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Oliveira Monteiro**

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em espécies de *Pinus* com diferentes graus de
suscetibilidade**

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Pinus species with different degrees of susceptibility**



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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, 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 do Doutor Artur Jorge da Costa Alves Investigador Principal do Departamento de Biologia da Universidade de Aveiro.

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“cada meta é uma nova linha de partida”

o júri

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palavras-chave

Doença do cancro resinoso, variação genética, interação planta-patogeno, mecanismos de defesa, performance fisiológica

resumo

A doença do cancro resinoso do pinheiro é causada por um agente patogénico - *Fusarium circinatum*, que afeta o género *Pinus* e também a espécie *Pseudotsuga menziesii*. Desde a sua descoberta em 1946, nos Estados Unidos da América, que a doença se tem dissipado pelo mundo provocando elevadas perdas económicas. Os sintomas característicos da doença são a descoloração, murchidão e queda das agulhas, o tombamento do ápice e a formação de cancros com exsudação visível de resina. Apesar das medidas de controlo aplicadas nos viveiros e plantações, ainda não existe um método eficaz para o controlo da doença sendo a seleção de material vegetal mais tolerante a ferramenta mais recomendada. Para tal, é crucial entender o mecanismo de infeção deste agente patogénico. Neste trabalho foram utilizadas três espécies de pinheiro, com diferentes graus de suscetibilidade - *P. radiata*, *P. pinaster* e *P. pinea*. Depois de 30 dias de aclimatização em câmara climática as plantas foram artificialmente inoculadas no caule com uma suspensão de esporos de *F. circinatum*. Para cada espécie foi implementado um grupo controlo (não inoculado). A amostragem foi realizada quando 50% dos indivíduos de um tratamento de cada espécie mostrou os sintomas característicos da doença – descoloração das agulhas, tombamento do ápice - tendo-se avaliado os seguintes parâmetros fisiológicos e bioquímicos: sobrevivência da planta, necrose relativa, trocas gasosas, relações hídricas, libertação de eletrólitos, pigmentos, prolina, açúcares solúveis totais e amido. Foram ainda realizados cortes histológicos para comparação anatómica do caule entre plantas controlo das três espécies. Após a infeção com *F. circinatum*, *P. radiata* foi a primeira espécie a mostrar sintomas – descoloração das acículas e tombamento do ápice, após 10 dias após inoculação, seguindo-se *P. pinaster* (17 dias) e por fim *P. pinea* (64 dias), onde apenas foi possível observar maior exsudação de resina e ramos laterais secos perto do ponto de inoculação. A inoculação com *F. circinatum* causou o aumento dos valores de necrose relativa em *P. radiata* e *P. pinaster* acompanhado com um aumento dos valores de libertação de eletrólitos. Em *P. pinea* não foram observadas estas alterações. Foi possível observar a resposta da planta a *F. circinatum* no que se refere ao seu impacto nas relações hídricas em *P. radiata* e *P. pinaster* através da diminuição do potencial hídrico e uma diminuição significativa no conteúdo relativo em água nesta última espécie, sem alterações em *P. pinea*. A prolina também teve um aumento significativo em *P. pinaster*. Nas espécies mais suscetíveis, *P. radiata* e *P. pinaster*, as trocas gasosas foram severamente afetadas ocorrendo um decréscimo na taxa de assimilação de CO₂, condutância estomática, transpiração e eficiência no uso de água, inversamente à concentração interna de CO₂ que registou um aumento. Observou-se um aumento das antocianinas, com maior incidência em *P. pinaster* e *P. pinea*. As diferenças a nível anatómico entre as três espécies estudadas, principalmente na espessura do córtex e no número de canais resiníferos, podem ser parte da explicação da tolerância encontrada em *P. pinea*. Os resultados apontam para uma maior plasticidade no ajuste osmótico em *P. pinaster* comparativamente a *P. radiata* que suporta o atraso verificado no aparecimento de sintomas apesar da maior proporção de necrose relativa observada. Com este estudo foi possível confirmar que a infeção com *F. circinatum* leva a uma resposta diferencial nas três espécies em estudo não só em termos temporais como aparentemente nos mecanismos de resposta que lhe estão associados.

keywords

Pitch canker disease, genetic variation, plant-pathogen interaction, defence mechanisms. physiological performance

abstract

Pitch canker disease (PCD) is caused by a pathogen – *Fusarium circinatum*, which affects *Pinus* genus and also the species *Pseudotsuga menziesii*. Since its first report in 1946, in the United States of America, the disease has spread worldwide provoking severe economic losses. The characteristic symptoms of the disease are the wilting and discoloration of the needles, that end up falling down, the tip dieback of the branches, and the formation of large cankers with visible resin exudation. Despite the control measures applied in nurseries and plantations, there is still no effective method for disease control, being the selection of tolerant plant material the most recommended tool. To do this, it is crucial to understand the pathogen infection mechanism. In this work three pine species with different degrees of susceptibility - *P. radiata*, *P. pinaster* and *P. pinea*, were used. After 30 days of an acclimatization period in a climatic chamber, plants were artificially inoculated in stems with a spore suspension of *F. circinatum*. A control group (not inoculated) was also implemented. Sampling was performed when 50% of the individuals of a treatment within each species showed the characteristic symptoms of the disease - needles discoloration, tip dieback - and the following physiological and biochemical parameters were evaluated: plant survival, relative necrosis, leaf-gas exchanges, water relations, electrolyte release, pigments, proline, total soluble sugars and starch. Histological sections were also performed between for comparison of control plants between the three species. After infection with *F. circinatum*, *P. radiata* was the first species to show symptoms, after 10 days-post-inoculation, followed by *P. pinaster* (17 days) and finally *P. pinea* (64 days), where it was only possible to observe a higher resin exudation and discoloured lateral branches near the inoculation point. The inoculation with *F. circinatum* caused the increase of the relative necrosis values in *P. radiata* and *P. pinaster*, where it was also possible to observe an increase on the values of electrolyte leakage. These changes were not observed in *P. pinea*. It was possible to observe the plants' response to *F. circinatum* infection regarding its impact on the plant-water relations in *P. radiata* and *P. pinaster* through a decreased water potential and a significant decrease in relative water content in this last species, without changes in *P. pinea*. Proline also had a significant increase in *P. pinaster*. In the most susceptible species, *P. radiata* and *P. pinaster*, leaf-gas exchanges parameters were severely affected, with a decrease of net CO₂ assimilation rate, stomatal conductance, transpiration and water-use efficiency, inversely to internal CO₂ concentration, which increased. An increase in anthocyanins was observed, with a higher incidence in *P. pinaster* and *P. pinea*. The anatomical differences between the three species studied, mainly in the thickness of the cortex and in the number of resin ducts, may be part of the explanation of the tolerance found in *P. pinea*. The results point to a greater plasticity in the osmotic adjustment in *P. pinaster* compared to *P. radiata* that supports the delay observed in the symptoms appearance despite the higher relative necrosis observed. With this study it was possible to confirm that the infection with *F. circinatum* leads to a differential response in the three species used, not only temporally related but also in the response mechanisms associated.

Table of contents

I. General introduction and aims	6
1. <i>Pinus</i> genus	6
2. <i>Fusarium circinatum</i> –taxonomy, biology and morphology	8
3. Pitch canker disease	9
4. The importance of plant genetic variation	13
5. Other management and disease control	15
6. Plant responses to pathogen attack	16
6.1. Mechanical barriers.....	17
6.2. Water relations	18
6.3. Photosynthetic performance.....	19
6.4. Carbohydrate mechanism.....	20
6.5. Nitrogen mechanism: the role of proline	21
6.6. Plant signalling and defence mechanisms: Oxidative stress, hormones and secondary metabolites	22
7. Aims	24
II. Physiological mechanisms behind <i>Fusarium circinatum</i> infection in three pine species with different degrees of susceptibility.	26
1. Introduction	26
2. Material and methods	30
2.1. Plant material	30
2.2. Fungal material	30
2.3. Experimental design and sampling	31
2.4. Histological sections	31
2.5. Visual aspect and disease symptomatology	32
2.6. Relative stem necrosis.....	32

2.7. Electrolyte leakage	32
2.8. Plant water status	33
2.9. Gas exchange parameters	33
2.10. Pigments quantification	34
2.11. Proline content	34
2.12. Total soluble sugars (TSS) and starch quantification	34
2.13. Statistical analysis	35
3. Results	36
3.1. Histological analysis	36
3.2. Visual aspect, growth, and plant disease symptoms	37
3.3. Relative necrosis	37
3.4. Electrolyte leakage	41
3.5. Plant water relations	42
3.6. Gas exchange related parameters	42
3.7. Pigments quantification	44
3.8. Total soluble sugars and starch quantification	45
3.9. Proline content	45
4. Discussion	46
4.1. Stem anatomical differences between species: does it matter?	46
4.2. Plant responses: a physiological approach	47
5. Conclusions	54
6. Acknowledgments	56
7. Future Perspectives	58
III. References	60

List of Figures

Part I

Figure 1 – Portuguese forestry.	6
Figure 2 – <i>Fusarium circinatum</i> spores.	8
Figure 3 - Worldwide dispersion of pitch canker disease.	10
Figure 4 - Plant symptomology.	11

Part II

Figure 1 – <i>Pinus-Fusarium</i> experimental design.	31
Figure 2 - Anatomical sections from <i>P. radiata</i> (a), <i>P. pinaster</i> (b) and <i>P. pinea</i> (c) healthy plants observed by optical microscope. All section were made under 40X (Ocular 10X and object-glass 4X) magnification.	36
Figure 3 - Plant symptomatology (%) in <i>P. radiata</i> , <i>P. pinaster</i> and <i>P. pinea</i> , control and inoculated with <i>F. circinatum</i> plants days post-inoculation (dpi).	37
Figure 4 - Plants visual aspect during sampling day of <i>P. radiata</i> (a), <i>P. pinaster</i> (b) and <i>P. pinea</i> (c), with associated treatments - control and plants inoculated with <i>F. circinatum</i> , respectively.	39
Figure 5 - <i>P. radiata</i> stem sections during necrosis length measurements.	40
Figure 6 - <i>P. pinaster</i> stem sections during necrosis length measurements.	40
Figure 7 - <i>P. pinea</i> stem sections during necrosis length measurements.	41
Figure 8 - Electrolyte leakage (%) in <i>P. radiata</i> , <i>P. pinaster</i> and <i>P. pinea</i> control and inoculated with <i>F. circinatum</i> plants.	41
Figure 9 - Water potential (ψ) (a) and Relative Water Content (RWC) (b) in <i>P. radiata</i> , <i>P. pinaster</i> and <i>P. pinea</i> control and inoculated with <i>F. circinatum</i> plants.	42
Figure 10 - CO ₂ assimilation rate (A) (a), internal CO ₂ concentration (ci) (b), stomatal conductance (gs) (c), transpiration rate (E) (d) and water use efficiency (A/E) (e) in <i>P. radiata</i> , <i>P. pinaster</i> and <i>P. pinea</i> control and inoculated with <i>F. circinatum</i> plants.	43
Figure 11- Total chlorophyll content (a), carotenoids (Car) (b) and anthocyanins (c) quantification in <i>P. radiata</i> , <i>P. pinaster</i> and <i>P. pinea</i> control and inoculated with <i>F. circinatum</i> plants.	44
Figure 12 - Total soluble sugars (TSS) (a) and starch quantification (b) in <i>P. radiata</i> , <i>P. pinaster</i> and <i>P. pinea</i> control and inoculated with <i>F. circinatum</i> plants.	45
Figure 13 - Proline content of <i>P. radiata</i> , <i>P. pinaster</i> and <i>P. pinea</i> in control and inoculated with <i>F. circinatum</i> plants.	45

List of Tables

Part II

Table 1 - Relative necrosis of *P. radiata*, *P. pinaster* and *P. pinea*, control and inoculated with *F. circinatum* plants, measured at the end of the experiment. Data are presented as mean \pm SE. Different asterisks indicate differences between treatments within each species ($p < 0.05$).

Table 2 - Two-way ANOVA summary table for morphological and physiological traits of non-inoculated and *F. circinatum* inoculated *P. radiata*, *P. pinaster* and *P. pinea* plants. Degrees of freedom (Df) and F value are shown for each source of variation; variance (MS) of the residual is also shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS, non-significant. Abbreviations: water potential (Ψ), net CO₂ assimilation rate (A), transpiration rate (E), stomatal conductance (gs), intercellular CO₂ concentration (Ci), water use efficiency (A/E) and total soluble sugars (TSS).

List of Abbreviations

·OH	Hydroxyl radical
ABA	Absciscic Acid
ATP	Adenoside Tri-Phosphate
CLA	Carnation Leaf Agar
DNA	Deoxyribonucleic Acid
E	Ethylene
ETI	Effectors Triggered Immunity
H ₂ O ₂	Hydrogen Peroxide
JA	Jasmonic Acid
MAMP's	Microbes-Associated Molecular Patterns
MDA	Malondialdehyde
NADPH	Dihydronicotinamide-adenine Dinucleotide Phosphate
NO	Nitric oxide
O ₂ ⁻	Superoxide radical
PAMP's	Pathogen-Associated Molecular Patterns
PCD	Pitch Canker Disease
PDA	Potato Dextrose Agar
PSII	Photosystem II
PTI	PAMP's Triggered Immunity
ROS	Reactive Oxygen Species
RWC	Relative Water Content
SA	Salicylic Acid
SM	Secondary Metabolites
SNA	Seawater Nutrient Agar
TCA cycle	Tricarboxylic acid cycle
TSS	Total Soluble Sugars
VOC's	Volatile Organic Compounds
WUE	Instantaneous Water-use efficiency

I. General introduction and aims

1. *Pinus* genus

Forests cover more than $4,1 \times 10^9$ hectares from the Earth surface, which represents about 30% from total world surface (Silva, 2010), covering 251 million hectares, in which 45% are covered by coniferous species (Rigo *et al.*, 2016). The genus *Pinus* (*Pinaceae*), considered to be the largest conifers genus, actually comprises over 100 recognised species and are considered to be economically relevant for human activities as an important source of wood, paper, resins, charcoal, food (nuts), and ornamentals (David M. Richardson & Rundel, 1998), where both forest resources, raw material and associated economical activities move around 1,2 billion euros (Mendes *et al.*, 2004; FAO 2007). Forests cover around 193 million hectares from the European territory, representing around 25% of the global forests territory, while in Portugal, according to ICNF (2013), the forestry landscape correspond to 3.154.800 hectares of all country, around 2% of all Europe forests panorama, where 977.988 were occupied only by resinous species (Figure 1).

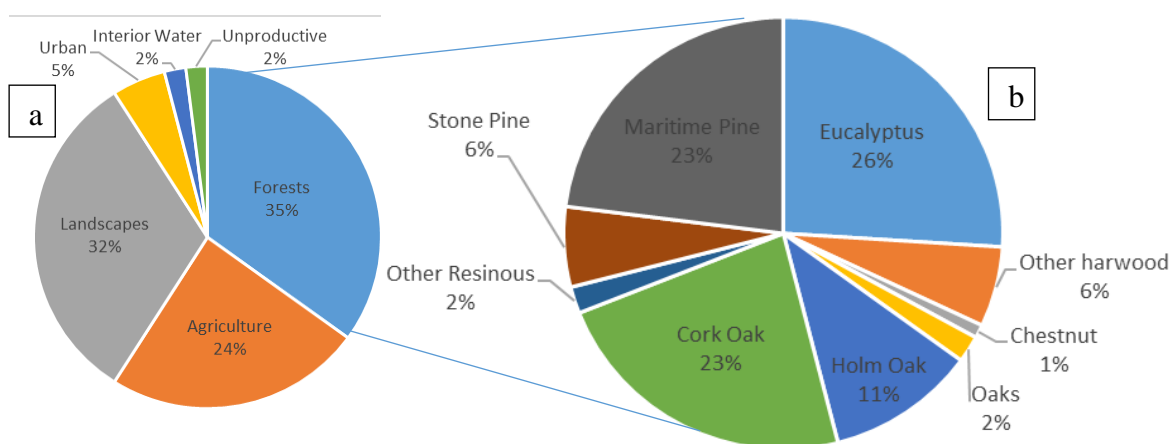


Figure 1 – **Portuguese forestry**. (a) Total Portuguese land use, in percentage; (b) representative species occupying forests sites, in percentage. Adapted from ICNF 2013.

Pinus radiata D.Don, or Monterey pine, is the most representative pine species planted worldwide – Chile, Australia, Spain or South Africa, Canada and United States of America (Lavery & Maed, 1998). The exact representation of *P. radiata* in the Portuguese landscape is not well known, belonging to the category “another resinous trees”, not having therefore an objective value for its land occupation (ICNF 2013). Due to its rapid growth, and medium-density softwood and suitability in a wide range of environments, it has been largely used in pine plantations. Besides the production of wood and fibre, *P.*

radiata plantations can also improve landscapes, reduce erosion, improve water quality, store carbon or harbour biodiversity. According to ICNF (2013), *Pinus pinaster* Aiton, also known as Maritime pine, occupied about 714 thousand hectares, being the most representative pine species, with an increased importance, being commonly used for reforestations, representing, in 2000, 11% of the total Portuguese exportation (Aguilar, Almeida, & Borralho, 2003). *Pinus pinea* L., also known as Stone pine, is responsible for the occupation of 175 thousand hectares, having an ecological, landscape and soil conservations importance together with its high economic impact – wood and pinion (nuts), which represents the most valuable product of this specie, providing significant incomes (Ayrlimis, Buyuksari, Avci, & Koc, 2009; Calama, Cañadas, & Montero, 2003). Both biotic and abiotic stresses are on the daily base of many plants' research papers due to their impact on global ecosystems and economy. Currently, both pine natural stands and plantations are often targets of different stress agents, both biotic and abiotic, being responsible for the decrement of pine productivity, and, therefore causing severe economic losses to countries which part of the economic scale relies on pine productivity. Furthermore, being accompanied by the climate changes occurring through these past decades, novel microorganisms started to appear, having devastating effects on pine plantations wellness, so it became mandatory to unveil the mechanisms behind stress tolerance in order to apply more efficient control measures (Reboredo, 2014). Several biotic agents, from insects – *Pissodes castaneus* (DeG.), *Ips sexdentatus* (Boern), *Orthotomicus erosus* (Woll.), *Tomicus piniperda* (L.) and *Tomicus destruens* (Boern), *Tomicus piniperda* (L.), *Thaumetopoea pityocampa* (Schiff), *Neodiprion sertifer* (Geoffroy), *Leucaspis pini* (Hartig) and *Leucaspis pusilla* (Loew), *Matsucoccus feytaudi* (Ducasse), *Pissodes validirostris* (Sahlberg), *Dyorictria mendacella* (Staudinger), *Cinara maritimae* (Dufour), *Bursaphelenchus xylophilus* (Steiner & Buhrer), to bacteria/fungi - *Cylindrocarpon* sp., *Botrytis cinereal* (Pers.: Fr.), *Lophodermium seditiosum* (Minter, Staley & Millar) and *Pestalotiopsis* sp., *Pestalotiopsis* sp., *Dothistroma* sp., *Diplodia sapinea* (Fr.) Fuckel and *Thyriopsis halepensis* (Cooke) Theiss. & Syd., *Neofusicoccum luteum*, *Neofusicoccum australe* and *Neofusicoccum parvum* and *Fusarium circinatum* (Nirenberg and O'Donnell) have been identified and considered to have nefarious effects on pines plantations (Branco *et al.*, 2014).

2. *Fusarium circinatum* –taxonomy, biology and morphology

The phytopathogenic fungi, *Fusarium circinatum* Nirenberg and O'Donnell, 1998 (teleomorph = *Gibberella circinata*) was first described as being an unidentified species from the *Leseola* section (Hepting & Roth, 1946). The technological evolution of equipment's and protocols allowed a better characterization, being nowadays included in the Ascomycota phylum. It is characterized by having a sexual stage, *Gibberella circinata*, and an asexual stage, *Fusarium circinatum*. In this species, only micro and macroconidia are produced, being characterized by the absence of chlamydospores. Macroconidia are usually 3-septate and possess a curved shape (Figure 2). Relatively to microconidia, they doesn't have septa (0-septate), having a rounded shape (oval/ovoid) (Leslie & Summerell, 2006) (Figure 2). The structural form/shape of the hyphae, micro- and macroconidia depends on the medium where *F. circinatum* is cultured. The most common mediums used are Carnation Leaf Agar (CLA), where rounded shaped microconidia are borne on mono- and polyphialides. It is possible to observe coiled hyphae, being more distinguishable when cultured on Seawater Nutrient Agar (SNA) due to higher degree of coiling hyphaes. The cultures on Potato Dextrose Agar (PDA) are characterizes by white and some violet pigmentation of the aerial mycelium (Leslie & Summerell, 2006).



Figure 2 – *Fusarium circinatum* spores. The white arrow represents macroconidia – 3-septate, while the black arrow represents microconidia, ovoid and 0-septate (photo: Pedro Monteiro).

Over evolutionary process fungi have developed several types of lifestyle, from biotrophic fungi, which live from healthy host tissues, to necrotrophic fungi, which produce different mycotoxins to kill their host, feeding from the nutrients released from death tissues, to withdraw the necessary nutrients from their hosts and fulfil their nutrient requirements (Horbach *et al.*, 2011). A more complex lifestyle is the hemibiotrophic fungi, which during their life cycle have a biotrophic period that feed on healthy tissues and, responding to a specify stimuli, is changed to a necrotrophic phase, as is the case of *F. circinatum* (Ma *et al.*, 2013).

3. Pitch canker disease

Fusarium circinatum is the causal agent of pitch canker disease, the most devastating disease affecting 57 species of *Pinus* species (Wingfield *et al.*, 2008) and *Pseudotsuga menziesii* (Douglas Fir) (Gordon *et al.*, 2006), being associated with high levels of tree mortality and a reduction in plant productivity. Since *Pinus* genus is used in a wide range of agricultural, construction and forestry companies, the proliferation of this disease can result in significant economic losses (Wingfield *et al.*, 2008). Being first reported in south-eastern areas of United States of America (Hepting & Roth, 1946), California (McCain *et al.*, 1987; Storer *et al.*, 2002), Florida (Dwinell & Phelps, 1977) it is nowadays spread around the world, being found in South Africa (Viljoen *et al.*, 1994; Coutinho *et al.*, 2007; Steenkamp *et al.*, 2014; Fru *et al.*, 2017), Chile (Vogler *et al.*, 2004; Jacobs *et al.*, 2007), Uruguay (Pfenning *et al.*, 2014), Brazil (Pfenning *et al.*, 2014), Colombia (Steenkamp *et al.*, 2012; Pfenning *et al.*, 2014), Portugal (Bragança *et al.*, 2009), Mexico (Britz *et al.*, 2001), Spain (Landeras *et al.*, 2005; Pérez-Sierra *et al.*, 2007; Berbegal *et al.*, 2013), Italy (Carlucci *et al.*, 2007), France (EPPO, 2007) Japan (Muramoto & Dwinell, 1990) and Korea (Lee *et al.*, 2000) (Figure 3).

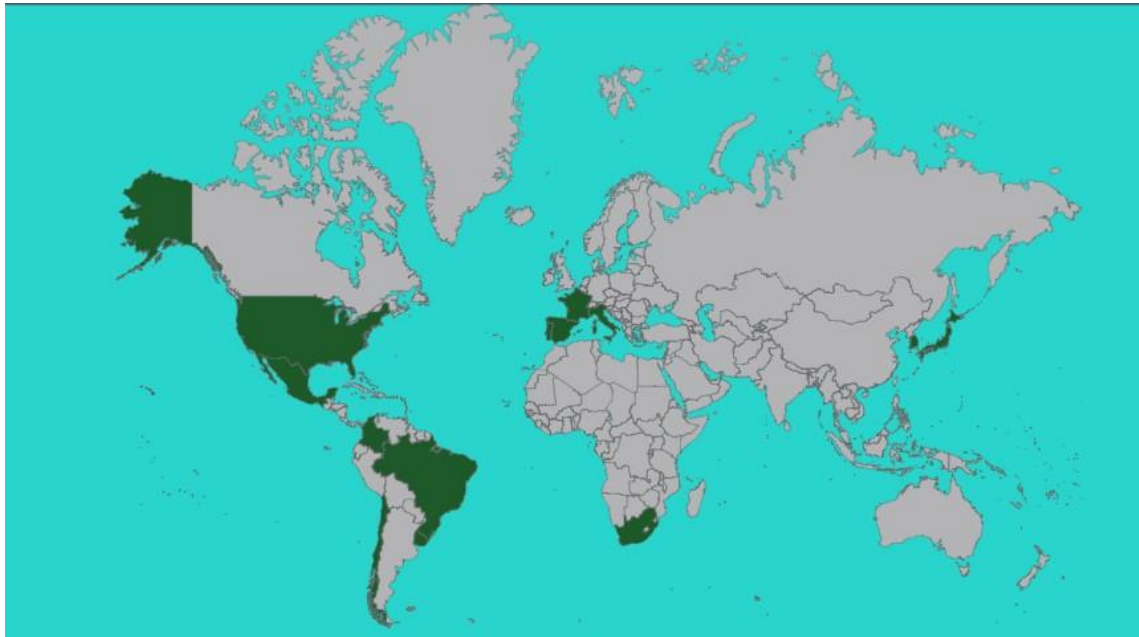


Figure 3 - **Worldwide dispersion of pitch canker disease.**

Being capable of infect either vegetative structures such as shoots, branches, stems and exposed roots and reproductive structures such as cones, seeds or flowers, of individuals of any age and at any time of the year, this pathogen's hosts show a characteristic set of disease symptoms which may lead to death. The first symptom to appear is the wilting of the needles, leading to their discolouration, passing from a green colour to a brown one, ending up to fall off (Figure 4a) (Storer *et al.*, 1995; Wingfield *et al.*, 2008). Without needles, branches end up dying – branch dieback. The progression of the disease normally occurs from the tip of the branch to the infection site, due to the obstruction of the plant's xylem causing a water flow blockage and a posterior branch's death as demonstrated by Solel and Bruck (1990). This obstructions are caused by the accumulation of resin on the plant's inner tissues, becoming soaked with resin, not deforming the plant tissues from external point of view, at the infection site causing resin cankers, helping therefore distinguishing this specific type of canker (Figure 4b) (Dwinell *et al.*, 1985). This resin accumulation is visible when damages are made in the bark through resin bleeding (Wingfield *et al.*, 2008). Tissues affected by the cankers are usually soaked in resin, causing resin bleedings, being yellow coloured (Storer *et al.*, 1995). The tree does not die by the infection of one branch only. The scenario becomes more serious when repeated infections occur within the same individual in different branches leading to an extensive canopy's dieback (Gordon *et al.*, 2001). When infected by *F. circinatum*, mature flowers

showed high mortality rate and, and deterioration or unviability of cones was visible (J. B. Barrows-Broaddus, 1990). Depending if the cones infection it is only superficial, or also internal, seeds may or may not germinate into asymptomatic seedlings since they have *F. circinatum* growing as an endophyte fungus but do not show pine pitch canker disease's characteristic symptoms, contributing to an easier dispersion of the disease to new places (Storer *et al.*, 1998). Viljoen *et al.*, (1994) also found that when colonized by this pathogen, *Pinus patula* seedlings presented rotten and necrotic roots that also leaded to death of individuals. Abiotic and biotic factors are considered to play major roles regarding the proliferation and dispersion of pine pitch canker disease agent around the world. These factors are known to be crucial for trees injuries and consecutively, allowing the entrance and infection of *F. circinatum* (Hammerbacher, 2005).



Figure 4 - **Plant symptomology.** (a) Needle discoloration and tip dieback (photo: Pedro Monteiro); (b) Pine pitch canker disease characteristic symptom - resin flow (photo: Michael Muller).

Among the abiotic factors, wind related events, such as hurricanes or tornadoes, are considered to be one of the most influent factors responsible for natural wounds on the tree branches. Human activities, such as cone removal, either manually or using tree shakers, are other factors which have to be taken into account due to the formations of fresh wounds on tree's branches, being the perfect environment for *F. circinatum* colonization and

infection (Dwinell & Barrows-Broadbent 1982). With the current environmental changes, studies were carried out in order to understand the effects that these could have, not only on fungal development, but also on the physiological state of the host. It is reported that the optimal growing temperatures for *F. circinatum* are within 14-26°C range (Leslie & Summerell, 2006). Other studies were focused on the importance of water availability in the host's susceptibility, and, with the decreasing of water availability, was found that pines were more susceptible to the disease (Dwinell *et al.*, 1985). Further studies were carried out, and air pollution can also play a key role in the propagation of pine pitch canker disease. High ozone concentrations were correlated with a higher development of the fungi within susceptible and resistant hosts (Carey & Kelly, 1994). In pine nurseries it is usual to apply fertilizers and nutrients to give better conditions for plant growth. It is documented that high levels of nitrogen (N) predispose pines to *F. circinatum*, being observed more severe symptoms and mortality rate (Lopez-Zamora *et al.*, 2007).

Regarding biotic factors, insects are considered to be the main vectors of the disease infection and dispersal. It is important to note that different locations means that exists specific endemic pine and insect species, that are present in some locations, being absent in others (Wingfield *et al.*, 2008). In South-eastern United States (SE-US), mechanical wounding is considered to be the most important vector of disease transmission, but insect also have an important role in disease dispersal. According to Blakeslee *et al.*, (1978) and Matthews (1962), *Pissodes nemorensis* Germas (Deodar weevil) and *Rhyacionia subtropica* Miller (subtropical pine tip moth), respectively, are the most relevant species responsible for tree's wounding, and thus, pitch canker disease progression. Other insects, have been reported as secondary vectors, such as *Contarinia* spp. (Needle midge), *Leptoglossus corculus* Say., *Tetraya bipunktulata* H.S.; worms, *Laspreyresia* spp.; and chalcids, *Megastionus atedius* Walker, can be responsible for the of *F. circinatum* infection on pines (Hammerbacher, 2005; Wingfield *et al.*, 2008). When it refers to California, south-western United States (SW-US), the genus *Ips* have the most representative slice when it comes to *F. circinatum* infections. *Ips mexicanus* Hopkins, *Ips paraconfusus* Lanier and *Ips plastographus* LeConte are the three main insect vectors present in California, SW-US, colonizing more debilitated individuals, either by naturally occurring wounds or by water scarcity. (Hammerbacher, 2005; Wingfield *et al.*, 2008). Other study carried out showed that there was a significant association between the presence and

infection of immature conelets of *P. radiata* through contact with different beetle species: *C. radiatae*, *E. punctulatus*, and/or *Pityophthorus* spp, moreover, the interspecific transmission of the inoculum between beetles species, occurred through the colonization of infected galleries (Hoover *et al.*, 1996). Other locations where *F. circinatum* was previously reported were object of studies of insects and fungi association. In South Africa, association between *F. circinatum* and *P. namorensis* (weevil) was found in insect's galleries on infected trees recovered from a plantation in Western Cape Province. The impact of *P. namorensis* on *F. circinatum* infection was previously described by Blakeslee *et al.*, (1998) (Coutinho *et al.*, 2007). In Spain, *F. circinatum* was recovered from several insect species; *Pityophthorus pubescens*, *Brachyderes incanus*, *Hylurgops palliates*, *Ips sexdentatus*, *Hypothenemus eruditus*, *Hylastes attenuates* and *Orthotomicus erosus* present in a *P. radiata* stands in Basque Country (Romón *et al.*, 2007).

The interaction of *F. circinatum* combined with other microorganisms, either fungi or bacteria, have been a target of the recently research interest (Hammerbacher, 2005). Several *in vitro* studies have been carried out and interesting results were found. *Cronartium quercum* f. sp. *fusiforme* is recognized as the infectious agent responsible for fusiform rust galls, and, pines when infected by this agent became more susceptible to a posterior colonization by *F. circinatum*. (Hepting, 1971; Dwinell *et al.*, 2001). Recent studies are using as guidelines the antagonistic effect of fungi as a potential method for management and control of the pitch canker disease (Hammerbacher, 2005).

4. The importance of plant genetic variation

The unavailability of plants to move upon handicap lead them to be able of re-organize their metabolism, which may lead to mutations in DNA and therefore changing their genotype in order to successfully overcome stress, arising resistant/tolerant genotypes (Roy & Kirchner, 2000). The selection of resistant genotypes to pathogens have been utilized as a management control other pests (Punja, 2001; Munkvold, 2003; Schmidt, 2003; Ratnadass *et al.*, 2012).

Despite the knowledge of the infection capacity of *F. circinatum* in several pine species, the symptom's severity varies among species, thus indicating that not all species have the same degree of vulnerability and respond in the same way. Several studies have already reported the differential response of different pine spp. to *F.*

circinatum infection, proving that genetic variance, both intra-specific (Storer *et al.*, 1999; Schmale *et al.*, 2003; Vivas *et al.*, 2012) and inter-specific (Gordon *et al.*, 1998a; Roux *et al.*, 2007; Kim *et al.*, 2008; Mitchell *et al.*, 2013) could be a promising alternative to the management and control of *F. circinatum*, replacing susceptible genotypes for resistant ones in areas that are most affected by PCD (Gordon, 2006; Wingfield *et al.*, 2008).

In 1983, Barrows-Broadbent & Dwinell led an investigation where analysed histological modifications of four different pine species, with different degrees of susceptibility to *F. moniliforme* var. *subglutinans*. In their study, through histopathological analysis, they observed that genetic variation among the four species resulted in a differential response in both survival and resistance mechanisms.

A screening over Central American and Mexican pine spp. together with *Pinus radiata* showed high susceptibility to *F. circinatum* (Hodge & Dvorak, 2000). Studies carried out by Storer *et al.*, (1999) demonstrated that depending on the spores' quantity, the time of the year and if the trees were grown in natural or planted sites, some of the individuals did not showed the characteristic symptom of the disease leading to further studies testing the inter-species resistance of other pine species to *F. circinatum*: *Pinus echinata* and *P. virginiana* showed to be more susceptible when compared with the other tested four different pine species and hybrids tested (Kim *et al.*, 2008). Roux *et al.*, (2007) investigate the tolerance among South African *Pinus* families and hybrids, observing that hybrids presented more tolerance to *F. circinatum*, revealing, therefore to be an alternative to pine plantations. *P. muricata* trees were also inoculated with the fungus, and the number of inoculations had an effect on the resistance of this species to *F. circinatum* (Schmale & Gordon, 2003). Studies carried out with *P. pinea* (Gordon *et al.*, 1998) and *P. pinaster* (Vivas *et al.*, 2012) showed higher resistance to the pitch canker disease fungus. Between these two species, in a study carried out by Iturriza *et al.*, (2013) demonstrated higher resistance of *P. pinea* when compared to both *P. radiata* and *P. pinaster*. Also intra-species variations in natural stands of *P. radiata*, showed that genetic variation and induced systemic resistance can be responsible for conferring resistance to *F. circinatum* (Storer *et al.*, 1999). In another study carried out by Enebak & Stanosz (2003), jack pine (*Pinus banksiana* Lamb.), red pine (*Pinus resinosa* Aiton), eastern white pine (*Pinus strobus* L.), Scots (*Pinus sylvestris* L.) and Austrian pine (*Pinus nigra* J.F.Arnold) were tested to assess the susceptibility of these species to *F. circinatum*. Two inoculations point were made, the

first in April 1999 and the second one in June 1999. The results demonstrated that Austrian and red pine were more tolerant to *F. circinatum*, showing less resin production, canker length and seedling mortality 12 weeks pot-inoculation, while the other three species demonstrated to be more susceptible to the pathogen.

5. Other management and disease control

The easy airborne dispersion of *F. circinatum*'s spores turns the management and control of the pitch canker disease, either in plantations/nurseries as in natural stands, a very difficult task. Therefore, it is mandatory to integrate and apply different management and control methods in order to reduce the economic impact of the disease and prevent the establishment of the fungi in new areas (Wingfield *et al.*, 2008).

Cultural, chemical and biological methodologies are the most commonly used, but none of them revealed entirely efficient against *F. circinatum* infections (Hammerbacher, 2005). When the pathogen is reported in a nursery, several phytosanitary and quarantine measures are applied. The use of clean seeds for germinations should be thoroughly confirmed. Beside, pathogen-free water, used for plant's irrigation, sterile soil and containers must be used in order to avoid pathogen proliferation within the nursery (Wingfield *et al.*, 2008). In pine plantations, if the pathogen has sporadically outbreaks, and only in a minor number of individuals, proper silvicultural methods, the pruning of the infected tips and/or branches – far from the infection site, can be applied, although it does not eradicate the disease from the area, existing the possibility of future outbreaks. The movement of infected plant material, between or within countries, plus the silvicultural tools/machinery should be limited in order to avoid propagation to clean areas and the complete destruction - chipping, debarked and burning - of infected branches and entire trees must be applied in order to avoid the dissemination of the disease through possible vectors. (Baker *et al.*, 2010; Storer *et al.*, 1995). In case of *F. circinatum* identification in a nursery, quarantine measure should be applied, being prohibited the movement of any seed species from the location (Baker *et al.*, 2010). Together with this practices, the management of *F. circinatum* can be achieved selecting resistant plant genotypes to the pathogen and/or alternative pine species (Wingfield *et al.*, 2008).

Chemical control – fungicides, systemic and non-systemic, insecticides or disinfectants - has often been used in order to eliminate the pathogen from seed surface (coatings).

Among fungicides, captan and thiram, both acting as non-systemic, showed the lowest phytotoxic effect on seed germination (Barnett & Varela, 2004). Regarding systemic fungicides, benomyl showed promising results regarding pathogen control and seed germination being as effective as hydrogen peroxide (Barnett *et al.*, 1999), but due to its secondary effects and the existence of resistant strains of *F. circinatum* to benomyl (Yan *et al.*, 1993), was commercially removed. Barnett & Mcgilvray (2002) carried out a study using thiophanate-methyl, showing that treatment of pine seeds with this chemical improved seedling production. Furthermore, authors also used thiophanate-methyl to treat pine seeds, and although it did not showed the same results as hydrogen peroxide, altering the concentration may be a plausible solution (Barnett & Varela, 2004). Runion & Bruck (1988) observed that the treatment of longleaf pine seeds, which were known to be contaminated with pitch canker fungus, with thiabendazole suspended in 10 % dimethyl sulfoxide decrease the *in vitro* growing of the pathogen.

In other studies, temperature shocks treatments, were applied to seeds surface, and evaluated as a potential, and effective treatment, against pitch canker disease. Since the temperature applied can penetrate into the seed, it should be raised, as far as possible, to the lethal temperature of the infectious organism (Agustí-Brisach, Pérez-Sierra, Armengol, García-Jiménez, & Berbegal, 2012). The same authors observed that, although the germination of pine seed was decrease compare to the control group, the application of a hot-water treatment (HWT) – 52° C to 55°C - to pine seeds that were infected with *F. circinatum*, respectively reduced the growing of conidia and mycelium in PDA media. In other study, the 90 seconds microwave exposure of *Pseudotsuga menziesii* (Douglas-fir) seeds, soaked in water (66.5° C) led to a significant reduction of *F. circinatum* infection levels (0.4 %). The germination of the seed was not affect with this temperature and time exposures. However, to obtain a total elimination of *F.circinatum* infection, was necessary a longer exposure period (120 seconds) in the microwave, having posteriorly effect on seed germination (Dumroese *et al.* 1998).

6. Plant responses to pathogen attack

When plants are confronted with a stress condition, such as a pathogen attack, unlike animals, which possess mobility to defend themselves, have to change their primary and secondary metabolisms in order to induced defence mechanisms (Fagard *et al.*, 2014).

Plant's innate immunity can be divided in two types of defence: pre-existing defences, which are non-specific, such as cell wall and cytoskeleton or present compounds with anti-microbial activity, and induced-defences (Rojas *et al.*, 2014). To activate induced-defences, plants use molecular patterns recognition associated to pathogens - Pathogen-Associated Molecular Patterns – PAMP's, such as chitin in case of fungi, or Microbe Associated Molecular Patterns - MAMPS, such as flagellin in microbes, giving origin to PAMP-triggered immunity (PTI) (Bolton, 2009). Through evolutionary processes, microorganisms became able to produce effectors proteins to suppress and overcome this first layer of plant defences. On the other hand, plants developed recognition mechanisms of these effectors, through the release of plant resistance (R) proteins that trigger effector-triggered immunity (ETI) (Chisholm *et al.*, 2006). After pathogen recognition and to avoid their spread, plants can have an early response, through morphological modifications, such as cell wall fortification (structural barriers), to biochemical re-arrangements and *de novo* synthesis of secondary metabolites – such as phytohormones, phytoalexins and plant volatiles, that can interacting directly with pathogens, be used indirectly, attracting natural pathogen's enemies, as signalling molecules, having their function distant from the infection site - systemic response, or react to pathogens through later events, such as transcription of pathogen-related proteins (PR-proteins) or induce programmed cell death (Bolton, 2009; Eyles *et al.*, 2010; Rojas *et al.*, 2014). Pathogen recognition will trigger modulation of plant's metabolism which will be reflected in changes at plant water relations, photosynthesis and carbohydrate metabolism, in order to overcome infection. Combining data obtained from direct pathogen infection parameters – visual aspect, disease symptomatology and necrotic lesions, together with parameters related to plants' physiology – electrolyte leakage (EL), water relations, photosynthetic capacity and leaf gas exchanges, and biochemical parameters related to plant metabolism – proline, total soluble sugars and starch content, it is possible to understand how plants react and respond upon a pathogen's attack.

6.1. Mechanical barriers

In a response to pathogens attack, plants possess non-specific barriers in order to prevent or contain pathogens colonization. Trees trunk is constituted by several layers of specialized cells. The outer layer, also called “bark”, is constituted by tissues external to

vascular cambium. The rhytidome – “outer bark”, is constituted by aggregates of death tissues, mainly layers of periderms and associated tissues is given, while the inner bark is constituted by living tissues – secondary phloem and periderm. (Biggs, 1992). Another characteristic chemical and physical defence of conifers is the induced production of resin, throughout an elaborate architecture of resin ducts present in wood and bark, upon injury or pathogen attack (Trapp & Croteau, 2001).

The investigation carried out by Barrows-Broaddus & Dwinell (1983) analysed histological modifications of four different pine species, with different degrees of susceptibility to *F. moniliforme* var. *subglutinans*. In their study, in susceptible species, higher values of active lesions and seed mortality. In the case of higher tolerant species, inoculation triggered cell wall lignification and regeneration of parenchyma layers, in order to content pathogen’s progression, presenting lower values of seed mortality and active lesions.

6.2. Water relations

In plants, water, together with CO₂ and light, plays a pivotal role for plants’ energy obtainment through photosynthesis (Atkin, *et al.*, 2000). Due to its high polarity characteristics, water is characterized by its capacity of transporting metabolites through the cell, delivering large quantities of ions and polar organic metabolites such as sugars, amino acids, and proteins. The two main parameters used to analyse plant water status are the plant water potential, through a thermocouple psychrometer or a pressure chamber, and relative water content, which are closely linked having the same profile response in plants (Turner, 1981). Studies proved that after inoculation *F. circinatum* proliferations caused phloem to collapse and fungal proliferation within the host occurred both upwards and downwards within the xylem vessels (Martin-Rodrigues *et al.*, 2013). Water movement along plants’ vessels is caused by water evaporation in leaves – transpiration, which in turn is regulated by stomatal opening/closure. This loss of water obligates nutrients and allows transport of nutrients from the soil to the root surface and of solutes from roots to storage organs in an interactive process (Lambers *et al.*, 2008). With the blockage of xylem a phloem observed by Martin-Rodrigues *et al.*, (2013), water flux may be compromised. Cerqueira *et al.*, (2017) observed a decrease in water potential values - approximately 1.3 times higher, reaching -0.370 MPa, upon *P. radiata* inoculation with *F. circinatum*.

6.3. Photosynthetic performance

The role of photosynthesis in plant defence is a fundamental. Upon a pathogen attack, plants have to adapt themselves and channel efforts to modify their metabolism to enhance defence strategies, with a determined energy cost associated (Rojas *et al.*, 2014). It should be expected a decrease in the photosynthesis rate due to: the appearance of necrotic lesions, as a result of pathogens' attack, indicating the absence of chloroplasts capable of photosynthesize, resulting in a decrease of photosynthetic capacity and consequently in the energy production or by a triggering on respiration process, in detriment of photosynthesis, in order to fulfil plant requirements to initiate defence processes (Berger *et al.*, 2007). The reduced photosynthetic rate, besides being reduced due to leaf necrotic lesions and therefore reduced leaf area, in case of pathogens which affect plant water relations, it could also be a result of colonization of transport vessels by the fungi or through a direct effect of the fungi on the host, through toxins emissions (Walters, 2015). Several studies have already used leaf-gas exchange measurements to evaluate physiological responses of plants to pathogens observing a decrement in the photosynthetic rate upon pathogen inoculation (Bowden & Rouse, 1991; Nogués *et al.*, 2002; Alves *et al.*, 2011; Cerqueira *et al.*, 2017).

.Through the *in vivo* analysis of CO₂ exchanges, using an infra-red gas analyser (IRGA), it is possible monitor changes in stomatal conductance, transpiration rate, and the overall plant capacity to photosynthesize, and therefore evaluate plants' fitness and how its reacting to a certain stress (von Caemmerer & Farquhar, 1981).

Being a non-invasive method, another valuable tool used to analyse plant photosynthesis is through chlorophyll fluorescence, where light absorbed by photosystem II (PSII) can proceed to three different destinations, being absorbed by the reaction centre chlorophyll, P680 or being dissipated through heat or fluorescence (Baker, 2008). Upon a stress condition, such as pathogen attack, down-regulation of the PSII florescence has been reported (Berger *et al.*, 2007). Bauriegel *et al.*, (2011) combined chlorophyll fluorescence with hyperspectral imaging to *in vivo* detect head blight disease in wheat (*Triticum aestivum* L. "Taifun") caused by *Fusarium culmorum*, observing a decrease in photosynthetic efficiency after fungal inoculation.

Being associated with photosynthetic performance, pigments - chlorophylls (*a* and *b*), which are the most abundant group in higher plants, are part of chloroplasts and responsible for the capture light energy and being the first electron donors (photoreceptors) (Katz *et al.*, 1978). Carotenoids, classified as tetraterpenes (isoprenoid family), are structural components of plants that have a function as protectors of the photosynthetic apparatus, by preventing photo-oxidation, have a determining function in pathogens' response (Davies, 2004; Lichtenthaler, 1987). During pathogen infection, the accumulation of ROS, either synthesized and used by plant as signalling molecules or as result of pathogen damage, is a common event in plant-pathogen interactions (Baxter *et al.*, 2014; Bastas, 2015). During excess light energy events, occurs the formation of excited triplet state of chlorophylls that will form singlet oxygen molecules and further photooxidation and photosynthetic apparatus damages. Carotenoids are able of absorb this excessive light energy from chlorophylls preventing the occurrence of damages in photosynthetic antennae (Graßmann, 2005).

Anthocyanins, one of the end product of phenylpropanoid biochemical pathway, beyond being responsible for plant colouring, have an important role in plant defences against both biotic and abiotic stress, such as osmotic regulators under drought and frost stress, antioxidants, phytoalexins and antimicrobial agents against pathogens attack (Kong *et al.*, 2003). Furthermore, they can also be used as protective agents of the photosynthetic apparatus due to its absorption capacity in the same wavelength as chlorophyll *b*, serving therefore as auxiliary agents preventing oxidative damages (Stintzing & Carle, 2004).

6.4. Carbohydrate mechanism

Sugars, which are the outcome of the photosynthetic process represent a source of energy, not only for the plants, but also for the pathogen which tries to manipulate plants' metabolism in their own benefit (Berger *et al.*, 2007). Beyond this, these molecules have been proved to be involved in plant signalling upon abiotic and biotic stresses (Moghaddam & Ende, 2012). Many researchers believe that sugars are involved in PTI and ETI, acting as priming molecules, enhancing plant resistance to pathogens (Morkunas & Ratajczak, 2014). In a normal situation, after photosynthesis, sugars are directed from photosynthetically active tissues, or source tissues, to storage organs, or sink tissues (Roitsch, 1999). Starch, which constituted by glucose, amylose and amylopectin is the

most common long-term storage product of higher plants (Zeeman *et al.*, 2010). During a pathogens attack the existence of a higher energy demand in order to overcome infection, together with and already discussed decrease in the photosynthetic rate, leads to a usage of the storage products by the host (Berger *et al.*, 2007). Both pathogens and plants are able to produce extracellular enzymes – cell wall invertase (CWI) - capable of degrading carbohydrates to their own benefits. Further studies have to be made in order to understand if the documented changes in the carbohydrate assimilates were from the pathogen, or from the plant, that produces enzymes responsible for the degradation of apoplastic sucrose into sucrose and glucose that are posteriorly transported into the cells and used to activate defence mechanisms. This transport also reduces the amount of available hexoses in the apoplast, reducing therefore the resources for the pathogen (Berger *et al.*, 2007; Bolton, 2009).

6.5. Nitrogen mechanism: the role of proline

Amino acids, which usually assimilate carbon into their structures, in a plant-pathogen scenario have to change their “chip”, routing carbon to energy production metabolism, like the tricarboxylic acid (TCA) cycle (Bolton, 2009). During plants metabolism occurs the production of the denominate non-protein amino acids (NPAAs). Proline is an amino acid essential for primary metabolism possessing a characteristic conformational rigidity together with an osmoprotective role and function as a chaperone protecting protein integrity and enhance different enzymes accumulation in both abiotic and biotic stress conditions (Szabados & Savoure, 2009). Alkaloids are recognized as another plant defence related family of compounds, being divided in three groups according to their structure, presence and location of nitrogen. True alkaloids, which are derived from amino acids, are cyclic compounds and nitrogen is present in the heterocyclic ring, on contrary to protoalkaloids, that despite their derivation from amino acids, the nitrogen is located outside the heterocyclic ring. Pseudo-alkaloids, do not derive from amino acids (Bennett & Wallsgrove, 1994a). Proline is considered to be involved in plant water status tolerance acting as an osmoprotective osmolyte, a ROS scavenging agent, a buffer to stabilize cellular redox potential or as a chaperone, stabilizing proteins structure, upon several described stresses such as salinity, drought, temperature variations, heavy metals and biotic interactions (Verbruggen & Hermans, 2008). Upon *P. radiata* seedling inoculation with *F. circinatum*, Cerqueira *et al.*, (2017) observed an increment in proline content.

6.6. Plant signalling and defence mechanisms:

Oxidative stress, hormones and secondary metabolites

During the normal functioning of plants cell, reactive oxygen species (ROS) – hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxyl radical ($\cdot\text{OH}$), and nitric oxide (NO) are being produced, accompanied by a proper elimination through scavenging mechanisms preventing the occurrence of oxidative damages (Bastas, 2015). During a pathogen attack, the occurrence of ROS production is one of the events to possible cause damages in proteins, DNA, membrane lipids, reduce photosynthesis, increase electrolyte leakage and cause cell death. Recent studies have also hypothesized ROS function as molecular signalling molecules during plant responses to stress (Bastas, 2015; Mittler *et al.*, 2011). To prevent this, when higher levels of ROS – oxidative burst - are present, plants initiate the biosynthesis of antioxidant molecules (Brien *et al.*, 2012). Damages in cell membranes can be assessed by electrolyte leakage estimation, which reflects the permeability of the membrane to electrolytes (Kombrink & Somssich, 1995). From the peroxidation of the unsaturated lipids present in the membranes, by the ROS species, occurs the liberation of peroxides of polyunsaturated fatty acids, such as malondialdehyde (MDA) responsible for cell membrane damages, being another possible indicator of oxidative damages (Davey *et al.*, 2005; Sharma *et al.*, 2012). Other compound have been related to the response of plant to pathogens attack. Plant hormones, such as salicylic acid (SA), jasmonic acid (JA), ethylene (E) abscisic acid (ABA) and auxins, are molecules that are present in the plant with diverse functions, from being important for growth and plant development and, on the other side, act as signalling molecules upon stress, , responsible for the *in planta* communication to enhance plant immune system upon pathogen invasion (Rojo *et al.*, 2003). The production of secondary metabolites secondary metabolites (SM) has an wide range of functionalities, from pollinator's attraction and reproduction to stress response and defence mechanisms, as happens upon a pathogen attack (Pusztahelyi *et al.*, 2015). Several classes of SM (cyanogenic glucosides, glucosinolates, alkaloids, phenolic compounds, phytoalexins, terpenoids) have been related to plants response to pathogens attack (Bennett & Wallsgrove, 1994).

7. Aims

Fusarium circinatum is the causal agent of the pitch canker disease affecting several conifers species and responsible for causing economic losses worldwide and since its discovery several studies have been carried out in order to characterize the pathogen and evaluate its pathogenicity on seeds and seedling together with worldwide occurrence and symptoms characterization (Gordon, 2006). Efforts have been made trying to find an effective mechanism to control pitch canker disease focusing on the response of both seeds and seedlings to several treatments, both chemical and biological, but, since exists a clear knowledge and information gap between plants' physiological response to *F. circinatum* infection and the underlying mechanisms it becomes difficult to find an efficient control method (Cerqueira *et al.*, 2017; Martin-Rodrigues *et al.*, 2013). To reduce the economic impact of this devastating disease on nurseries, wood and pulp industries, it is mandatory to have an integrative approach of physiological, morphological and biochemical techniques and experimental data to have a better insight on infection mechanisms. The differential response of pine species to the pathogen have been documented and selection of the most tolerant species is another recommend method to use in both nurseries and plantations (Wingfield *et al.*, 2008).

The main goal of this work is to contribute to decipher the plant's responses using three pine species - with different degree of susceptibility (*P. radiata*, *P. pinaster* and *P. pinea*) to the pathogen, *F. circinatum*, by linking morphological, physiological and biochemical data.

II. Physiological mechanisms behind *Fusarium circinatum* infection in three pine species with different degrees of susceptibility.

1. Introduction

Forests cover more than $4,1 \times 10^9$ hectares from the Earth surface, representing about 30% from total world surface (Silva, 2010). In the Portuguese context, according to ICNF (2013), the forestry landscape correspond to 3.154.800 hectares of all country, around 2% of all Europe forests panorama, where 977.988 were occupied by resinous species. The genus *Pinus* (*Pinaceae*), comprises over 100 recognised species and are considered to be economically relevant for human activities as an important source of wood, paper, resins, charcoal, food (nuts), and ornamentals (Richardson & Rundel, 1998), moving around 1,2 billion euros (Mendes *et al.*, 2004; FAO 2007). Due to its rapid growth, and medium-density softwood and suitability in a wide range of environments, improving landscapes and water quality, reducing erosion, storing carbon or harbour, *Pinus radiata* D. Don, or Monterey pine, has been the most representative pine species planted worldwide – Chile, Australia, Spain or South Africa, Canada and United States of America (Lavery & Maed, 1998). The exact representation of *Pinus Radiata* in the Portuguese landscape is not well documented, belonging to the category “another resinous trees” category, not having therefore an objective known value for its landscape occupation (ICNF, 2013). In the case of *Pinus pinaster* Aiton, also known as Maritime pine, its occupation rounds the 714 thousand hectares, being the most representative pine species, with an increased importance of being commonly used for reforestations and representing, in 2000, 11% of the total Portuguese exportation (Aguiar *et al.*, 2003; ICNF 2013). Regarding *Pinus pinea* L., also known as Stone pine, is responsible for occupying 175 thousand hectares, having an ecological, landscape and soil conservations impact together with its high economic importance – wood and pinion (nuts), which represents the most valuable product of this specie, providing significant incomes (Ayırmis *et al.*, 2009; Calama *et al.*, 2003).

Currently, both pine natural stands and plantations are often targets of different stress agents, both biotic and abiotic, being responsible for the decrement of pine productivity, and, therefore causing several economic losses to both producers, industries and countries

(Wingfield *et al.*, 2008). Furthermore, accompanied by the climate changes occurring through these past decades, novel microorganisms appeared, having devastating effects on pine plantations wellness, becoming mandatory to unveil the mechanisms behind stress tolerance in order to apply efficient control measures (Reboredo, 2014). Being considered a quarantine fungi, which is subjected to specific management rules (EPPO 2009), *Fusarium circinatum* Nirenberg and O'Donnell, 1998 (teleomorph = *Gibberella circinata*) was first described as being an unidentified species from the *Leseola* section (Hepting & Roth, 1946). The pitch canker disease (PCD), the most devastating disease affecting 57 species of *Pinus* spp. (Wingfield *et al.*, 2008) and *Pseudotsuga menziesii* (Douglas Fir) (Gordon *et al.*, 2006) with high levels of tree mortality and reduction in plant production resulting in significant economic losses (Wingfield *et al.*, 2008). Being first reported in south-eastern areas of United States of America, it is nowadays spread worldwide (Gordon, 2006; Wingfield *et al.*, 2008). Being capable of infect either vegetative structures such as shoots, branches, stems and exposed roots and reproductive structures such as cones, seeds or flowers, of individuals of any age and at any time of the year, this pathogen's hosts show a characteristic set of symptoms that leads to death. Needles discoloration, tip dieback of branches and resin cankers, with excessive resin exudation are considered the characteristic symptomatology of pitch canker disease (PCD) (Wingfield *et al.*, 2008).

The infection capacity of *F. circinatum* in pine species, the severity varies among species, thus indicating that not all species have the same degree of vulnerability and respond in the same way (Gordon *et al.*, 2015). It is known that *P. radiata* is the most susceptible pine specie to the pitch canker fungus (Gordon *et al.*, 1998; Martínez-Álvarez *et al.*, 2014), but studies carried out by Storer *et al.*, (1999) demonstrated that depending on the spores' quantity, the time of the year and if the trees were grown in natural or planted sites, some of the individuals did not showed the characteristic symptom of the disease. Studies carried out with *P. pinea* (Gordon *et al.*, 1998) and *P. pinaster* (Vivas *et al.*, 2012) showed higher resistance to the pitch canker disease. Between these two species, *P. pinea* is considered to be more tolerant when compared to *P. pinaster* (Iturrutxa *et al.*, 2013).

The management and control of the PCD, both in plantations/nurseries as in natural stands, is a very difficult task, being used an application of different management and control methods – both cultural, chemical and biological, in order to reduce the economic impact of the disease and prevent the establishment of the fungi in new areas (Hammerbacher,

2005). Due to the scarcity of physiological studies on *Pinus-Fusarium* interaction, together with the fact that the current status of disease control methods are inefficient, turns the selection of more resistance plant material an emergent issue.

Beyond the pre-existing defences, mechanical barriers or resin production (Woodward, 1992), changes in photosynthetic efficiency and carbohydrate metabolism (Berger *et al.*, 2007), water relations (Walters, 2015), carbohydrates status (Morkunas & Ratajczak, 2014) and secondary defence mechanisms activation (Bennett & Wallsgrove, 1994a) have been intimately linked to pathogenic responses. The impact of *F. circinatum* in gas exchange parameters are well documents for *P. radiata*, being observed a negative impact on net CO₂ assimilation rate , stomatal conductance) and transpiration rate associated with a decrease in water potential and internal CO₂ concentration values (Cerqueira *et al.*, 2017). Through confocal microscopy, Martin-Rodrigues *et al.*, (2013) studied fungal colonization in *P. radiata* by *F. circinatum*, observing the xylem and phloem vessels colonization and blockage by fungal hyphae's. The blockage caused by hyphae's progression unable water to move from roots to plant leaves, resulting in a lower water content in the leaves, and therefore, being possible to observe an decrease in water potential, similar to what is observed under water stress conditions (Wingfield *et al.*, 2008). Upon this changes in water relations plant responses will be dependent of their capacity of osmotic adjustment which may also differ among species (Liu *et al.*, 2011). Osmolytes, such as amino acids, soluble sugars and other metabolites, play a pivotal role regarding plants osmotic adjustment (Bohnert & Jensen, 1996). Proline is among the most studied amino acid responsible for plant response upon water deficit (Yoshiba *et al.*, 1997). Soluble sugars have been recognized to also have an important role in plant signalling and osmotic adjustment upon water stress situations (Hare *et al.*, 1998).

Being responsible for light harvesting during photosynthesis and being the first donor in the electron transporter chain, acting as antioxidants and scavenging mechanisms under stress, preventing photo-oxidation, pigments proved to have a crucial role in the photosynthetic fitness maintenance (Lichtenthaler, 1987; Chalker-Scott, 1999;).

In this work, by linking morphological, physiological and biochemical traits we aim to decipher how three pine species – *P. radiata*, *P. pinaster* and *P. pinea*, with different degrees of susceptibility respond to *F. circinatum* and the impact on the most important physiological processes.

2. Material and methods

2.1. Plant material

Ninety plants from three different species of pine (30 plants each): *Pinus radiata* -Turkey provenience (LOT: 16/17-1PNR), *Pinus pinaster* – Portuguese provenience (Region 2) (LOT: 15-16/1PNB) and *Pinus pinea* - Portuguese provenience (Region 5) (LOT:15-16/1PNM) seedlings were obtained from a Portuguese nursery, Melo & Cancela (Anadia, Portugal). Plants were placed in plastic pots (200 mL) filled with a 3:2 (w/w) peat:perlite mixture and kept under controlled conditions in a climate chamber (Fitoclima D1200, Aralab, Portugal): 16 h light/8 h darkness, 25 °C/20 °C (day/night), 65/60 % (day/night) relative humidity, 500 $\mu\text{mol m}^{-2}\text{s}^{-1}$ photon flux density (PFD) for an acclimatization period of 30 days. During this period plants were well-watered regularly and fertilized once a week with nutritive solution N:P:K 5:8:10 (NUTRIQUISA 5-8-10®, Agroquisa, Portugal). During the entire experiment, in order to minimize the effects of environmental heterogeneity, pots were randomly organized and periodically moved to neighbouring position.

2.2. Fungal material

Fungal material, *Fusarium circinatum* (FcCa 6) was obtained from the Forest Entomology and Pathology Lab collection, from the University of Valladolid. *F. circinatum* was grown on potato dextrose agar (PDA; Scharlau®, Spain) at 25 °C in the dark, until the mycelium covers at least 90 % of the Petri dish. Three to five pieces of mycelium (about 5 mm of diameter) were cut and grown under agitation on potato dextrose broth medium (PDB; VWR Chemicals, USA) for at least 24 h before plant inoculation. Spore suspension was filtered and spores were counted using a haematocytometer. For the inoculation procedure, wounds were made on pines stem and spores' concentration, previous adjusted to 1×10^6 spores/mL with sterile distilled water were placed on the wound. Control plants were inoculated with distilled water. All plants' wounds were sealed with Parafilm ®.

2.3. Experimental design and sampling

Plants were divided into two groups, with 15 individuals each one – Non-inoculated plants (C) and inoculated plants with *F. circinatum* (F). Sampling procedure was carried out when 50% of the plants from a treatment, from each species, showed symptoms (Figure 1). At each sampling point, immediate measurements were performed - leaf gas-exchange parameters, height and water potential. Stem necrosis, electrolyte leakage and relative water content (RWC) parameters were also accessed.

Needles from the apical portion were collected and immediately frozen in liquid nitrogen and stored at -80 °C for further analysis of several biochemical parameters: pigments, carotenoids and anthocyanins quantification, proline content and total soluble sugars (TSS) and starch quantification. To confirm Koch's postulates, cuts of the stem were placed in PDA (CONDA, Spain) and incubated at 20 ± 2 °C for 7 days. All experimental design ran under the acclimatization period conditions mentioned above.

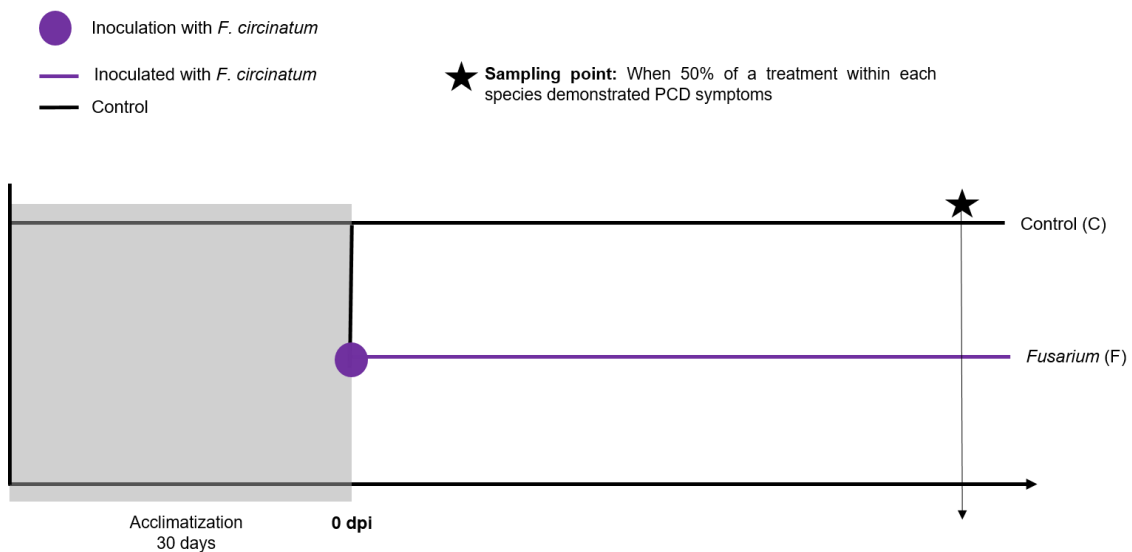


Figure 1 – ***Pinus-Fusarium* experimental design**. In a climate chamber, 30 plants, from *P. radiata*, *P. pinaster* and *P. pinea* each, were submitted to a 30 days acclimatization period. After this, within each species, plants were divided into control non-inoculated plants (C) and into inoculated with *F. circinatum* (F), with 15 plants each.

2.4. Histological sections

Histological sections were performed according to the method described by Lopes *et al.* (2016). Briefly, stem sections were cut and conserved in 70% ethanol. Sections were embedded in paraffin and cut with a microtome at 50 µm thickness. Samples were clarified in a solution of sodium hypochlorite, neutralized in acetic water 1% (5 minutes) and finally

washed with distilled water. The sections were then double-stained with iodine green (5-10 seconds) and alum carmine (10 minutes) and dehydrated: 70% (2-3 minutes), 90% (2-3 minutes), absolute ethanol and absolute ethanol:xilol (1:1)/(v/v) (5 minutes). Samples were mounted in glass microscope slides using Entellan. Definitive preparations were observed under an optical microscope (DS-U3, Nikon Instruments Europe B.V., Netherlands) coupled with a high-resolution digital microscope camera (DS-Ri1, Nikon Instruments Europe B.V., Netherlands). Histological sections were performed in three biological replicates from non-inoculated plants.

2.5. Visual aspect and disease symptomatology

Plants were evaluated daily throughout the experiment by visual observation to assess disease symptoms (tip dieback and needle wilting) until fifty per cent (50 %) of a treatment displayed disease symptoms, within each species.

2.6. Relative stem necrosis

Progression of *F. circinatum* inside the plants' stem was measured on five to seven independent biological replicates per treatment. Stem was cut above the soil and with a scalpel, carefully opened longitudinally and internal lesion (IL) was measured with a rule. The height of ten biological replicates from each specie and treatment was recorded at the end of each sampling point. Images of the necrosis were observed and photographed using a zoom stereomicroscope (SMZ1500, Nikon Instruments Europe B.V., Netherlands) coupled to a high-resolution digital microscope camera (DS-Ri1, Nikon Instruments Europe B.V., Netherlands) and its controller (DS-U3, Nikon Instruments Europe B.V., Netherlands). Relative Necrosis (%) was calculated through the following equation: $(IL/Height)*100$.

2.7. Electrolyte leakage

Electrolyte leakage (EL) was measured according to Escandón *et al.* (2016). One hundred mg of needle pieces (1 cm long) from six biological replicates from each treatment were collected. Then, 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 $EL\% = [(C_{exp} - C_i / C_t - C_{ii})] \times 100$, where C is water conductivity under control condition (C_i), control condition after autoclaving (C_{ii}), experimental condition (C_{exp}) and after autoclaving (C_t).

2.8. Plant water status

Plant's shoot water potential (ψ , MPA) was measured above the inoculation point with a Scholander-type pressure chamber (PMS Instrument Co., OR) in six independent biological replicates per treatment.

For relative water content (RWC), five needles per plant individual were collected and immediately weighted (FW). Plant material was submersed in distilled water and kept overnight in dark, at 4 °C, and then, excess of water was carefully removed from plant material and turgid weight (TW) was accessed. Needles were therefore completely dried at 80 °C and dry weight (DW) was recorded. After this process, RWC was calculated using the following equation: $RWC (\%) = (FW - DW) / (TW - DW) \times 100$. This process was repeat in six biological replicates per treatment.

2.9. Gas exchange parameters

Net CO₂ assimilation rate (A , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), stomatal conductance (g_s , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), transpiration rate (E , $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), and sub-stomatal CO₂ concentration (C_i , vpm) were measured with a IRGA (LCpro-SD, ADC BioScientific Limited, Hertfordshire, UK) with a conifer type chamber (LCP-010/C, ADC BioScientific Limited, Hertfordshire, UK). Inside the chamber, the following conditions were set for all the measurements: 200 $\mu\text{mol s}^{-1}$ air flux, and $\approx 25.13 \pm 2.46$ °C block temperature. The C_a (ambient CO₂ concentration were approximately 407, $52 \pm 9.81 \mu\text{mol m}^{-2} \text{ s}^{-1}$). To find out the saturation light intensity light response curves of CO₂ assimilation (A/PPFD) were performed with the following photosynthetic photon flux density (PPFD): 2500, 2000, 1500, 1000, 750, 500, 250, 100, 50 and 0 $\mu\text{mol m}^{-2} \text{ s}^{-1}$. Measurements were performed at 1500 $\mu\text{mol m}^{-2} \text{ s}^{-1}$. Data were recorded when the measured parameters were stable (2–6 min). Instantaneous water use efficiency (WUE) was calculated through the ratio between Net CO₂ assimilation rate (A) and transpiration rate (E). Six independent biological replicates per treatment were measured.

2.10. Pigments quantification

Total chlorophyll content and carotenoids were quantified according to Sims & Gamon, (2002). Briefly, 50 mg of fresh plant material were grounded in liquid nitrogen using a pestle and mortar. 1.5 mL of extraction buffer, (80:20) Acetone:Tris 50 Mm, pH = 7.8, was added to the homogenate. The supernatants were used to read absorbance at 663, 537, 647, 440 nanometres. Chlorophyll *a* and *b* and carotenoids were calculated according to the equations proposed by the authors. This process was repeat in six biological replicates per treatment.

Anthocyanins content were evaluated according to Sanchez-Zabala *et al.* (2013). Acidified methanol (methanol:HCl (99:1)) was used as extraction buffer of 50 mg of fresh plant material. Samples were submersed in boiling water during 1.5 minutes and then left in the dark, at 4 °C, for 24 hours. After a centrifugation step (10.000g, 4 °C, 10 minutes), the absorbance was read at 530 and 657 nanometres. Six independent biological replicates were measured.

2.11. Proline content

For proline content measurements, the protocol established by Bates *et al.* (1973) was followed, with slight modifications. Briefly, 100 mg of fresh plant material were homogenized with 1.5 mL of 3% (w/v) sulphasalicylic acid. After a centrifugation (10.000 g, 10 minutes, 4 °C), 1 mL of supernatant was collected to a new tube, and 1 mL of ninhydrin acid and 1 mL of glacial acetic acid were added. After an incubation at 100 °C for 60 minutes, tubes were cooled on ice and 2 mL of toluene were added to each sample. 30 minutes were given to accomplished phase separation and the upper phase was collected to absorbance readings at 520 nanometres. For proline content absolute quantification, L-Proline standard curve (250-0 µg/mL) was made. Six biological replicates were used per treatment.

2.12. Total soluble sugars (TSS) and starch quantification

The extraction and quantification procedures of total soluble sugar (TSS) and starch were made according to Chow & Landhäusser (2004). Briefly, 50 mg frozen leaves were extracted using 80% (v/v) ethanol. The extract was incubated in boiling water (95 °C) for 15 minutes, and the centrifuged for 15 minutes, at 4 °C and 10.000 g. Then the supernatant

was transferred to new falcon tubes. This procedure was repeated 3 times. Quantification was made according to Irigoyen *et al.* (1992) where the 100 μ L of supernatant was mixed with 1.5 mL of anthrone and incubated at 100 °C during 10 min. Absorbance was read at 625 nm and TSS content was calculated against a D-glucose standard curve (1.25-0 mg/mL).

The pellet resultant from the extraction procedure was used to quantify starch. As described by Osaki *et al.* (1991). The pellet was incubated with 5mL of 30% (v/v) perchloric acid at 60 °C during 1h. The mixture was centrifuged and 1.5 mL anthrone was added to 150 μ L of supernatant. After heating the mixture at 100 °C for 10 min, absorbance was read at 625 nm and starch content was determined according to a d-glucose standard curve (1.25-0 mg/mL).

2.13. Statistical analysis

Statistical analysis was carried out through two-way analysis of variance (ANOVA) tests followed by post-hoc tests comparisons between treated with *Fusarium* (F) and non-treated plants – control (C), to access interaction of *F. circinatum*'s infection within each different species on all morphological, physiological and biochemical traits analysed. Normality and homogeneity of variances were both tested in each analysis by Shapiro and Bartlett tests. When neither of these assumption was violated, classical two-way ANOVA and Tukey's HSD tests were applied, otherwise, when at least one of the assumptions was violated, robust statistical methods were applied through heteroscedastic two-way ANOVAs using the generalized Welch procedure and a 0.1 trimmed mean transformation (García-Pérez, 2005). When a negative interaction was found, a T-test was performed between treatments, in case of normality failure, a Mann-Whitney U Test was performed, within each species using SigmaPlot for Windows (Systat Software for Windows v. 12.0 Systat Software Inc., Chicago, IL, USA).. Results are presented as means \pm standard errors (SE) of six or more biological replicates. These analyses were carried out using the "Wilcox' Robust Statistics (WRS2)" package implemented in the R software environment. In every case, asterisk indicate significant differences ($p < 0.05$) between control and inoculated plant within each pine species.

3. Results

3.1. Histological analysis

Stem sections from pine species (*P. radiata*, *P. pinaster* and *P. pinea*) visualized by optical microscopy revealed structural differences among species (Figure 2). Regarding *P. radiata* and *P. pinaster*, the cortical area (vi) is less thick when compared to *P. pinea*. The first two species also presented fewer resin ducts in this area. Comparing the pitch area (i), *P. radiata* showed accented protrusions oriented towards cortical area, which were not observed in *P. pinaster* and *P. pinea*.

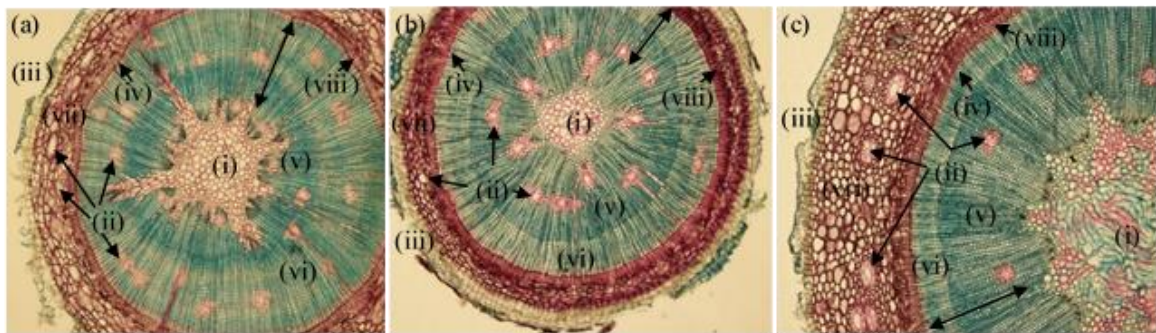


Figure 2 – Transversal anatomical sections from *P. radiata* (a), *P. pinaster* (b) and *P. pinea* (c) healthy plants observed by optical microscope. All section were made under 40X (Ocular 10X and object-glass 4X) magnification. Legend: (i) – pine pitch; (ii) – resin ducts; (iii) – suber; (iv) – vascular cambium; (v) – primary xylem (vi) – secondary xylem; (vii) – cortical parenchyma; (viii) – phloem; double-headed arrow – resin rays.

3.2. Visual aspect, growth, and plant disease symptoms

Visual symptoms (tip dieback and needles discoloration) (Figure 4) appeared in *P. radiata* 10 days post-inoculation (dpi). In *P. pinaster* took 17 dpi to show the same symptomatology, while *P. pinea* only showed symptoms - wilting and discoloured branches with resin exudation - on lateral branches near inoculation point 64 dpi (Figure 3).

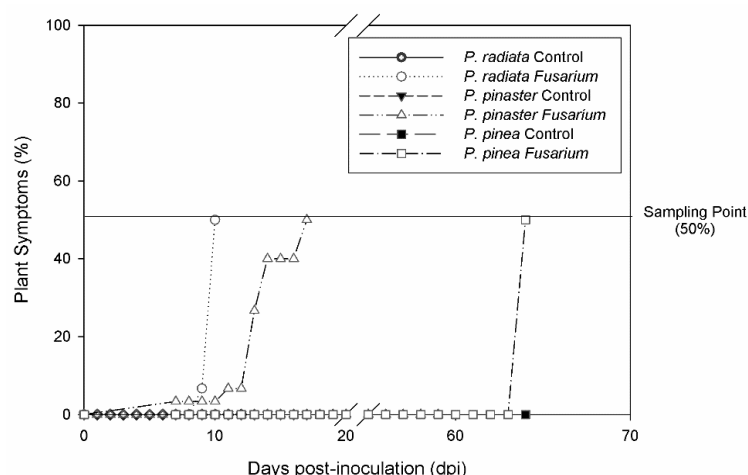


Figure 3 - Plant symptomatology (%) in *P. radiata*, *P. pinaster* and *P. pinea*, control and inoculated with *F. circinatum* plants days post-inoculation (dpi). T=0 represents inoculation day.

3.3. Relative necrosis

Upon inoculation with *F. circinatum*, relative necrosis was significantly higher in *P. radiata* (Figure 5) and *P. pinaster* (Figure 6) when compared to *P. pinea* (Figure 7) (Table 1). Both species ($p < 0.02$) and inoculation factor ($p < 0.001$) were significant and an interaction was found between them ($p < 0.02$) (Table 2).

Table 1 - Relative necrosis of *P. radiata*, *P. pinaster* and *P. pinea*, control and inoculated with *F. circinatum* plants, measured at the end of the experiment. Data are presented as mean \pm SE. Asterisk represents significant differences between inoculated and control group within same species ($p < 0.05$).

<u>Relative necrosis</u>		
	<u>Control</u>	<u>Fusarium</u>
<u><i>P. radiata</i></u>	0.000 \pm 0.000	17.046 \pm 1.068 *
<u><i>P. pinaster</i></u>	0.000 \pm 0.000	21.337 \pm 3.873 *
<u><i>P. pinea</i></u>	0.000 \pm 0.000	4.997 \pm 0.508

Table 2 - Two-way ANOVA summary table for morphological and physiological traits of non-inoculated and *F. circinatum* inoculated *P. radiata*, *P. pinaster* and *P. pinea* plants. Degrees of freedom (Df) and F value are shown for each source of variation; variance (MS) of the residual is also shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS, non-significant. Abbreviations: water potential (Ψ), net CO₂ assimilation rate (A), transpiration rate (E), stomatal conductance (gs), intercellular CO₂ concentration (Ci), water use efficiency (A/E) and total soluble sugars (TSS).

<i>Parameter</i>		<i>Source of Variation</i>	<i>Df</i>	<i>F</i>	<i>p-value</i>	<i>Significance</i>
<i>Relative Necrosis</i>		<i>Species</i>	2	24.4772	0.002	**
		<i>Fungal Inoculation</i>	1	86.9770	<0.001	***
		<i>Interaction</i>	2	24.4781	0.002	**
<i>Water Potential</i>	Ψ	<i>Species</i>	2	33.0567	<0.001	***
		<i>Fungal Inoculation</i>	1	23.3640	0.003	**
		<i>Interaction</i>	2	90.2056	<0.001	***
<i>Relative Water Content</i>	<i>RWC</i>	<i>Species</i>	2	23.8922	<0.001	***
		<i>Fungal Inoculation</i>	1	17.0397	<0.001	***
		<i>Interaction</i>	2	52.0547	<0.001	***
<i>Electrolyte Leakage</i>	<i>EL</i>	<i>Species</i>	2	29.6837	0.002	**
		<i>Fungal Inoculation</i>	1	10.6006	0.009	**
		<i>Interaction</i>	2	9.2986	0.058	NS
<i>Gas exchange related parameters</i>	<i>A</i>	<i>Species</i>	2	1239.9966	<0.001	***
		<i>Fungal Inoculation</i>	1	939.5561	<0.001	***
		<i>Interaction</i>	2	523.0712	<0.001	***
	<i>E</i>	<i>Species</i>	2	164.5113	<0.001	***
		<i>Fungal Inoculation</i>	1	261.3165	<0.001	***
		<i>Interaction</i>	2	275.8993	<0.001	***
	<i>gs</i>	<i>Species</i>	2	258.1266	<0.001	***
		<i>Fungal Inoculation</i>	1	61.2500	<0.001	***
		<i>Interaction</i>	2	116.6724	<0.001	***
	<i>Ci</i>	<i>Species</i>	2	41.7201	<0.001	***
		<i>Fungal Inoculation</i>	1	64.7487	<0.001	***
		<i>Interaction</i>	2	41.0426	<0.001	***
<i>Pigments</i>	<i>Total Chlorophylls</i>	<i>Species</i>	2	92.9822	<0.001	***
		<i>Fungal Inoculation</i>	1	17.5727	<0.001	***
		<i>Interaction</i>	2	3.5101	0.207	NS
	<i>Carotenoids</i>	<i>Species</i>	2	6.0881	0.074	NS
		<i>Fungal Inoculation</i>	1	0.1130	0.741	NS
		<i>Interaction</i>	2	0.6297	0.741	NS
	<i>Anthocyanins</i>	<i>Species</i>	2	14.28	<0.001	***
		<i>Fungal Inoculation</i>	1	14.72	<0.001	***
		<i>Interaction</i>	2	12.68	<0.001	***
<i>Proline</i>		<i>Species</i>	2	19.1964	0.004	**
		<i>Fungal Inoculation</i>	1	11.7765	0.012	*
		<i>Interaction</i>	2	12.1152	0.020	*
<i>Total Soluble Sugars</i>	<i>TSS</i>	<i>Species</i>	2	34.0493	<0.001	***
		<i>Fungal Inoculation</i>	1	3.7641	0.087	NS
		<i>Interaction</i>	2	1.6670	0.481	NS
<i>Starch</i>		<i>Species</i>	2	14.4956	0.006	**
		<i>Fungal Inoculation</i>	1	0.1400	0.714	NS
		<i>Interaction</i>	2	1.3056	0.547	NS



Figure 4 - Plants visual aspect during sampling day of *P. radiata* (a), *P. pinaster* (b) and *P. pinea* (c), with associated treatments - control and plants inoculated with *F. circinatum*, respectively. Zoom in on visual symptomatology of *P. pinea* lateral branches near inoculation point (d).



Figure 5 - *P. radiata* stem sections during necrosis length measurements. (a) and (b) correspond to control plant and (c) and (d) to inoculated with *F. circinatum*. (a) and (c) are longitudinal sections while (b) and (d) are transversal sections.



Figure 6 - *P. pinaster* stem sections during necrosis length measurements. (a) and (b) correspond to control plant and (c) and (d) to inoculated with *F. circinatum*. (a) and (c) are longitudinal sections while (b) and (d) are transversal sections.



Figure 7 - *P. pinea* stem sections during necrosis length measurements. (a) and (b) correspond to control plant and (c) and (d) to inoculated with *F. circinatum*. (a) and (c) are longitudinal sections, while (b) and (d) are transversal sections.

3.4. Electrolyte leakage

Upon inoculation with *F. circinatum*, *P. radiata* and *P. pinaster* showed an increasing tendency, although not statistically different, in electrolyte leakage values. *Pinus pinea* remained constant (Figure 8). Despite both species ($p < 0.002$) and inoculation ($p < 0.009$) factors being statistically different, no interaction was found (Table 2).

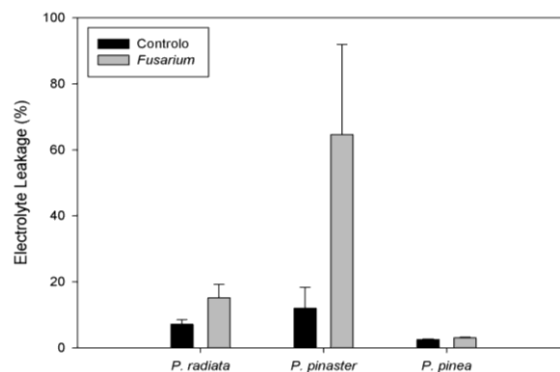


Figure 8 - Electrolyte leakage (%) in *P. radiata*, *P. pinaster* and *P. pinea* control and inoculated with *F. circinatum* plants. Data are presented as mean \pm SE. Black bars represent control non-inoculated plants and grey bars represent plants inoculated with *F. circinatum*.

3.5. Plant water relations

Relative to water potential (ψ), both inoculated *P. radiata* and *P. pinaster* showed a decrement in this parameter when compared with their respective controls, contrarily to *P. pinea*. *Pinus pinaster* presented lower values than *P. radiata* (Figure 9a). *Pinus radiata* and *P. pinea* maintained constant relative water content (RWC) values, while *P. pinaster* presented significantly lower values in this parameter (Figure 9b). Regarding water potential, both species ($p < 0.001$) and inoculation ($p < 0.003$) factors were significant, revealing an interaction between them ($p < 0.001$). Regarding RWC, a similar profile was observed ($p < 0.001$) (Table 2).

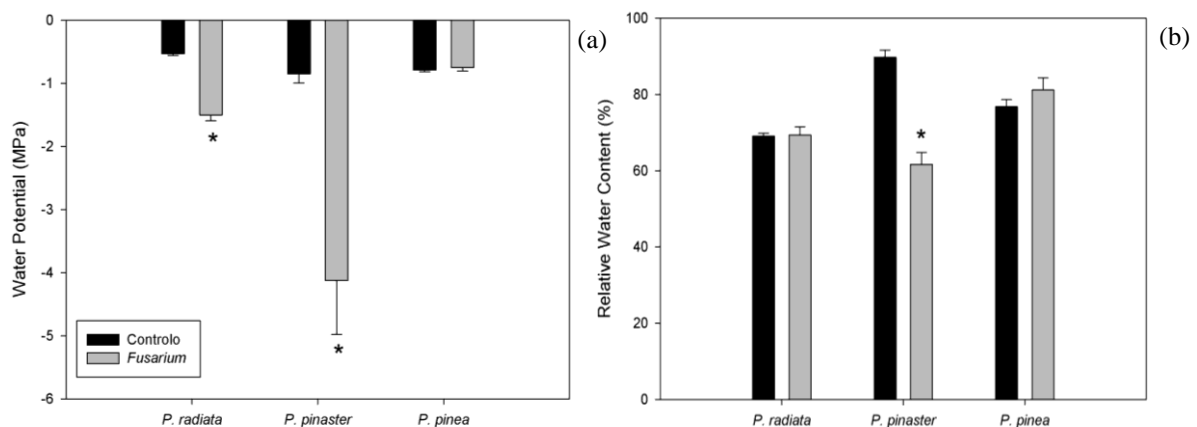


Figure 9 - **Water potential (ψ) (a) and Relative Water Content (RWC) (b) in *P. radiata*, *P. pinaster* and *P. pinea* control and inoculated with *F. circinatum* plants.** Data are presented as mean \pm SE. Black bars represent control non-inoculated plants and grey bars represent plants inoculated with *F. circinatum*. Asterisk represents significant differences between inoculated and control group within same species ($p < 0.05$).

3.6. Gas exchange related parameters

For CO_2 assimilation rate (A), a significant decrease in *P. radiata* and *P. pinaster* inoculated plants was observed, while *P. pinea* did not show differences. Regarding the internal CO_2 concentration (c_i), all species showed a significant increment. *Pinus radiata* and *P. pinaster* showed a similar response of lower transpiration rate (E) and stomatal conductance (g_s) upon *F. circinatum* inoculation, contrarily to *P. pinea*, which revealed higher values. Upon *F. circinatum* inoculation, water use efficiency (WUE) reflected a significant decrease in all three species (Figure 10). Species variation and inoculation revealed to be crucial factors, together with the interaction *Pinus-Fusarium* (Table 2).

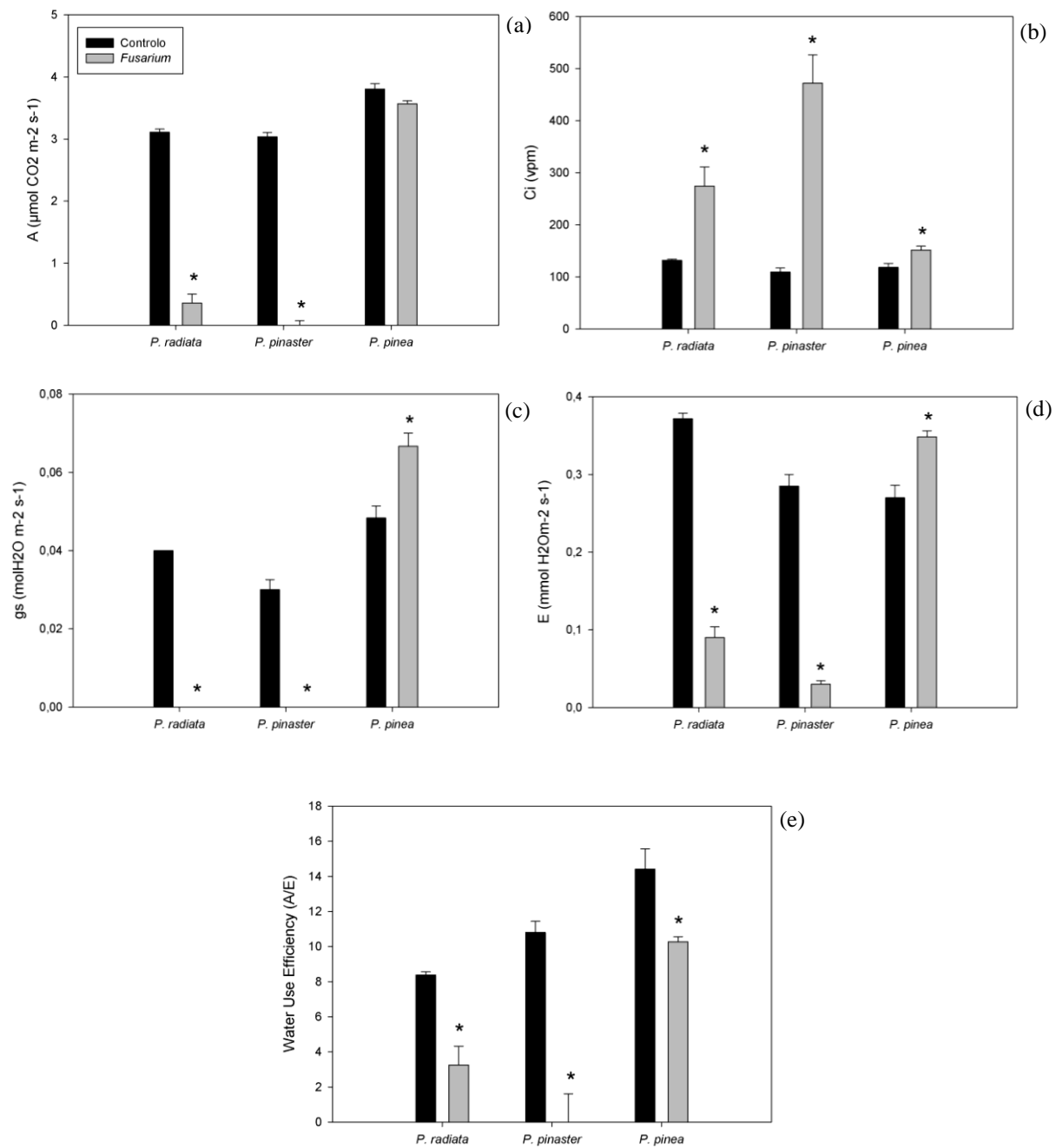


Figure 10 - CO₂ assimilation rate (A) (a), internal CO₂ concentration (ci) (b), stomatal conductance (gs) (c), transpiration rate (E) (d) and water use efficiency (A/E) (e) in *P. radiata*, *P. pinaster* and *P. pinea* control and inoculated with *F. circinatum* plants. Data are presented as mean \pm SE. Black bars represent control non-inoculated plants and grey bars represent plants inoculated with *F. circinatum*. Asterisk represents significant differences between inoculated and control group within same species.

3.7. Pigments quantification

Regarding total chlorophylls, despite the effect of *F. circinatum* inoculation on *P. radiata*, *P. pinaster* and *P. pinea*, no interaction between these two factors was found (Table 2 and Figure 11a).

In concern to carotenoids quantification, there was no significant differences between treatments in all three species (Figure 11b) and both factor, species and fungal inoculation revealed to not have any interaction (Table 2).

Regarding anthocyanins quantification, this showed to be significantly higher in *P. pinaster* and *P. pinea* after *F. circinatum* inoculation, while *P. radiata* remained constant (Figure 11c). Both factors revealed significant and an interaction between them was found (Table 2).

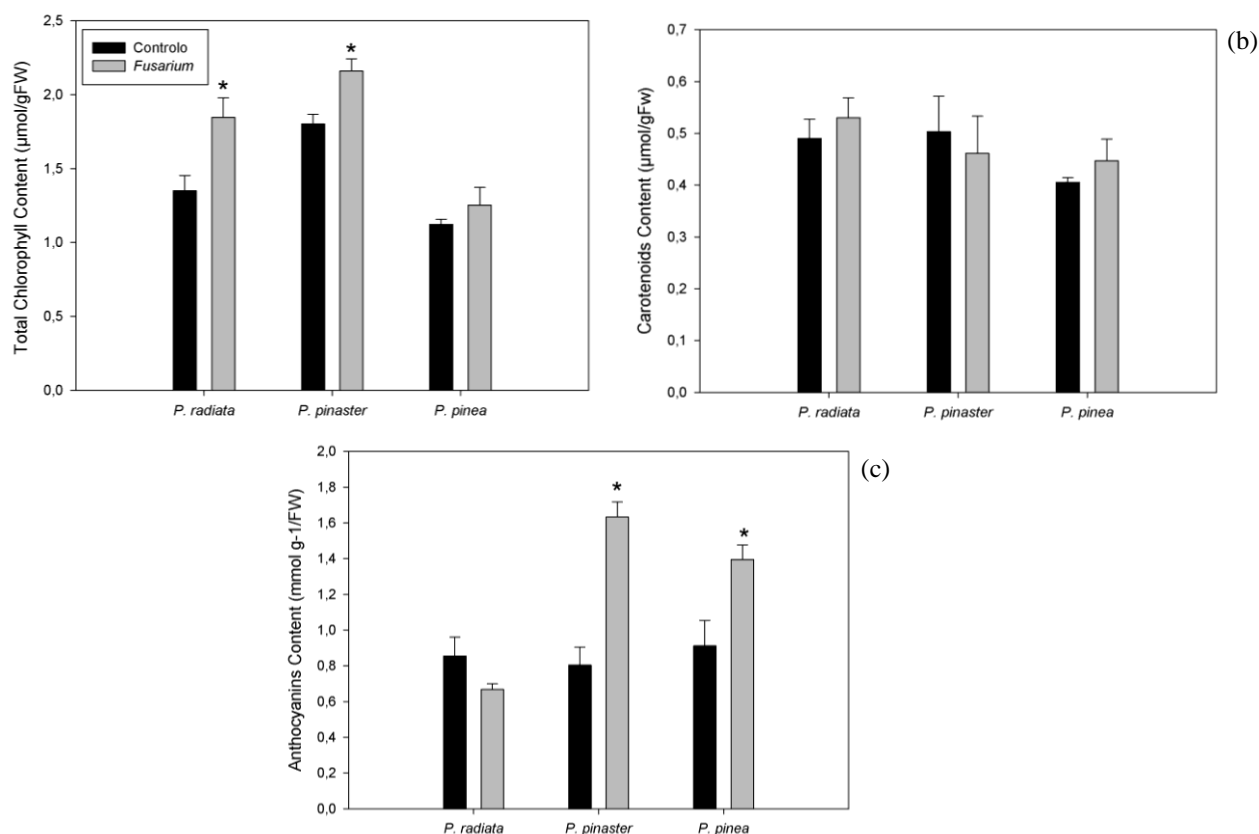


Figure 11- Total chlorophyll content (a), carotenoids (Car) (b) and anthocyanins (c) quantification in *P. radiata*, *P. pinaster* and *P. pinea* control and inoculated with *F. circinatum* plants. Data are presented as mean \pm SE. Black bars represent control non-inoculated plants and grey bars represent plants inoculated with *F. circinatum*. Asterisk represents significant differences between inoculated and control group within same species.

3.8. Total soluble sugars and starch quantification

Different species revealed to be a determinant factor in total soluble sugars (TSS) content (Figure 12a) and starch quantification (Figure 12b), while upon inoculation with *F. circinatum*, no statistical differences were found, revealing a negative interaction in TSS and starch quantification between the different species and *F. circinatum* inoculation (Table 2).

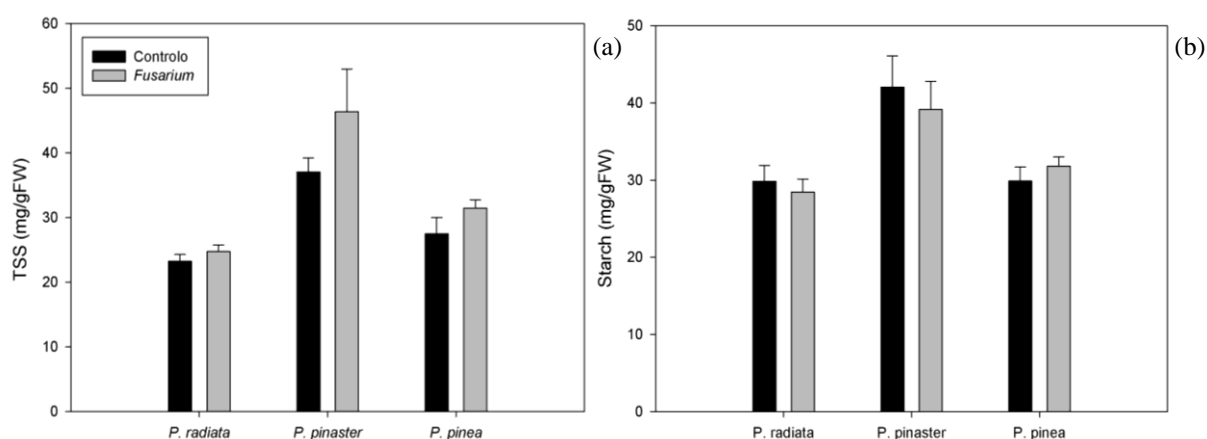


Figure 12- **Total soluble sugars (TSS) (a) and starch quantification (b) in *P. radiata*, *P. pinaster* and *P. pinea* control and inoculated with *F. circinatum* plants.** Data are presented as mean \pm SE. Black bars represent control non-inoculated plants and grey bars represent plants inoculated with *F. circinatum*. Asterisk represents significant differences between inoculated and control group within same species.

3.9. Proline content

Regarding proline content, upon fungal inoculation with *F. circinatum*, only *P. pinaster* plants had a significant increment (eight times higher concentration in F when compared to control plants) in this parameter (Figure 13). Both factors revealed significant and an interaction between them was found (Table 2).

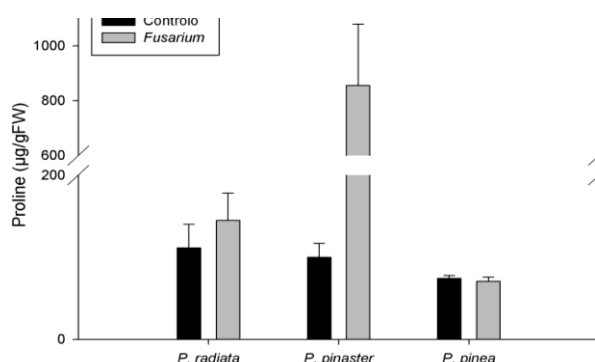


Figure 13 - **Proline content of *P. radiata*, *P. pinaster* and *P. pinea* in control and inoculated with *F. circinatum* plants.** Data are presented as mean \pm SE. Black bars represent control non-inoculated plants and grey bars represent plants inoculated with *F. circinatum*. Asterisk represents significant differences between inoculated and control group within same species.

4. Discussion

Since the discovery of PCD and its devastating effects on pine populations, several studies have been made in order to describe the underlying responses of hosts to this pathogen, using therefore distinct species and hybrids with different degrees of susceptibility (Enebak & Stanosz, 2003; Roux *et al.*, 2007; Kim *et al.*, 2008; Mitchell *et al.*, 2012; Iturritya *et al.*, 2013). Despite this intense research on pine susceptibility differences to *F. circinatum*, little research has been made to fill in the gap between pine susceptibility variances and morphological and physiological/biochemical responses. Despite this intense research on pine susceptibility differences to *F. circinatum*, little research has been made to fill in the gap between pine susceptibility variances and morphological and physiological/biochemical responses. In this study we connect physiological and biochemical responses to metabolic adjustments in three different species, *Pinus radiata*, *Pinus pinaster* and *Pinus pinea*, having different degrees of susceptibility to pine pitch canker fungus, in order to understand which metabolic pathways are involved in the response of these species to *Fusarium circinatum* infection.

4.1. Stem anatomical differences between species: does it matter?

Mechanical barriers, such as architectural structure, oleoresin production and callose, are among the first line of plants' defence against pathogens (Hückelhoven, 2007; Phillips & Croteau, 1999). From visual symptomatology observations, *P. pinea* showed higher resin secretions at inoculation point when compared to *P. radiata* and *P. pinaster* which may indicate a defence response to *F. circinatum* inoculation. Besides this, through histological stem sections observation, it is possible to conclude that *P. pinea* has a thicker cortical area together with a higher amount of resin ducts, becoming therefore a physical barrier enabling fungi to reach the pine pitch. Barrows-Broaddus & Dwinell (1983) analysed histological modifications of four different pine species, with different degrees of susceptibility to *F. moniliforme* var. *subglutinans*. In the case of higher tolerant species, inoculation triggered cell wall lignification and regeneration of parenchyma layers, in order to content pathogen's progression, presenting lower values of seed mortality and active lesions. Kim *et al.* (2010), demonstrated that wounded and wounding prior to inoculation with *F. circinatum* stimulated resin and lignin biosynthesis in *P. densiflora* and *P. rigida*.

In an experiment carried out by Valluri & Soltes (1990), using *Pinus elliotti* seedlings, was found higher callose production in both in vitro (tissue-cultured) and greenhouse cultured, upon inoculation with *Fusarium subglutinans*.

4.2. Plant responses: a physiological approach

Upon *F. circinatum* inoculation, *P. radiata* was the first specie to show the disease characteristic visual symptoms (needle discoloration and tip dieback) followed by *P. pinaster* and finally *P. pinea*. These results corroborates the finding made by Gordon *et al.* (1998) in a greenhouse experiment with 3 to 4 years old trees, where *P. radiata* revealed to be the most susceptible showing higher lesions length and infection in both lateral branches and main stem when compared to *P. pinea* and other pine species – *P. thunbergiana*, *P. canariensis*, *P. halepensis*. In other study carried out by Bragança *et al.* (2009), the inoculation of seedling of 5-, 9- and 8- month-old, *P. pinaster* and *P. radiata* and *P. pinea*, respectively, showed a clear differentiation among susceptibility to *F. circinatum* of these three species, where symptomatology observed 8 days post-inoculation (dpi) in *P. radiata*, 30 days dpi 70% of *P. pinaster* plants were dead, and regarding *P. pinea* only 2% of these plants presented wilting of the terminal shoot. These symptoms were accompanied by a higher relative necrosis, fungal colonization both upwards and downwards from inoculation point, in *P. radiata* and *P. pinaster*, but not existing in *P. pinea* inoculated plants. This corroborates the study carried out by Martin-Rodrigues *et al.* (2013), that demonstrated a chronological overview of the fungal progression in *P. radiata* inoculated plants through confocal microscopy observing both internal progression towards the pith and progression within the outer layers of the host (phloem and cortex). *Fusarium circinatum* progression was coupled with an increasing tendency, on electrolyte leakage (EL) values, higher in *P. radiata* and *P. pinaster*, which corroborate the existence of internal damages observed in relative necrosis found in those species. Electrolyte leakage is associated with oxidative stress burst during plants' hypersensitive response to pathogen, which will lead to further damages in membranes (Kombrink & Somssich, 1995). In a study carried out by Cerqueira *et al.* (2017) also showed increased electrolyte leakage values in *P. radiata* plants upon inoculation with *F. circinatum*. Using banana plants (*Musa acuminata*) inoculated with *Fusarium oxysporum* f. sp. *cubense*, both Dong *et al.* (2012) and Anthony *et al.* (2017) also found an increment in EL upon fungal

infection. *Fusarium subglutinans* culture filtrate was used to inoculate two different varieties of pineapple (*Ananas comosus* (L.) Merr) - Smooth Cayenne and Perolera. From electrolyte leakage measurements, Hidalgo *et al.* (1998) demonstrated that Perolera was more resistant, having no significant alterations in EL values in the presence of fungal culture filtrate, when compared to Smooth Cayenne that showed highly significant electrolyte leakage in the presence of *Fusarium subglutinans* culture filtrate.

From this findings it is possible to assume that exists clear differences on the susceptibility to *F. circinatum* among the three pine species used in this study. The differences in symptoms appearance, relative necrosis and electrolyte leakage values may indicate the occurrence of more complex re-arrangements in physiological and biochemical machinery in order to successfully overcome infection.

Water enters in plant system via roots and travels through xylem until reaches plants' leaves (Steudle, 2000) where it is used during photosynthesis (Atkin *et al.*, 2000).

In 1990, Solel and Bruck, using different families of *P. patulta* observed correlated the disease severity with the interference of the fungi with water movement within plant vessels, concluding that tip dieback result from the water movement obstruction in xylem. Martin-Rodrigues *et al.* (2013) used confocal microscopy to observe *P. radiata* xylem and phloem colonization by *F. circinatum*. Upon inoculation, fungal hyphae's proliferates inside host tissues in two different ways, progressively moving towards the centre of pine stem, until reach the pine pith (xylem), and a more external projection along the phloem and cortex. This characteristic grow blocks water and nutrient flux to reach the apical region of the plants, provoking a "water stress" like situation in plants, resulting in needle discolouration and, in more advanced disease states, tip dieback (Wingfield *et al.*, 2008). The observed lower water potential and relative water content values present in the needles of *P. pinaster* corroborate this "water stress" like situation. Water potential values observed in *P. radiata* were not so severe and relative water content remained stable. In *P. pinaster*, these parameters reveal us that water is not being able to be transported to plants' leaves, leading to possible alterations on gas exchange parameters. In *P. pinea* these alterations in plant water status were not observed supporting the assumption of this species as the most tolerant among the three. Inoculated *P. pinaster* plants with *F. circinatum* demonstrated lower values of water potential (Otero *et al.*, 2016). Same pattern was found in tomato plants (*Lycopersicon esculentum* cv. Roma) upon inoculation with

Fusarium oxysporum f. sp. *lycopersici* race 1 (FOL-1), where a decrease in photosynthetic rate upon inoculation, accompanied by a decrease in water potential and a decrease in relative water content were observed (Nogués *et al.*, 2002)

Decreasing in water content leads to an interference with the turgor pressure within plant cells, which, as a response, have developed several mechanisms in order to maintain turgor pressure within plant cells (Morgan, 1984). The enhanced production of cellular compatible solute is a common response in plant adaptation to water deprivation situations (Blum, 2017). Proline is an amino acid involved in plant water status tolerance acting as an osmoprotective osmolyte, ROS scavenging agent, a buffer to stabilize cellular redox potential or as a chaperone (Verbruggen & Hermans, 2008). The proline increment found in *P. pinaster* demonstrate the resilience of this species in trying to fight *F. circinatum* colonization, in the opposition to what happened in *P. radiata*, unchanging its proline levels upon inoculation which may contribute to its higher susceptibility. Concerning *P. pinea*, the progression of the fungi was not so extended to be observed alterations in proline content. Proline quantification was also accessed by Cerqueira *et al.* (2017) finding higher values of proline in plants upon inoculation with *F. circinatum*, which were ameliorated after potassium phosphite application. Seeve (2010) also found an up-regulation of 1,4 times-fold in proline biosynthesis gene (P5CR), which may lead to proline accumulation in *Pinus taeda* L. plants inoculated with *F. circinatum*. Using wheat (*Triticum aestivum* L.) inoculated with *Fusarium culmorum*, Akbari-Vafaii *et al.* (2014) found an accumulation of proline upon inoculation, which was reduced by the previous application of methyl-jasmonate (MeJA) application.

The response of *F. circinatum* infection lead to changes in the photosynthetic performance and gas exchange parameters. Both *P. radiata* and *P. pinaster* had decrease their capacity in CO₂ assimilation resulting in lower values of net CO₂ assimilation rate. To prevent higher water loss, plants close their stomata, resulting in a decrement of stomatal conductance, which will lead to a decrease in transpiration rate, observed with more intensity in *P. radiata* and *P. pinaster*. Together with this, the values of internal CO₂ concentration were found higher upon inoculation in *P. radiata* and *P. pinaster* meaning that CO₂. These findings may indicate that decrement in net CO₂ assimilation rate is a result of both stomatal limitations, through stomata closure, and non-stomatal limitations, observed in the increasing CO₂ concentration which suggest that plant have available

internal CO₂, but are not able to use it to photosynthesize. This may indicate further damages on the enzymes involved in photosynthesis. Wu *et al.* (2008) studied the direct effect of a mycotoxin (fusaric acid), directly extracted from *Fusarium oxysporum* f. sp. *niveum* on the leaves of water melon seedling, observing a decrease on the photosynthetic parameters. In *P. pinea* leaf-gas exchange parameters were not affected by *F. circinatum* inoculation, proving its tolerance to this pathogen. Our results are corroborated by Bolton (2009) review which defends that upon pathogen attack, the occurrence of photosynthetic adjustments may lead to a decrease in photosynthesis rate redirecting energy supplies for plant defence mechanisms. Instantaneous water-use efficiency, is a parameter that have been largely used in crop production and a marker used upon drought stress (Anyia & Herzog, 2004), reflects the ratio between net CO₂ assimilation rate and water consumption - transpiration (Monclus *et al.*, 2006). In this case, it's observed a decrement in this parameter for all three species, which, analysing both factors net CO₂ assimilation rate and transpiration separately, reveals that net CO₂ assimilation rate suffers a reduction, due to photosynthetic apparatus shut down upon *F. circinatum* inoculation. Our results go in accordance with experiments carried out by Cerqueira *et al.* (2017), after *P. radiata* inoculation with *F. circinatum*, a negative effect on the hydric relationships of the plants, with a decrease in water potential accompanied by a decrease in gas exchange parameters – stomatal conductance, transpiration and net CO₂ assimilation rate, confirmed by an increment in the CO₂ concentration, revealing that despite CO₂ availability, plants were unable to photosynthesize due to both stomatal or non stomatal limitations.

Chlorophylls are pigments involved in photosynthetic apparatus responsible for light harvesting, acting as the first photon receptor (Katz *et al.*, 1978; Niyogi, 2000). Upon infection by pathogenic agents, most of the studies had reported a decrease in chlorophyll content (Ei-Khallal, 2007; Vesonder *et al.*, 1992). In this case, despite having a significant decrease in CO₂ assimilation rate, since total chlorophyll content did not change, it is possible to assume that this decrement is not caused by photosynthetic damages, but instead due to resource focus on defence mechanisms (Scharte *et al.*, 2005). Our results showed an increment in the total chlorophylls content in *P. radiata* and *P. pinaster*, while in *P. pinea* it remained stable. The decrease of water potential, accompanied by the lower values in relative water content in *P. pinaster* results in a decrease of total amount of water in plants, presenting therefore a

higher concentration of total chlorophylls, supporting the hypothesis that the lower values in leaf-gas exchange parameters are result of stomatal limitations. This profile was previous seen in plants under water stress conditions (Correia *et al.*, 2014; Jesus *et al.*, 2015; Berenguer *et al.*, 2017) giving strength to the hypothesis of “water stress” status caused by *F. circinatum* infection.

Carotenoids, beyond being responsible for attract insects and animals for pollination, are considered to be accessory pigments responsible for harvesting light during photosynthesis also acting as photo-protectors in high light conditions (Lu & Li, 2008). Across the three species carotenoids content remained stable, which may indicate that photo-oxidation and therefore oxidative damage of the photosynthetic apparatus is still functional, therefore, the observed limitation is photosynthetic rate occur due to stomatal limitations.

Another point to be account in *Pinus* response to *Fusarium* infection is the anthocyanins content. Being considered to be accessory pigments involved in plant response to a wide range of stress factors, anthocyanins are also involved in the protection of chloroplast from photo-oxidative damages, scavenging free radicals and ROS (Gould, 2004). The higher concentrations of anthocyanins found in *P. pinaster* and *P. pinea* show an activation of the defence machinery which did not occurred in *P. radiata*, and revealed insufficient in *P. pinaster* due to its earlier symptomatology appearance when compared to *P. pinea*. In Sorghum plants (*Sorghum bicolor*), as a response to inoculation with *Fusarium thapsinum* and *Fusarium proliferatum*, was observed an accumulation of anthocyanin's upon inoculation (Huang & Backhouse, 2005). Using *Brassica rapa* inoculated with fungal pathogens, both Abdel-Farid *et al.* (2009) and Rostás *et al.* (2002) found an increase of flavonoids (kaempferol and quercetin), which are the precursors of anthocyanins in phenylpropanoid pathway and increase in the anthocyanin content observed in fungus-infected leaves, respectively. Both concluded that these induction were related to plants response to fungal infection.

With the decrement in photosynthesis, and therefore a decrement in carbohydrates production, plants have to get their resources from storage organs products (Berger *et al.*, 2007). Upon fungal inoculation it should be expected an increase in total soluble sugars content resulting from reserves degradation in order to supply energy to plants' defence mechanisms, acting also as signalling molecules stimulating the activation of several defence related pathways (cell wall lignification, production of pathogen-related proteins

and flavonoids) (Morkunas & Ratajczak, 2014). Our results do not show any alterations in both TSS and starch content thus indicating that despite the decrease of photosynthesis, observed in leaf-gas exchange parameters, it is still providing the necessary energy for plants metabolism. Another possible function of soluble sugars, that, working together with other osmolytes molecules (such as amino acids), are responsible for the osmotic pressure maintenance, under water-stress conditions (Bohnert and Jensen 1996; Epron and Dreyer 1996). This may explain that despite the lower values of water potential and relative water content, together with the decrement in leaf-gas exchange parameters, *P. pinaster* survived longer in comparison with *P. radiata*. This goes into accordance with the finding made by Cerqueira *et al.* (2017) where no alterations were found in total soluble sugars content in *P. radiata* upon inoculation with *F. circinatum*.

From the discussed above it is possible to hypothesize that in the case of *P. radiata* the infection by the pathogen occur in a more direct and aggressive way, probably due to toxins produced by fungi in the presence of *P. radiata*, leading to an earlier death when compared with both *P. pinaster* and *P. pinea*. Regarding *Pinus* genus, it is known that basal metabolism, and consequently secreted metabolites, differs between pine species (Roussis *et al.*, 1995; Chen *et al.*, 2006; Santos *et al.*, 2006). These metabolic profile differences between species have to be taken into account in the plant-pathogen interaction, where they may modulate fungal metabolism, changing both secreted proteins and metabolites. This alteration may influence the infection mechanism and severity, being translated in plant's differential response between species upon inoculation by the same pathogen. This hypothesis was observed by Stepień *et al.*, (2015) evaluated the differential fumosins (toxins) production by *Fusarium proliferatum* in presence of six different crop plant species extracts, having also an effect on fungal biomass. More studies focusing on plant-pathogen interaction must be made to better understand which metabolic pathways are being modulated upon infection.

From the discussed above, it is possible to admit that the three species used in this study have different response mechanisms to *F. circinatum* infection.

5. Conclusions

From this work it was possible to confirm the differences on susceptibilities among the three pine species used, where *P. radiata* proved to be the most susceptible, followed by *P. pinaster* and *P. pinea*.

Plants' anatomical traits, such as the thickness of the cortical area and the amount of resin ducts, differs among *Pinus* spp. where it stands out the higher advantages to *P. pinea*. Upon inoculation, higher relative necrosis, and electrolyte leakage were found in *P. radiata* and *P. pinaster* revealing the colonization and damage made by *F. circinatum* inoculation. The blockage of xylem and phloem vessels in both *P. radiata* and *P. pinaster* led to a decrease of water potential values and a decrease in relative water content, demonstrating that water flux was not reaching higher leaves. To prevent higher water losses, stomata were closed, visible in stomatal conductance decrease accompanied by a decrement in transpiration rate values in both species. An increment in proline content was observed in *P. pinaster*, acting as an osmoprotective amino acid protecting plants from the “water-stress” caused by the blockage of xylem and phloem vessels by the pathogen. One possible defence mechanism activation in response to *F. circinatum* was visible in *P. pinaster* and *P. pinea* through the increment anthocyanins content, translated in the later symptomatology appearance, where it revealed insufficient in *P. pinaster*. In the case of *P. radiata*, anthocyanins remained unchanged giving strength to its higher susceptibility. The rate of the pathogen progression in *P. radiata* may indicate that *F. circinatum* may have acted directly in *P. radiata* metabolism through metabolite or protein emission, causing an earlier symptomatology appearance when compared to *P. pinaster* and *P. pinea*, an issue that deserve more research. Relatively to *P. pinea*, its structural advantages coupled successful activation of signalling mechanism (e.g. anthocyanins) counts for the differential response to *F. circinatum*. These results point to a higher plasticity and capacity of osmotic adjustments of *P. pinaster* in comparison to *P. radiata*, supported by the delayed symptoms appearance despite presenting a higher relative necrosis.

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7. Future Perspectives

It is recognized that *F. circinatum* is a threat to worldwide forests and plantations, causing several economic losses for both producers and industries. Up to date, there is no effective management method to control pitch canker disease. The three species used showed to have different anatomical structure which led to the differences observed in the susceptibility to *F. circinatum*, where *P. pinea* revealed as the most tolerant, followed by *P. pinaster* and *P. radiata*.

The aim of this work was to decipher the interaction mechanisms of *F. circinatum* in three pine spp. with different degrees of susceptibility. In *P. pinea*, the structural advantages and successful activation of defence mechanisms was translated in its tolerance to this pathogen, in the opposition to what occur in *P. pinaster* and *P. radiata*. Between them, it was visible the progression of the pathogen within the hosts, and its effect on plant-water relations and photosynthetic machinery. The main differentiation point was the activation of defence mechanisms, where there was a visible increment of anthocyanins in *P. pinaster*, contrarily to *P. radiata*.

The use of molecular approaches, such as genomics, proteomics and metabolomics studies, already applied in other plant-pathogen interactions, had revealed essential to better understand infection and defence activation mechanisms. In this case, to have a better insight of *Pinus-Fusarium* interaction, both plant and fungal point-of-views must be carefully analysed.

From this work it is possible to conclude that cultural methods – genetic selection, choosing the most resistant species, based on the different resistances against *F. circinatum*, can be applied in nurseries and natural plantations in order to have a better management and control of the disease.

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