mechanical damages were reported, and the photosynthetic performance and chlorophyll fluorescence were ameliorated, pigment content increased, and a decrease total soluble sugars content was observed. At $Phi_{4\%}$, mechanical damages on the needles were noticed, and no differences in all parameters except gas exchange parameters, lipid peroxidation and total soluble sugars content were noticed. In terms of survival rates, Phi pre-treated plants displayed a delay of symptoms in a dose-response manner. On untreated plants, *Fusarium* inoculation led to

null values of gas exchange parameters and stomatal conductance, and a decrease on chlorophyll fluorescence. The application of Phi ameliorated these effects, and pre-treated plants positive results, independently of the concentration used. Despite the damages, plants pre-treated with $Phi_{4\%}$ displayed signs of defense priming (lower of lipid peroxidation). In addition, Phi presents an effect on the pathogen growth proven by *in vitro* assays, which that may be due to effects on hyphal growth.

Future studies must focus on the mechanisms of action of *F. circinatum* aiming for its metabolome, proteome and genome, and the specificity of the host. This will allow to fulfill the absence of information about *Fusarium* genus, more specifically about *Fusarium circinatum*, and help to develop more efficient protection strategies. Regarding Phi application, $Phi_{4\%}$ is questionable for plants of this age. Further experiments must be with intermediate concentrations, older plants as well as plants of different species, and other routes of Phi application must be carried out in order to find the best way for disease control on *Pinus* spp.

In conclusion, this study allows the elucidation of the effects of *F. circinatum* and Phi application on *P. radiata* physiology, and presents a viable alternative for the control of the pitch canker.

References

Ali, Z., Smith, I. & Guest, D.I., 1999. Effect of potassium phosphonate on root rot of *Pinus radiata* caused by *Phytophthora cinnamomi*. *Australasian Plant Pathology*, 28(2), p.120.

Ashraf, M., & Harris, P. J. C. (2013). Photosynthesis under stressful environments: an overview. *Photosynthetica*, *51*(2), 163-190.

Atkinson, N. J., & Urwin, P. E. (2012). The interaction of plant biotic and abiotic stresses: from genes to the field. *Journal of Experimental Botany*, *63*(10), 3523-3543.

Berbegal, M., Pérez-Sierra, A., Armengol, J., & Grünwald, N. J. (2013). Evidence for multiple introductions and clonality in Spanish populations of *Fusarium circinatum*. *Phytopathology*, *103*(8), 851-861.

Berger, S., Sinha, A. K., & Roitsch, T. (2007). Plant physiology meets phytopathology: plant primary metabolism and plant–pathogen interactions. *Journal of Experimental Botany*, *58*(15-16), 4019-4026.

Bock, C. H., Brenneman, T. B., Hotchkiss, M. W., & Wood, B. W. (2012). Evaluation of a phosphite fungicide to control pecan scab in the southeastern USA. *Crop Protection*, *36*, 58-64.

Bolton, M.D. (2009). Primary metabolism and plant defense - fuel for the fire. *Molecular Plant-Microbe Interactions: MPMI*, 22(5), pp.487–497.

Bragança, H., Diogo, E., Moniz, F., & Amaro, P. (2009). First report of pitch canker on pines caused by *Fusarium circinatum* in Portugal. *Plant Disease*,*93*(10), 1079-1079.

Britz, H., Couhnho, T. A., Gordon, T. R., & Wingfield, M. J. (2001). Characterisation of the pitch canker fungus, *Fusarium circinatum*, from Mexico.*South African Journal of Botany*, *67*(4), 609-614.

Carlucci, A., Colatruglio, L., & Frisullo, S. (2007). First report of pitch canker caused by *Fusarium circinatum* on *Pinus halepensis* and *P. pinea* in Apulia (Southern Italy). *Plant Disease*, *91*(12), 1683-1683.

Conejero, M. V. (2013). *Susceptibility of Pinus pinaster Ait. to Fusarium circinatum Nirenberg and O'Donnell: variability and maternal effects*. Doctoral Dissertation. Sustainable Forest Management Research Institute. Department of Plant Production and Forest Resources. University of Valladolid. 208 pp.

Conrath, U., Pieterse, C.M.J. & Mauch-mani, B. (2002). Priming in plant – pathogen interactions. *Trends in Plant Science*, 7(5), pp.210–216.

Conrath, U., Beckers, G. J., Langenbach, C. J., & Jaskiewicz, M. R. (2015). Priming for enhanced defense. *Annual Review of Phytopathology*, *53*, 97-119.

Cook, P. J., Landschoot, P. J., & Schlossberg, M. J. (2009). Inhibition of *Pythium* spp. and suppression of *Pythium* Blight of turfgrasses with phosphonate fungicides. *Plant Disease*, *93*(8), 809-814.

Dalio, R. J., Fleischmann, F., Humez, M., & Osswald, W. (2014). Phosphite protects *Fagus sylvatica* seedlings towards *Phytophthora plurivora* via local toxicity, priming and facilitation of pathogen recognition. *PloS one*, *9*(1), e87860.

Dangl, J. L., & Jones, J. D. (2001). Plant pathogens and integrated defence responses to infection. *Nature*, *411*(6839), 826-833.

Daniel, R. & Guest, D., (2006). Defence responses induced by potassium phosphonate in *Phytophthora palmivora*-challenged *Arabidopsis thaliana*. *Physiological and Molecular Plant Pathology*, 67(3-5), pp.194–201.

Deliopoulos, T., Kettlewell, P.S. & Hare, M.C., (2010). Fungal disease suppression by inorganic salts: A review. *Crop Protection*, 29(10), pp.1059–1075.

de Paiva, D. S. C. S. (2011). *Detecção de Fusarium circinatum Nirenberg & O'Donnell em coníferas da região central de Portugal*. Tese de Mestrado em Biodiversidade e Biotecnologia Vegetal. Faculdade de Ciência - Universidade de Coimbra. 64 pp.

Dreaden, T. & Smith, J. (2010). *Pitch canker disease of pines*. University of Florida Extension, IFAS Extension.

Dick, M. A., Williams, N. M., Bader, M. K. F., Gardner, J. F., & Bulman, L. S. (2014). Pathogenicity of *Phytophthora pluvialis* to *Pinus radiata* and its relation with red needle cast disease in New Zealand. *New Zealand Journal of Forestry Science*, *44*(6), 1-12.

Dodds, P. N., & Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant–pathogen interactions. *Nature Reviews Genetics*, *11*(8), 539-548.

Donoso, A., Rodriguez, V., Carrasco, A., Ahumada, R., Sanfuentes, E., & Valenzuela, S. (2015). Relative expression of seven candidate genes for pathogen resistance on *Pinus radiata* infected with *Fusarium circinatum*. *Physiological and Molecular Plant Pathology*, *92*, 42-50.

EFSA Panel on Plant Health (PLH): Risk assessment of *Gibberella circinata* for the EU territory and identification and evaluation of risk management options. EFSA Journal 2010; 8(6):1620. [93 pp.]. doi:10.2903/j.efsa.2010. Available online: www.efsa.europa.eu.

European and Mediterranean Plant Protection Organization. 2005. Giberella circinata. Available at https://www.eppo.int/QUARANTINE/data_sheets/fungi/DS_Gibberella_circinata.pdf.

European and Mediterranean Plant Protection Organization. 2009. Giberella circinata. Available at http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2338.2009.02317.x/full.

Eyles, A., Bonello, P., Ganley, R., & Mohammed, C. (2010). Induced resistance to pests and pathogens in trees. *New Phytologist*, *185*(4), 893-908.

Fagard, M., Launay, A., Clément, G., Courtial, J., Dellagi, A., Farjad, M., & Masclaux-Daubresse, C. (2014). Nitrogen metabolism meets phytopathology. *Journal of Experimental Botany*, *65*(19), 5643-5656.

Farjon, A. 2013. *Pinus radiata*. The IUCN Red List of Threatened Species 2013: e.T42408A2977955. http://dx.doi.org/10.2305/IUCN.UK.2013-1.RLTS.T42408A2977955.en. Downloaded on 07 May 2016.

Fitza, K. N., Payn, K. G., Steenkamp, E. T., Myburg, A. A., & Naidoo, S. (2013). Chitosan application improves resistance to *Fusarium circinatum* in *Pinus patula*. *South African Journal of Botany*, *85*, 70-78.

Gernandt, D. S., López, G. G., García, S. O., & Liston, A. (2005). Phylogeny and classification of *Pinus*. *Taxon*, *54*(1), 29-42.

Ghildiyal, S. K., Sharma, C. M., & Gairola, S. (2010). Variation in morphological characters of mycorrhizal seedlings of various provenances of *Pinus roxburghii* Sargent. *New York Science Journal*, *3*, 1-8.

Göhre, V., Jones, A. M., Sklenár, J., Robatzek, S., & Weber, A. P. (2012). Molecular crosstalk between PAMP-triggered immunity and photosynthesis. *Molecular Plant-Microbe Interactions*, *25*(8), 1083-1092.

Gordon, T. R., Storer, A. J., & Wood, D. L. (2001). The pitch canker epidemic in California. *Plant Disease*, *85*(11), 1128-1139.

Gordon, T. R. (2006). Pitch canker disease of pines. *Phytopathology*, *96*(6), 657-659.

Gozzo, F., & Faoro, F. (2013). Systemic acquired resistance (50 years after discovery): moving from the lab to the field. *Journal of Agricultural and Food Chemistry*, *61*(51), 12473-12491.

Hammerbacher, A. (2006). *Biology and Epidemiology of Fusarium circinatum.* Master thesis in Agriculture. Faculty of Agricultural and Natural Sciences, Department of Microbiology and Plant Pathology - University of Pretoria. 139 pp.

Hammerbacher, A., Wright, L. P., Wingfield, B. D., Wingfield, M. J., & Coutinho, T. A. (2008). Factors affecting pine pitch canker modelled on Michaelis-Menten kinetics. *Botany*, *87*(1), 36-42.

Heil, M. & Baldwin, I.T. (2002). Fitness costs of induced resistance: Emerging experimental support for a slippery concept. *Trends in Plant Science*, 7(2), pp.61–67.

Hua, J. (2013). Modulation of plant immunity by light, circadian rhythm, and temperature. *Current Opinion in Plant Biology*, *16*(4), 406-413.

ICNF, 2013a. Informação estatística sobre o setor florestal e sobre as fileiras florestais 2000 a 2012 [pdf], 94pp, Divisão de Apoio à Produção Florestal e Valorização de Recursos Silvestres (DAPFVRS), Instituto da Conservação da Natureza e das Florestas. Lisboa.

ICNF, 2013b. IFN6 – Áreas dos usos do solo e das espécies florestais de Portugal continental. Resultados preliminares. [pdf], 34 pp, Instituto da Conservação da Natureza e das Florestas. Lisboa.

ICNF, 2014. Prospeção e monitorização do cancro resinoso do pinheiro - *Fusarium circinatum* Nirenberg & O'Donnell. Relatório síntese. [pdf], 18 pp, Instituto de Conservação da Natureza e das Florestas. Lisboa.

Inman, A. R., Kirkpatrick, S. C., Gordon, T. R., & Shaw, D. V. (2008). Limiting effects of low temperature on growth and spore germination in *Gibberella circinata*, the cause of pitch canker in pine species. *Plant Disease*, *92*(4), 542-545.

Ioannou, E., Koutsaviti, A., Tzakou, O., & Roussis, V. (2014). The genus *Pinus*: a comparative study on the needle essential oil composition of 46 pine species. *Phytochemistry Reviews*, *13*(4), 741-768.

Iturritxa, E., Ganley, R. J., Wright, J., Heppe, E., Steenkamp, E. T., Gordon, T. R., & Wingfield, M. J. (2011). A genetically homogenous population of *Fusarium circinatum* causes pitch canker of *Pinus radiata* in the Basque Country, Spain. *Fungal Biology*, *115*(3), 288-295.

Iturritxa, E., Mesanza, N. & Brenning, A. (2015). Spatial analysis of the risk of major forest diseases in Monterey pine plantations. *Plant Pathology*, pp.880–889.

Jung, S. C., Martinez-Medina, A., Lopez-Raez, J. A., & Pozo, M. J. (2012). Mycorrhiza-induced resistance and priming of plant defenses. *Journal of Chemical Ecology*, *38*(6), 651-664.

Kangasjärvi, S., Neukermans, J., Li, S., Aro, E. M., & Noctor, G. (2012). Photosynthesis, photorespiration, and light signalling in defence responses.*Journal of Experimental Botany*, err402.

Landeras, E., García, P., Fernández, Y., Braña, M., Fernández-Alonso, O., Méndez-Lodos, S. & Beltrán, R. (2005). Outbreak of pitch canker caused by *Fusarium circinatum* on *Pinus* spp. in northern Spain. *Plant Disease*, *89*(9), 1015-1015.

Landi, M., Tattini, M., & Gould, K. S. (2015). Multiple functional roles of anthocyanins in plant-environment interactions. *Environmental and Experimental Botany*, *119*, 4-17.

Landschoot, P., & Cook, J. (2005). Sorting out the phosphonate products. *Golf Course Management*, *73*(11), 73-77.

Lobato, M. C., Olivieri, F. P., Altamiranda, E. G., Wolski, E. A., Daleo, G. R., Caldiz, D. O., & Andreu, A. B. (2008). Phosphite compounds reduce disease severity in potato seed tubers and foliage. *European Journal of Plant Pathology*, *122*(3), 349-358.

López-Arredondo, D.L. & Herrera-Estrella, L., 2012. Engineering phosphorus metabolism in plants to produce a dual fertilization and weed control system. *Nature Biotechnology*, 30(9), pp.889–93.

Lopez-Zamora, I., Bliss, C., Jokela, E. J., Comerford, N. B., Grunwald, S., Barnard, E., & Vasquez, G. M. (2007). Spatial relationships between nitrogen status and pitch canker disease in slash pine planted adjacent to a poultry operation. *Environmental Pollution*, *147*(1), 101-111.

Lyon, G.D. & Newton, A. C., (1997). Do resistance elicitors offer new opportunities in integrated disease control strategies?. *Plant Pathology*, 46(5), pp.636–641.

Ma, L. J., Geiser, D. M., Proctor, R. H., Rooney, A. P., O'Donnell, K., Trail, F., & Kazan, K. (2013). *Fusarium* pathogenomics. *Annual Review of Microbiology*, *67*, 399-416.

Machinandiarena, M. F., Lobato, M. C., Feldman, M. L., Daleo, G. R., & Andreu, A. B. (2012). Potassium phosphite primes defense responses in potato against *Phytophthora infestans*. *Journal of Plant Physiology*, *169*(14), 1417-1424.

Martín-Rodrigues, N., Espinel, S., Sanchez-Zabala, J., Ortíz, A., González-Murua, C., & 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 Phytologist*,*198*(4), 1215-1227.

Massad, T. J., Dyer, L. A., & Vega, G. (2012). Costs of defense and a test of the carbon-nutrient balance and growth-differentiation balance hypotheses for two co-occurring classes of plant defense. *PloS one*, *7*(10), e47554.

Mead, D.J. (2013). Sustainable management of *Pinus radiata* plantations. FAO Forestry Paper No. 170. Rome, FAO.

Mitchell, R. G., Steenkamp, E. T., Coutinho, T. A., & Wingfield, M. J. (2011). The pitch canker fungus, *Fusarium circinatum*: implications for South African forestry. *Southern Forests: a Journal of Forest Science*, *73*(1), 1-13.

Muramoto, M., & Dwinell, L. D. (1990). Pitch canker of *Pinus luchuensis* in Japan. *Plant Disease*, *74*(7).

Nirenberg, H. I., & O'Donnell, K. (1998). New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex. *Mycologia*, 434-458.

O'Brien, J. A., Daudi, A., Butt, V. S., & Bolwell, G. P. (2012). Reactive oxygen species and their role in plant defence and cell wall metabolism. *Planta*, *236*(3), 765-779.

Oliver, R. P., & Ipcho, S. V. (2004). *Arabidopsis* pathology breathes new life into the necrotrophs vs. biotrophs classification of fungal pathogens. *Molecular Plant Pathology*, *5*(4), 347-352.

Pastor, V., Luna, E., Mauch-Mani, B., Ton, J., & Flors, V. (2013). Primed plants do not forget. *Environmental and Experimental Botany*, *94*, 46-56.

Percival, G. C., & Banks, J. M. (2015). Phosphite-induced suppression of *Pseudomonas* bleeding canker (*Pseudomonas syringae* pv. aesculi) of horse chestnut (*Aesculus hippocastanum* L.). *Arboricultural Journal: The International Journal of Urban Forestry*, *37*(1), 7-20.

Pereira, V. F., Resende, M. L. V. D., Ribeiro Júnior, P. M., Regina, M. D. A., Mota, R. V. D., & Vitorino, L. R. R. (2012). Potassium phosphite on the control of downy mildew of grapevine and physicochemical characteristics of Merlot grapes. *Pesquisa Agropecuária Brasileira*, *47*(11), 1581-1588.

Pérez-Sierra, A., Landeras, E., León, M., Berbegal, M., García-Jiménez, J., & Armengol, J. (2007). Characterization of *Fusarium circinatum* from *Pinus* spp. in northern Spain. *Mycological Research*, *111*(7), 832-839.

Pfenning, L. H., Costa, S. D. S., Melo, M. P. D., Costa, H., Ventura, J. A., Auer, C. G., & Santos, Á. F. D. (2014). First report and characterization of *Fusarium circinatum*, the causal agent of pitch canker in Brazil. *Tropical Plant Pathology*, *39*(3), 210-216.

Pieterse, C. M., Leon-Reyes, A., Van der Ent, S., & Van Wees, S. C. (2009). Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology*, *5*(5), 308-316.

Richardson, D. M., and P. W. Rundel. (2000). Ecology and biogeography of *Pinus*: an introduction. pp. 3–46 *in* D. M. Richardson, ed. Ecology and biogeography of *Pinus*. Cambridge Univ. Press, Cambridge, U.K.

Rogers, D. L. (2004). *In situ* genetic conservation of a naturally restricted and commercially widespread species, *Pinus radiata*. *Forest Ecology and Management*, *197*(1), 311-322.

Rojas, C. M., Senthil-Kumar, M., Tzin, V., & Mysore, K. S. (2014). Regulation of primary plant metabolism during plant-pathogen interactions and its contribution to plant defense. *Front. Plant Sci*, *5*(17), 10-3389.

Rolfe, S. A., & Scholes, J. D. (2010). Chlorophyll fluorescence imaging of plant–pathogen interactions. *Protoplasma*, *247*(3-4), 163-175.

Sakamoto, J.M. & Gordon, T.R., (2006). Factors influencing infection of mechanical wounds by *Fusarium circinatum* on Monterey pines (*Pinus radiata*). *Plant Pathology*, 55(1), pp.130–136.

Santana, Q. C., Coetzee, M. P. A., Wingfield, B. D., Wingfield, M. J., & Steenkamp, E. T. (2015). Nursery-linked plantation outbreaks and evidence for multiple introductions of the pitch canker pathogen *Fusarium circinatum* into South Africa. *Plant Pathology*, 65, 357–368

Satish, S., Raveesha, K. A., & Janardhana, G. R. (1999). Antibacterial activity of plant extracts on phytopathogenic *Xanthomonas campestris* pathovars.*Letters in Applied Microbiology*, *28*(2), 145-147.

Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany*, *2012*.

Schultz, J. C., Appel, H. M., Ferrieri, A. P., & Arnold, T. M. (2013). Flexible resource allocation during plant defense responses. *Front Plant Sci*, *4*, 324.

Schweigkofler, W., O'Donnell, K. & 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. *Applied and Environmental Microbiology*, 70(6), pp.3512–20.

Scott, P. M., Dell, B., Shearer, B. L., Barber, P. A., & Hardy, G. S. J. (2013). Phosphite and nutrient applications as explorative tools to identify possible factors associated with *Eucalyptus gomphocephala* decline in South-Western Australia. *Australasian Plant Pathology*, *42*(6), 701-711.

Scott, P.M., Barber, P. A. & Hardy, G.E.S.J., (2015). Novel phosphite and nutrient application to control Phytophthora cinnamomi disease. *Australasian Plant Pathology*, 44(4), pp.431–436.

Serrano, Y., Elvira-Recuenco, M., Conde, M., Troya, M. T., & Raposo, R. (2015). *In vitro* evaluation of Wood Preservatives to Prevent Dispersal of Pitch Canker Pathogen, F*usarium circinatum*. *Journal of Phytopathology*, *163*(9), 783-786.

Silva, O. C., Santos, H. A. A., Dalla Pria, M., & May-De Mio, L. L. (2011). Potassium phosphite for control of downy mildew of soybean. *Crop Protection*,*30*(6), 598-604.

Sørhagen, K., Laxa, M., Peterhänsel, C., & Reumann, S. (2013). The emerging role of photorespiration and non-photorespiratory peroxisomal metabolism in pathogen defence. *Plant Biology*, *15*(4), 723-736.

Stael, S., Kmiecik, P., Willems, P., Van Der Kelen, K., Coll, N. S., Teige, M., & Van Breusegem, F. (2015). Plant innate immunity–sunny side up?. *Trends in Plant Science*, 20(1), 3-11.

Stone, C., Penman, T. & Turner, R., 2012. Managing drought-induced mortality in *Pinus radiata* plantations under climate change conditions: A local approach using digital camera data. *Forest Ecology and Management*, 265, pp.94–101.

Szabados, L., & Savoure, A. (2010). Proline: a multifunctional amino acid.*Trends in Plant Science*, *15*(2), 89-97.

Swett, C. L., Kirkpatrick, S. C., & Gordon, T. R. (2016). Evidence for a Hemibiotrophic Association of the Pitch Canker Pathogen *Fusarium circinatum* with *Pinus radiata*. *Plant Disease*, *100*(1), 79-84.

Tatagiba, S. D., DaMatta, F. M., & Rodrigues, F. Á. (2015). Leaf gas exchange and chlorophyll a fluorescence imaging of rice leaves infected with *Monographella albescens*. *Phytopathology*, *105*(2), 180-188.

Thao, H. T. B., & Yamakawa, T. (2009). Phosphite (phosphorous acid): fungicide, fertilizer or bio-stimulator?. *Soil Science and Plant Nutrition*, *55*(2), 228-234.

Vallad, G. E., & Goodman, R. M. (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop science*, *44*(6), 1920-1934.

Viljoen, A., Wingfield, M. J., & Marasas, W. F. O. (1994). First report of *Fusarium subglutinans* f. sp. pini on pine seedlings in South Africa. *Plant Disease*, *78*(3), 309-312.

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

Vos, I.A., Pieterse, C.M.J. & Van Wees, S.C.M., 2013. Costs and benefits of hormone-regulated plant defences. *Plant Pathology*, 62(S1), pp.43–55.

Walters, D. & Heil, M., 2007. Costs and trade-offs associated with induced resistance. *Physiological & Molecular Plant Pathology*, 71(1-3), pp.3–17.

Wingfield, M. J., Jacobs, A., Coutinho, T. A., Ahumada, R., & Wingfield, B. D. (2002). First report of the pitch canker fungus, *Fusarium circinatum*, on pines in Chile. *Plant Pathology*, *51*(3), 397-397.

Wingfield, M. J., Hammerbacher, A., Ganley, R. J., Steenkamp, E. T., Gordon, T. R., Wingfield, B. D., & Coutinho, T. A. (2008). Pitch canker caused by *Fusarium circinatum*—a growing threat to pine plantations and forests worldwide. *Australasian Plant Pathology*, *37*(4), 319-334.

Zou, J., Rodriguez-Zas, S., Aldea, M., Li, M., Zhu, J., Gonzalez, D. O., & Clough, S. J. (2005). Expression profiling soybean response *to Pseudomonas syringae* reveals new defense-related genes and rapid HR-specific downregulation of photosynthesis. *Molecular Plant-Microbe Interactions*,*18*(11), 1161-1174.