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Metabolic shifts associated with drought and drought-like symptoms induced by pine pitch canker disease

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Mestrado em Biologia dos Recursos Vegetais

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2023

Abstract

Pine pitch canker (PPC) is a serious disease that affects pines worldwide caused by the fungus *Fusarium circinatum*. As there are no means to eradicate the disease once established, PPC leads to significant economic losses when valuable species like *Pinus pinaster* are affected. It is known that PPC leads to the appearance of drought-like symptoms in *P. pinaster*, and recent studies have reported further resemblances between the plant's response to the fungus and to drought. Nevertheless, a direct comparison between pine response to *F. circinatum* and to drought has never been conducted. Thus, the aim of this work was to evaluate the similarities and divergences between the mechanisms of response to drought and *F. circinatum* in *P. pinaster* in the moment of tip-wilting appearance, through a holistic approach combining morphological, physiological, hormonal, and biochemical data. In this study we found a similar severe drought-like response in both *P. pinaster* infected with *F. circinatum* and subjected to drought, evidenced by a decrease in water potential, a non-stomatal photosynthesis impairment and the accumulation of starch in the needles, possibly due to phloem collapse functioning. However, there was a greater accumulation of osmolytes and antioxidants in water-stressed plants while fungus-infected pines presented a greater decrease in water potential and a less prolongated stomatal closure, suggesting that *F. circinatum* disrupts the water allocation more abruptly than drought. These findings are in accordance with a previous hypothesis that drought similarities found in PPC are due to tissue breakdown caused by the fungus, but it still does not explain the positive correlation between tolerance to both conditions. We anticipate that the knowledge obtained in this thesis will provide some useful insight on *P. pinaster* response to *F. circinatum*, thus contributing to more efficient management strategies of PPC in the future.

Keywords: *Fusarium circinatum*; *Pinus pinaster*; drought; stress response; isohydric species.

Resumo alargado

O cancro resinoso do pinheiro é uma das doenças de pinheiro mais relevantes a nível mundial, causada pelo fungo patogénico, hemibiotrófico e heterotálico pertencente ao filo *Ascomycota*, *Fusarium circinatum*. Este fungo tem a capacidade de infetar várias espécies, incluindo mais de 60 do género *Pinus*, em todas as fases do ciclo de vida da planta. Em plantas adultas, esta doença caracteriza-se por provocar cancros com escorrimento abundante de resina no tronco e ramos, seca dos ramos e queda das agulhas e, finalmente, morte da árvore. Em plantas jovens de viveiro, ocorre tombamento apical, murchidão e coloração castanho avermelhada das agulhas, levando à morte das plantas. Esta doença está espalhada um pouco por todo o mundo, podendo ser encontrada no continente americano, Ásia, África e Europa, incluindo Portugal. Atualmente não existem meios para erradicar a doença uma vez estabelecida, pelo que as medidas de gestão disponíveis são principalmente preventivas, como monitorização da doença, suspensão das exportações de material possivelmente infetado, substituição de espécies suscetíveis por espécies ou híbridos mais resistentes e adoção de programas de melhoramento. Uma vez estabelecido, o cancro resinoso do pinheiro leva a elevados prejuízos devido à mortalidade das plantas de viveiro, dificuldade do seu estabelecimento nas plantações, e redução da produtividade das plantações estabelecidas, prejudicando as indústrias que dependem da matéria-prima resultante. Estas perdas económicas são especialmente significativas quando espécies valiosas do ponto de vista industrial e ambiental, como o pinheiro-bravo, são afetadas.

Pinus pinaster, comummente conhecido por pinheiro-bravo, é uma espécie perenifólia resinosa de porte arbóreo pertencente à família *Pinaceae*, que se distribui pela bacia ocidental do mar Mediterrânico e pela costa atlântica de Portugal, Espanha e França. Atualmente, 22,1% da área florestal do território de Portugal Continental está ocupada por esta espécie, tornando-a a conífera mais comum em Portugal. Esta espécie é utilizada por várias indústrias e contribui de forma significativa para a economia do país, especialmente na indústria florestal onde é responsável por 80% dos postos de trabalho, 88% das empresas e 38% das exportações de bens desse setor. Apesar da sua ampla distribuição, a área ocupada por *P. pinaster* tem diminuído nos últimos anos, o que coloca sérios desafios na satisfação das exigências da indústria portuguesa de madeira que atualmente enfrenta um défice de madeira de pinho. O aparecimento do cancro resinoso do pinheiro em Portugal apresenta-se como uma séria ameaça perante esta conjuntura, sendo essencial encontrar estratégias de gestão mais eficientes para esta doença.

Devido à relevância do cancro resinoso do pinheiro, vários estudos têm sido desenvolvidos de modo a expandir o conhecimento atual relativo a esta doença e desvendar possíveis mecanismos de defesa contra *F. circinatum*. Nesse âmbito, vários investigadores reportaram paralelismos entre a resposta de *Pinus* spp. à infeção por *F. circinatum* e à seca. De facto, os sintomas visuais característicos da doença assemelham-se a uma situação de stress hídrico e diferentes mecanismos típicos de resposta à seca parecem estar ativos em plantas sintomáticas. Nestas plantas verifica-se a diminuição do potencial hídrico, redução da condutância estomática e da taxa de transpiração, assim como uma redução da taxa fotossintética. Também se observa um aumento de osmólitos, como prolina e açúcares solúveis, e um aumento no conteúdo de ácido abscísico. De facto, já foi proposto que o catabolismo do ácido abscísico poderá estar envolvido na resistência a *F. circinatum*. Para além disso, já foi sugerido que a resistência ao stress hídrico poderá ser induzida durante o mecanismo de defesa contra o cancro resinoso do pinheiro, e há uma alta correlação positiva entre a resistência de *P. pinaster* ao défice hídrico e a *F. circinatum*. Alguns investigadores também propuseram que a progressão do fungo pelos tecidos do tronco leva ao colapso generalizado do fluxo de água, levando à falência hídrica e gerando os sintomas e respostas semelhantes à seca. Apesar da grande especulação científica associada à relação entre esta

doença e a seca, nunca foi realizada uma comparação direta entre a resposta dos pinheiros ao *F. circinatum* e ao défice hídrico.

O objetivo deste estudo foi comparar as semelhanças e diferenças das duas condições para a planta no momento do aparecimento do sintoma de tombamento apical. Através de uma abordagem holística, foram analizados parâmetros morfológicos, como crescimento e observações de cortes histológicos, parâmetros fisiológicos, nomeadamente relacionados com as trocas gasosas e potencial hídrico, parâmetros hormonais, com ênfase no ácido abscísico, jasmonatos, ácido salicílico e auxinas e marcadores bioquímicos como pigmentos fotossintéticos, carboidratos não-estruturais, metabolitos secundários e níveis de malondialdeído. Ambos os stresses causaram uma resposta semelhante de seca severa nos pinheiros. Em ambos os grupos houve uma diminuição do potencial hídrico, algo que ocorre apenas em situações próximas da morte, em espécies isohídricas como *P. pinaster*. Além disso, as plantas pareciam ter um bloqueio na fotossíntese de origem não-estomática, uma vez que a concentração interna de CO_2 aumentou, mas a taxa líquida de assimilação de CO_2 era negativa, e apresentavam uma acumulação de amido nas agulhas, que pode ser resultante de uma interrupção no transporte do floema causada pelo aumento da viscosidade da seiva floémica. Contudo, o fungo aparentemente perturba a alocação de água de forma mais abrupta do que a seca, uma vez que as plantas infetadas têm uma maior diminuição do potencial hídrico e parecem fechar os estomas posteriormente, enquanto a seca leva a uma maior acumulação de osmólitos e metabolitos secundários com propriedades antioxidantes, como compostos fenólicos, flavonoides e carotenoides. Estas diferenças na magnitude de resposta sugerem que as plantas sujeitas a seca tentam manter a homeostasia hídrica assim que percecionam o défice hídrico, enquanto as plantas infetadas com *F. circinatum* possivelmente presenciam uma situação semelhante a défice hídrico, mas de forma mais abrupta, num estágio mais avançado da infeção, chegando a apresentar um estado hídrico mais deteriorado. Ainda assim, ambos os stresses parecem levar a uma resposta típica de seca severa no momento de aparecimento dos sintomas. Além disso, o perfil hormonal de *P. pinaster* sujeito a ambos os stresses aparentam ser semelhantes, mas considerando que as hormonas vegetais estão envolvidas na resposta a vários stresses, não é claro se esse perfil hormonal tem a mesma origem.

Os resultados obtidos neste estudo são concordantes com a hipótese pré-existente de que o fungo degrada e obstruí os tecidos vasculares das plantas à medida que a doença progride, levando ao colapso do fluxo de água e simulando uma situação de seca. Assim, as plantas infetadas percecionariam o estímulo de défice hídrico no momento de interrupção da translocação, de forma abrupta e severa. No entanto, a relação entre o cancro resinoso do pinheiro e seca aqui proposta não explica a correlação positiva entre a tolerância de *P. pinaster* à infeção por *F. circinatum* e à seca. Para melhor compreender esta correlação, poderá ser importante avaliar ao longo do tempo, a resposta de genótipos com diferentes níveis de resistência perante uma situação de défice hídrico e infeção por *F. circinatum*. Além disso, o facto das plantas terem sido recolhidas para análise com base no aparecimento de sintomas, resultando em diferentes momentos de amostragem, e a possível variabilidade genotípica do material vegetal utilizado, poderão ter dificultado a deteção de outro tipo de respostas envolvidas. Não obstante, esta tese contribui para a expansão do conhecimento atual sobre o cancro resinoso do pinheiro, particularmente sobre a possível sobreposição entre a resposta de *P. pinaster* a *F. circinatum* e à seca, que poderá ser aplicado futuramente em estratégias de gestão mais eficientes contra esta doença.

Palavras-chave: *Fusarium circinatum*; *Pinus pinaster*; seca; resposta ao stress; espécie isohídrica.

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A net CO_2 assimilation rate

ABA abscisic acid

B bark

C mock-inoculated control

Ca cambium

Car carotenoids

CBL calcineurin B-like proteins

CDPK calcium-dependent protein kinase

CERK1 chitin elicitor receptor kinase 1

Chl*a* chlorophyll *a*

Chl*b* chlorophyll *b*

Ci sub-stomatal CO_2 concentration

CIPK CBL-interacting protein kinases

Co cortex

COI1 coratine insensitive 1

DAMPs damage-associated molecular patterns

DPA dihydrophaseic acid

dpi days post-inoculation

DW dry weight

E transpiration rate

ET ethylene

ETI effector-triggered immunity

F *Fusarium circinatum*-inoculated plants

FAA free amino acids content

FcCa6 *Fusarium circinatum* isolated from *Pinus radiata* from Comillas, Cantabria

FW fresh weight

gs stomatal conductance

HI height increment

HR hypersensitive response

IAA indole-3-acetic acid

ISR induced systemic resistance

iWUE intrinsic water-use efficiency

JA jasmonic acid

JA-Ile / JA_Ile jasmonic acid-isoleucine

JAZ jasmonate-zim-domain protein

LysM-RLKs lysine motif receptor-like kinases

MAMPs microbe-associated molecular patterns

MAPK mitogen-activated protein kinase

MAPKK MAPK kinase

MAPKKK MAPKK kinase

MDA malondialdehyde

MeOH methanol

NLRs nucleotide-binding leucine-rich repeat receptors

OPDA oxo-phytodienoic acid

P p-value

PA phaseic acid

PAL phenylalanine ammonia-lyase

PAMPs pathogen-associated molecular patterns

PCA principal component analysis

PDA potato dextrose agar

PDB potato dextrose broth

Ph phloem

PHP photosynthetic pigments

Pi pith

PNB-RP IV Provenance region IV of maritime pine

PP2C type 2C protein phosphatases

PPC pine pitch canker

PR pathogenesis-related proteins

PR1 pathogenesis-related protein 1

PR2 β-1,3-glucanases

PR3 chitinases

PR5 thaumatin-like proteins

PR9 peroxidases

PR10 pathogenesis-related protein 10

PR14 pathogenesis-related protein 14

PRRs pattern-recognition receptors

PTI pattern-triggered immunity

R resistance proteins

rChl ratio chlorophyll *a*/*b*

RD resin ducts

RD22 responsive to dehydration 22 protein

RIN relative internal necrosis

ROS reactive oxygen species

RWC relative water content

SA salicylic acid

SAG salicylic acid glucoside

SAR systemic acquired resistance

SE standard error

SNP single nucleotide polymorphism

SnRK sucrose non-fermenting 1-related protein kinase

SnRK2.6 Snf1-related protein kinase 2.6

STA starch content

SWC soil water content

tChl total chlorophyll

TF transcription factors

TFL total flavonoids content

TPC total phenolic compounds

TSS total soluble sugars content

VvLYK1-1 *Vitis vinifera* LysM-RLK1-1

VvLYK1-2 *Vitis vinifera* LysM-RLK1-2

WP / Ψmd midday water potential

WS water-stressed plants

WUE instantaneous water-use efficiency

WW well-watered control

X xylem

Chapter 1

Introduction

1.1. *Pinus pinaster*

1.1.1. Botanical and biological characterization

Pinus pinaster Aiton, also known as maritime pine, is a resinous evergreen tree belonging to the *Pinaceae* family. This monoecious, diploid tree (2n=24) can reach 40 meters in height and has green needle-like leaves grouped in pairs (1). It is distributed throughout the western basin of the Mediterranean Sea and the Atlantic coast of Portugal, Spain, and France (2). *P. pinaster* occurs in various types of substrates (3) and in places characterized by a Mediterranean climate, especially in areas with Atlantic influence since they are wetter than the typical Mediterranean conditions (4).

This conifer is considered to be a pioneer species (5), with rapid growth and shade intolerance (6). It generally forms open forests with dense shrubs and mixed forests with oaks and other pine species, and occasionally monospecific forests (7). *P. pinaster* plants with their canopy above the ground are tolerant to low-intensity surface fires (8).

1.1.2. Economic, social, and environmental importance of maritime pine

The maritime pine was a frequent choice in reforestation projects in Portugal, such as in the North and Center mountain ranges and in the fixing of dunes in coastal regions, due to its ability to grow in non-colonized areas and being a fast-growing tree (6,9). As a result, of the 36.2% of the mainland Portugal territory covered by forest, 713.3 thousand ha are occupied by maritime pine, which corresponds to 22.1% of the total forest area (10), making it the most common conifer in Portugal. In fact, *P. pinaster* is one of the tree species that occupies the largest area in the forests of mainland Portugal, surpassed only by *Eucalyptus globulus* (26.2%) and *Quercus suber* (22.3%) (10). Central Portugal is the region with the highest incidence of *P. pinaster* in the country, since 42.1% of its total forest area is covered by this species (10). Therefore, maritime pine is of great economic, social, and environmental importance for this region.

In Portugal, 80% of the jobs and 88% of the companies in the forestry industries are directly dependent on maritime pine. In addition, the pine cluster represents 3.4% of total national exports of goods and 38% of forest industries-related exports of goods (11). In 2021 alone, *P. pinaster* generated more than 2000 M€ in exports, an increase of 25% over the previous year. That same year, the main asset exported in terms of economic value was furniture, yielding 795 M€ (11).

This species is used by various industries, contributing significantly to the country's economy (9). In the timber industry, maritime pine is used for various purposes such as the production of wood for the construction industry, joinery, furniture, wood panels and packaging (12). In the resin production industry, *P. pinaster* is the most important species for resin extraction, being the only species used for this purpose in the Western Mediterranean countries (13). Moreover, *P. pinaster* is the main species used to produce wood pellets for biomass heating systems in Portugal (14). In addition to the direct economic value, maritime pine forest areas also create indirect value through complementary activities, such as mushroom production, beekeeping, pastoralism, hunting, and ecotourism (12).

1.1.3. Current threats to *P. pinaster* forests

Despite its previously mentioned wide distribution, the area covered by *P. pinaster* in Portugal decreased 264.7 thousand ha between 1995 and 2015, causing pine forests to become the forest ecosystem with the greatest decrease in area in Portugal (10). In face of the current deficit of pine wood in the Portuguese industry (11), the reduction of maritime pine availability poses significant challenges to meet industry demands. The Portuguese governmental entity *Instituto da Conservação da Natureza e das Florestas* (10,15) identified forest fires, increased rural abandonment, lack of management of forest areas, increasingly frequent droughts, and pests such as *Thaumetopoea pityocampa* and *Rhyacionia buoliana* as the causes of the decline of *P. pinaster* in Portugal.

In Spain, Gea-Izquierdo et al. (16) observed, in a mixed forest dominated by *P. pinaster*, that the maritime pine presented a high mortality and very low regeneration in comparison with other species, which could lead to important changes in forest composition. The researchers concluded that the decline of this species may be associated with biotic agents and drought (16), as in Portugal. It is known that drought is the main limiting factor for *P. pinaster* growth in Mediterranean climate regions (17), characterized by hot, dry summers (18). According to climate projections, the Mediterranean area will have increasingly frequent drought, due to increased heat coupled with decreased precipitation (19), which could lead to greater susceptibility of isohydric species such as the maritime pine (20), to insects and pathogens due to carbon starvation (21).

In addition, two other threats to *P. pinaster* are of major concern, *Bursaphelenchus xylophilus*, the causal agent of pine wilt disease, in Portugal, and *Fusarium circinatum*, the causal agent of pine pitch canker disease, in the Iberian Peninsula (15,22). Both biotic agents are quarantine organisms and are responsible for causing substantial damage (15).

1.2. Pine pitch canker (PPC) disease

Pine pitch canker (PPC) disease is one of the most relevant pine diseases worldwide (23) and it is caused by *Fusarium circinatum* Nirenberg & O'Donnell, a hemibiotrophic and heterothallic pathogenic fungus belonging to the *Ascomycota* phylum (24,25). It is currently known that this fungus can infect 67 species and 18 hybrids of the genus *Pinus*, 6 species of trees of other genera and 15 species of herbaceous plants and grasses (26) and can infect the plant during all its life cycle phases (27). The disease is characterized by provoking damping-off, tip-wilting, dieback, and death of nursery seedlings and dieback of infected branches, cankers with an overflow of abundant resin and, in some cases, the death of the whole tree in adult plants (Figure 1.1) (23,28,29).

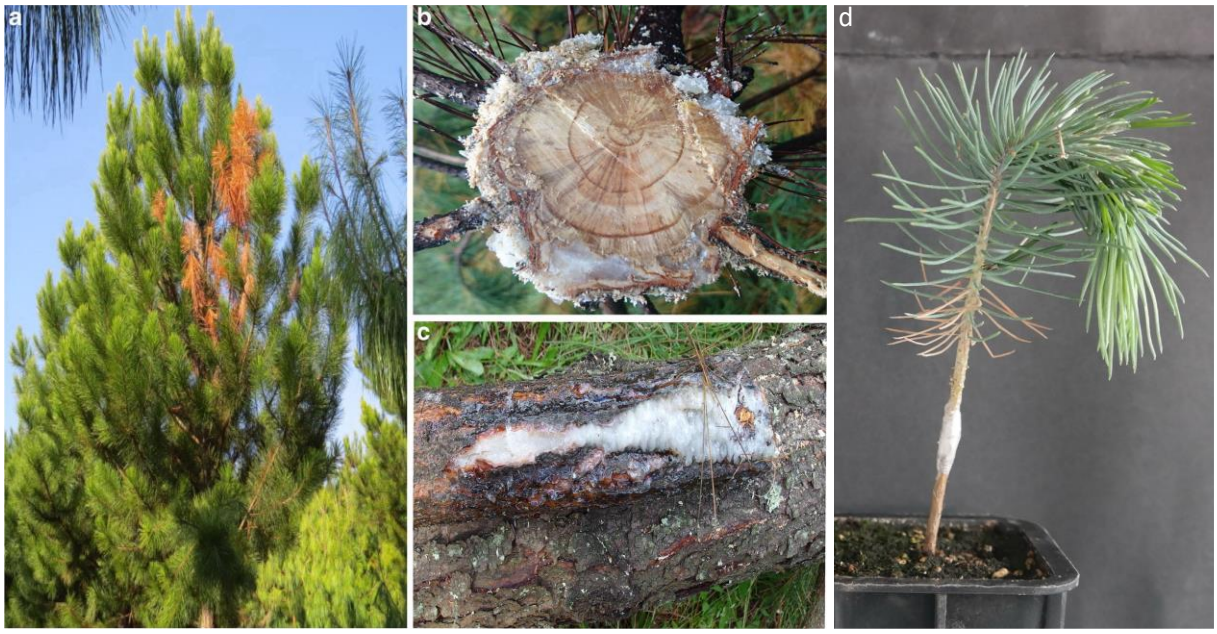

Figure 1.1 | Characteristic symptoms of pine pitch canker disease. Adult *Pinus greggii* trees with a) flagging of an infected branch, b) resin-soaked xylem and c) resinous stem canker on the main stem. d) *P. pinaster* seedling with tip-wilting. Adapted from Fru et al. (30).

PPC was first reported in the United States in 1945 (31), but it is thought that *F. circinatum* originates from Mexico, because it is in this region where it reaches its maximum diversity (32). Currently, as a consequence of international trade in plant products (33), the disease is spread all over the world (Figure 1.2), and can be found, in the American continent, in 13 states of the United States, Haiti, Mexico, Chile, Uruguay, Colombia and Brazil, in Asia, present in Japan and South Korea and, in Africa, present in South Africa (26). In Europe, the disease was formally reported for the first time in a nursery in Spain, in *Pinus radiata* and *P. pinaster* (22), followed by France (34), Italy (35) and Portugal (36). However, it seems that the disease has been eradicated in France and Italy (26).

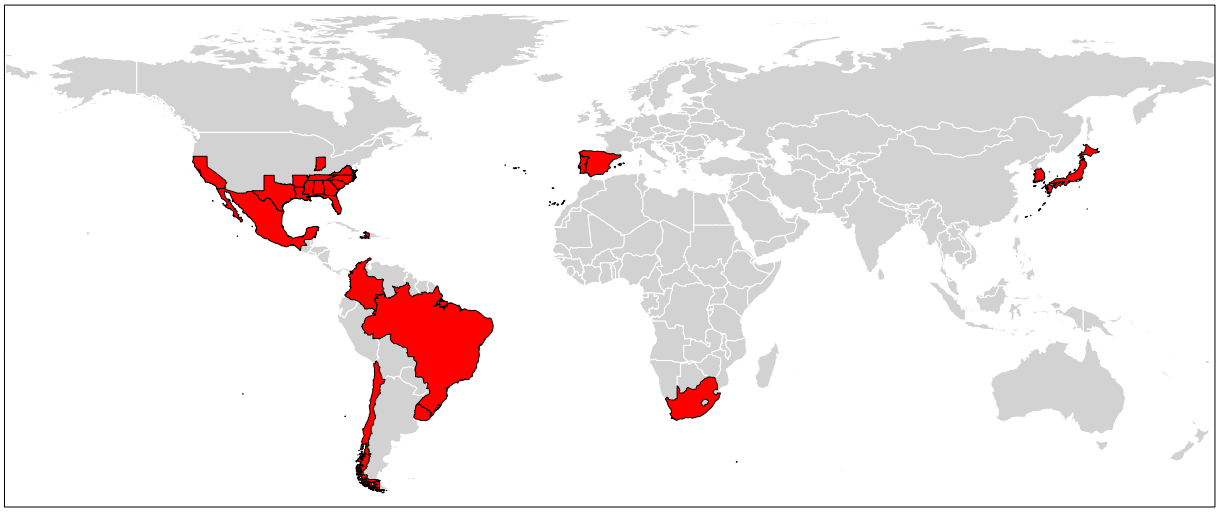

Figure 1.2 | Global distribution of *F. circinatum*. The regions where the pathogen is present are colored in red.

Although *F. circinatum* is primarily seen as a pathogenic fungus of pine trees, this may not represent its main ecological activity (37). There are several studies that report endophytic colonization of this fungus in herbaceous plants of the *Poaceae* (37–40), *Asteraceae*, *Lamiaceae*, and *Rosaceae* families (41). This is in accordance with the fact that *F. circinatum* belongs to the American Clade of *Fusarium fujikuroi* species complex along with several other species known to colonize *Poaceae* (42–

44). In addition, this fungus is tolerant to 2-benzoxazolinone resembling corn endophytes (45). As a result, Swett & Gordon (37) hypothesized that the pathogenic relationship between this fungus and pine species is a recent evolutionary innovation, resulting from the recent jump of *F. circinatum* from grasses to pines. More recently, studies report a hemibiotrophic nature of *F. circinatum* evidenced in the observation of an endophytic stage of this fungus in *P. radiata* (25,46), where it colonizes the intercellular space without evident damage to the surrounding cells (25). These sequences of new discoveries point to the possibility that the main ecological activity of *F. circinatum* is the endophytic colonization of a wide variety of plant species (47). Thus, the typical view of *F. circinatum* as a pathogenic fungus may result from a biased point of view where this fungus is only observed in situations that cause disease, not representing its main ecological activity (37). However, the new findings may represent greater difficulties in combating PPC since a plant colonized by *F. circinatum* that presents an endophytic lifestyle can act as a source of inoculum, since the same genotype can adopt a pathogenic or endophytic lifestyle (37,39–41,48).

1.3. PPC-associated problems and management strategies

The PPC disease is responsible for significant economic losses due to the high mortality of seedlings in nurseries and the difficulty of establishing them in plantations, as well as the reduced productivity of established plantations, which jeopardizes industries that depend on the resulting raw material (23,27,29).

As there are currently no measures to control or eradicate the disease once it is established (49), the management measures available are mainly preventive. In the short term, the measures aim to stop the spread of the pathogen to areas with the presence of unaffected susceptible hosts (29). According to the Portuguese law *Dec. Lei n.º 294/2013 de 27 de setembro*, when the presence of *F. circinatum* is detected, the lot to which the sample belongs to should be eliminated as well as all symptomatic plants. In addition, no plant material of the host species existent in the place of production can be marketed until two consecutive years have elapsed without a new presence of the fungus being detected on the site. During these two years, all host species undergo frequent intensive phytosanitary inspection in order to detect possible new infections (50). These disease management measures end up aggravating the economic losses associated with the PPC, since they require high costs resulting from the monitoring and control of the disease and the suspension of exports (27).

In addition to the immediate measures mentioned above, it is possible to incorporate long-term measures such as the replacement of susceptible species with more resistant species or hybrids as well as the adoption of breeding programs to obtain more resistant genotypes (28,51). *P. pinaster* is a species that presents a great diversity of responses to *F. circinatum* (52), thus the adoption of breeding programs aimed at obtaining more resistant genotypes may be the appropriate strategy to be chosen in this species. By implementing the management measures for the PPC it is possible to reduce the associated economic impact (23).

1.4. Pathogen dispersal and infection mechanism

The dispersion of *F. circinatum* can occur naturally, through air, rain, and insects, or facilitated by humans (Figure 1.3) (33). In certain regions like California (53) and North-Western Spain (54), *F. circinatum* inoculum can be found in the air throughout the entire year. In these cases, the inoculum responsible for the dispersion seems to be the conidiospores, asexual spores (53). *F. circinatum* may also be present in seeds, both inside and on their surface, especially when coming from symptomatic branches (46). Thus, the pathogen can infect unaffected seeds during seed storage (55). Another possible source of inoculum are plants in which the fungus has an endophytic lifestyle (37,39–41,48), as

mentioned in 1.2. section. Some insect species have been confirmed as possible vectors of *F. circinatum*, such as *Ernobius punctulatus* or *Tomicus piniperda* (56,57). However, the presence of this fungus in several other insect species has also been confirmed (58,59).

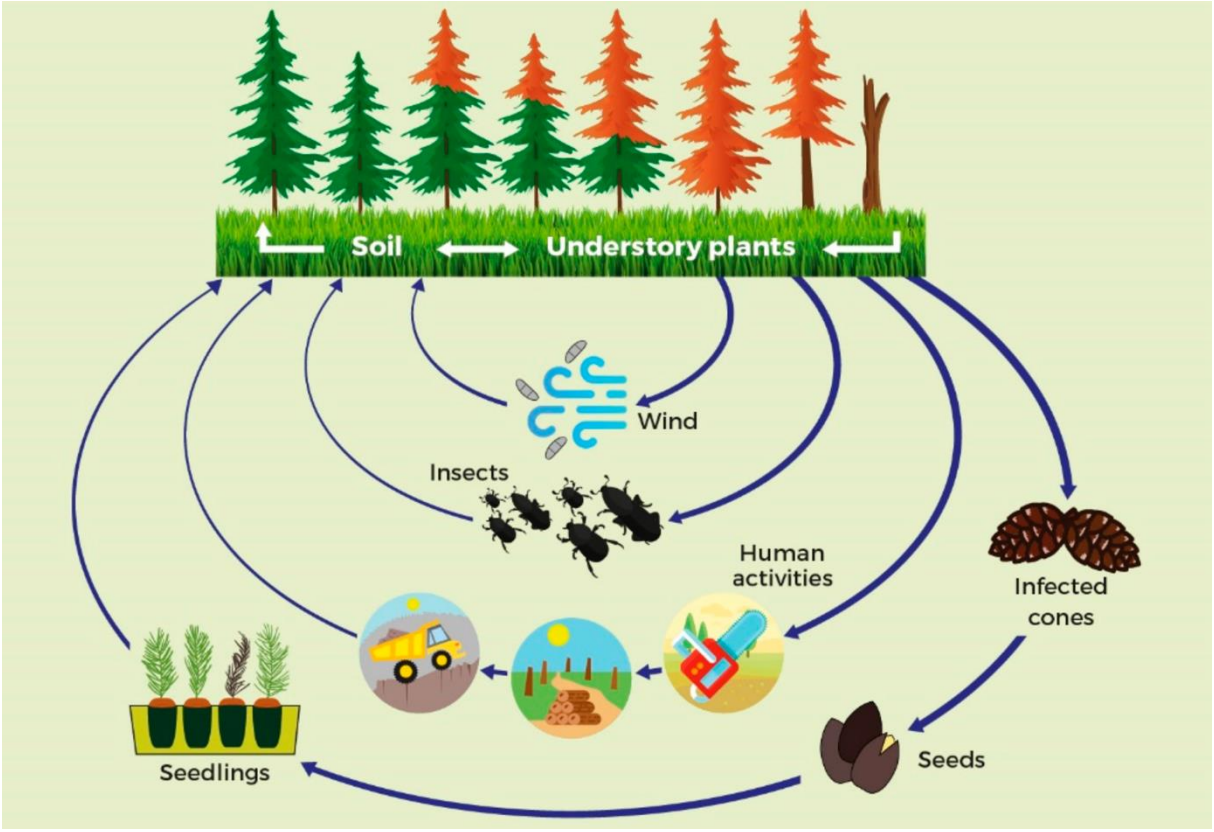

Figure 1.3 | Spreading pathways of *F. circinatum* in the pine pitch canker disease (33).

Infection with *F. circinatum* can occur through any type of wound, such as wounds resulting from forestry practices, weather events, and wounds resulting from insects feeding (60–62). The success of an infection can be determined by several parameters such as the size of the wounds, with larger wounds benefiting the pathogen, the timing of infection, since inoculations at the end of the day and on more recent wounds increase the likelihood of infection, and the concentration of *F. circinatum* spores at the wound site (63). Although wounds were thought to be the main way of *F. circinatum* infection, there is evidence of successful infections through natural openings, such as the site of emergence of new roots and the insertions of needles into branches, or through direct penetration without the need for wounds, possibly using penetration structures such as hyphopodia (64,65).

1.5. Tissue colonization

Initially, colonization by *F. circinatum* occurs through the intercellular spaces in the radial direction towards the pith and in the tangential direction between the most superficial layers of the cortex and phloem. In the more advanced stages, vertical colonization of plant tissues occurs through the pith tissues, resiniferous ducts, and axial tracheids of the xylem and by the outermost layers of the cortex and phloem, resulting in the characteristic necrotic appearance. When the fungus reaches the starch reserves in the pith, it develops conidiophores in the cavities of the pith, leading to the production of conidiospores that will allow the perpetuation of the disease cycle (66). To obtain nutrients, the fungus is thought to secrete into the apoplast cell wall-degrading enzymes such as endopolygalacturonase, a protein whose coding sequence is present in the *F. circinatum* genome (66,67). It has also been

suggested that the fungus manipulates energy production pathways such as fermentation, β-oxidation of fatty acids and the tricarboxylic acid cycle to obtain amino acids for its benefit (68).

During *F. circinatum* infection, the plant produces traumatic resiniferous ducts (66), increasing the amount of resin as a defense mechanism (69). However, the fungus maintains its growth in both the constitutive and traumatic resiniferous ducts (66), since *F. circinatum* is highly resistant to resin components (70). Finally, the progression of the fungus through the stem tissues obstructs the conducting vessels due to the large amounts of exuded resin, generating a generalized collapse of the flow of water and nutrients, leading to water failure, and contributing to the death of the tree (66).

1.6. *Pinus-F. circinatum* pathosystem from a cellular and molecular perspective

1.6.1. Zigzag coevolutionary model

The zigzag coevolutionary model initially proposed by Jones & Dangl, (71) describes the plant-pathogen interaction at the cellular and molecular level after the non-specific physical and chemical constitutive defenses of plants have been surpassed (72,73). According to this model, pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) released by the pathogen and damage-associated molecular patterns (DAMPs) resulting from plant cell damage are recognized by the pattern-recognition receptors (PRRs) present on the plant's plasma membrane extracellular surface, triggering pattern-triggered immunity (PTI) (71,72,74). In order to subvert PTI, the pathogen interferes with the recognition of molecular patterns by PRRs at the plasma membrane level or releases into the cytosol effector proteins that modulate plant's physiology. These proteins modify the plant's signaling or resistance response. However, plants can recognize effector proteins through R proteins, mostly nucleotide-binding leucine-rich repeat receptors (NLRs), thus triggering effector-triggered immunity (ETI). In fact, the pathogen can also develop effectors to subvert the ETI (71,72,75).

In the *Pinus-F. circinatum* pathosystem the responses triggered by the plant may occur in a similar way to those described in the previously mentioned model. Hernandez-Escribano et al. (76) observed that during the early stage of *F. circinatum* infection, *P. pinaster* upregulates genes encoding for lysin motif receptor-like kinases (LysM-RLKs). LysM-RLKs are a family of PRRs responsible for recognizing oligosaccharidic PAMPs/MAMPs (77). For example, CERK1 in *Arabidopsis thaliana* and VvLYK1-1 and VvLYK1-2 in *Vitis vinifera* are homologous molecules of the LysM-RLK family, responsible for recognizing chitin and chitosan and triggering PTI (78–80). Also, in the resistant *Pinus pinea* it was observed the upregulation of several genes coding for PRRs (49).

After the recognition of molecular patterns by PRRs, cell signaling mechanisms are triggered promoting local and systemic defense responses, such as Ca^{2+} influx, production of extracellular reactive oxygen species (ROS), and activation of calcium-dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK) signaling cascades (74). The activation of cell signaling mechanisms may differ between *Pinus* species according to their level of susceptibility to *F. circinatum*. In *P. pinea*, there is an upregulation of several MAPK kinases (MAPKKs) and MAPKK kinases (MAPKKKs) while in the susceptible *P. radiata* the induction of PRRs is practically non-existent and the upregulation of MAPK and CDPK is not observed (49). MAPKs and CDPKs are responsible for activating transcription factors (TFs) that regulate defense-related genes, triggering the biosynthesis of antimicrobial compounds and defense-related hormones (81,82), hence the lack of expression of MAPK and CDPK in *P. radiata* may be related to its susceptibility (49).

There is still no evidence to support the existence of ETI in the *Pinus-F. circinatum* pathosystem (83). In fact, ETI is only effective against pathogens with biotrophic behavior, being ineffective in pathogens with necrotrophic behavior (84), as *F. circinatum*. This is because the recognition of effectors

by the plant's R proteins that characterizes ETI often triggers programmed cell death around the site of infection, a mechanism known as hypersensitive response (HR) (84–86). While HR is effective in defending against biotrophs, this mechanism can do nothing against pathogens with necrotrophic behavior, which actively release toxins and effectors that cause cell death, often taking advantage of HR in order to feed on the dead tissues (84,86). In fact, in this pathosystem, HR activation can be observed in *Pinus patula*, a susceptible species (87,88).

1.6.2. Defense-related hormones

Phytohormones play an important role in plant defense in the *Pinus*-*F. circinatum* pathosystem. In fact, several researchers have already suggested that resistance to *F. circinatum* may be associated with the coordinated induction of salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and auxins, since when analyzing the transcriptome of species with different levels of susceptibility to this fungus they noticed an increase in the expression of genes inducing these hormones (49,76,87,88). In general, the hormones cytokinin, gibberellic acid and brassinosteroid appear to be suppressed in this pathosystem (76,88). In addition, Amaral et al. (89) proposed that the catabolism of abscisic acid (ABA) in phaseic acid (PA) may be associated with resistance after observing the accumulation of PA in *P. pinea*. The role of ABA in this pathosystem is described in the 1.7. section. Plant hormones trigger the activation of systemic resistance: SA activates systemic acquired resistance (SAR), an important defense mechanism against pathogens with biotrophic behavior, and JA/ET are responsible for the activation of induced systemic resistance (ISR), a mechanism activated by mutualistic microorganisms and pathogens of necrotrophic behavior (84,90,91).

Defense-related hormones seem to act at different stages in the response to *F. circinatum*. Visser et al. (88) reported that the resistant species *Pinus tecunumanii* activates the ET and auxin biosynthesis pathways in the early stages, while the activation of JA and SA occurs later in the process. In fact, JA-mediated signaling is suppressed in the early stages through the upregulation of JAZ and JA hydroxylase and the absent expression of JA biosynthesis-related genes, which enables a greater ET defense response in *P. tecunumanii* (88). Moreover, there seems to be a coordinated role of ET in the defense of the resistant species *P. pinea* (49) and *Pinus oocarpa* (87), which also highlights the possibility that this hormone may be important in the resistance against the fungus. However, in the early stages of infection in *P. oocarpa* JA biosynthesis and auxin suppression seem to occur (87), contradicting a previous report (88). This suggests differences in the timing of hormone action between different resistant species (87).

Due to the crucial role that phytohormones play in defending against *F. circinatum*, it is expected that this fungus presents mechanisms that aim to subvert the defenses to its advantage. In fact, by studying the response of *P. pinaster* to *F. circinatum*, Hernandez-Escribano et al. (76) found that the fungus can disrupt the plant's ET homeostasis by expressing genes associated with ET biosynthesis throughout the infection process, especially at the end. One of these genes, 2-keto-4-methylyhiobutyrate-dependent ethylene-forming enzyme coding gene, seems to be associated with the virulence of the fungus because it presents 90% similarity with a *Fusarium oxysporum* gene that decreases the virulence of this fungus in tomato plants when knocked out (76,92). Hernandez-Escribano et al. (76) also suggests two additional *F. circinatum* mechanisms interfering with plant defenses, the expression of genes coding for enzymes of the isochorismatase hydrolase family that prevent the biosynthesis of salicylic acid through inhibition of the chorismate pathway, and the potential blocking of JA signaling by suppression of coronatine insensitive 1 (COI1), since the COI1 gene was downregulated in *P. pinaster* at 5- and 10-days post inoculation (dpi). Also, Zamora-Ballesteros et al. (49) noted decreased expression of COI1 in *P. pinea*, supporting this hypothesis. Further evidence of *F. circinatum* subverting plant defenses was observed in *Pinus greggii*, a susceptible species, since early

active defense responses were weakening over time (87), and in *P. patula*, another susceptible species, as there was a downregulation of hormones (88) that could be explained as an inhibition of the defenses by the fungus.

1.6.3. Pathogenesis-related proteins

The hormones SA, JA and ET induced by pathogen attack or abiotic stress, as well as HR and SAR lead to the accumulation of pathogenesis-related (PR) proteins, essential in plant defense against pests and pathogens (93,94). In the *Pinus*-*F. circinatum* pathosystem, it has been reported that the hormones JA and SA induce PR3 (95). In *P. pinaster* infected with *F. circinatum*, biosynthesis levels of SA, JA and ET increase, leading to the upregulation of PR proteins including PR1, PR2, PR3, PR5 and PR9 (76). PR1 has antimicrobial activity, possibly by sequestering the sterol that the pathogen needs (96), amplifying the defense signal and recognizing sterols and effectors (97). It is known that *F. circinatum* presents the biosynthesis of ergosterol impaired when it infects the resistant species *P. tecunumanii* since the genes involved are downregulated, which may increase the susceptibility of the fungus to PR1 (88). PR2 (β-1,3-glucanases) hydrolyze β-1,3-glucan and PR3 (chitinases) hydrolyze the glycosidic bonds β-1,4 that bind the N-acetylglucosamine residues of chitin. These PRs are responsible for degrading the fungus cell wall and releasing oligosaccharides with elicitor potential during the process (94). Finally, PR5 (thaumatin-like proteins) cause osmotic rupture of the fungus cells by generating pores in the plasmalemma, while PR9 (peroxidases) limit the expansion of the pathogen by promoting cell wall reinforcement, producing ROS and reactive nitrogen species, as well as antimicrobial metabolites such as phytoalexins (94,98).

It is thought that PR proteins may be related to *Pinus* resistance or susceptibility to *F. circinatum*. Zamora-Ballesteros et al. (49) reported that both *P. pinea* and *P. radiata* expressed the coding genes for the PR proteins mentioned above, as well as PR10 and PR14, but the resistant species had the highest expression levels, and *P. radiata* did not express PR2. In another study, researchers compared *P. radiata* genotypes with different susceptibility levels and reported an overall increase in PR1, PR3, PR5 and PR9 after inoculation with *F. circinatum*, but the less susceptible genotype significantly increased the expression of PR1 compared to the more susceptible genotype (99). Donoso et al. (100) also compared genotypes of *P. radiata* with different susceptibilities to *F. circinatum* and noted that the less susceptible genotypes had higher expression of PR5 and PR9. On the other hand, Amaral et al. (101) reported that PR5 gene expression increased in *P. pinaster* and PR3 increased in *P. pinaster* and *P. radiata* but remained unchanged in *P. pinea*. Although this result is not in agreement with the previously mentioned studies, the *P. pinea* sampling was performed later than the other *Pinus* species sampling, which may corroborate the hypothesis that resistant hosts have a rapid response immediately after inoculation and a subsequent return to baseline levels, while susceptible hosts are slow to activate defense responses (100). Therefore, when the material was collected, the PR gene expression in *P. pinea* could have already returned to baseline.

1.6.4. Phenylpropanoid pathway and sulfur metabolism

In addition to the defense mechanisms mentioned above, the phenylpropanoid pathway is also important in the plant defense. This pathway begins with the deamination of L-phenylalanine by L-phenylalanine ammonia-lyase (PAL) (102). After inoculation with *F. circinatum*, PAL expression is upregulated in resistant species such as *P. pinea* and *P. tecunumanii* (49,88,101) and in the moderate susceptible species *P. pinaster* (76,101). Susceptible species such as *P. radiata* and *P. patula* appear to have greater variation in PAL transcription after *F. circinatum* infection, as it has already been reported an upregulation (88,100,101), a downregulation (103) and a non-alteration (49) of the expression of this gene. It has also been observed that more susceptible *P. radiata* genotypes may express higher PAL

levels than less susceptible genotypes in the first days after inoculation, but those high expression levels decrease shortly after (100).

The phenylpropanoid pathway branches into different biochemical pathways, generating several compounds, such as lignin, stilbenes, or flavonoids (102). Flavonoids are essential molecules that, among other functions, act in defense against biotic and abiotic factors (104,105). In this pathosystem, resistant species have a higher constitutive expression of flavonoid biosynthesis genes, which may contribute to resistance (87). In the resistant species *P. pinea* it was observed the upregulation of flavonoid biosynthesis associated genes in response to infection (49). Lignin is an important component of the plant cell wall responsible for increasing wall rigidity and protecting against pests and pathogens (106). The lignification seems to be a relevant step in resistance against *F. circinatum*, since *P. pinea* upregulates enzymes responsible for lignin biosynthesis as a response to the fungus while *P. radiata* has a weak regulation of genes associated with this process (49). In that same study, it was also found that *F. circinatum* increased the expression of genes related to cell wall degradation, such as genes related to the degradation of lignin and the enzyme that degrades polysaccharides into fermentable sugars, 1,5-alpha-L-arabinosidase, while it was infecting *P. pinea*. In *P. radiata* this is not observed which can be due to the different wall composition between the two hosts (49). Another product that results from the phenylpropanoid pathway is pinosylvin, a phenolic compound belonging to the stilbenes family with antimicrobial properties (107). Pinosylvin may also be important in the resistance to *F. circinatum* since it was observed a greater increase in the expression of pinosylvin synthase in the more resistant *P. radiata* genotype comparing to the more susceptible one (100).

Besides flavonoid biosynthesis associated genes, genes related to sulfur metabolism are also constitutively more expressed in resistant species (87). Sulfur is important to produce resistance metabolites, such as camalexin (108), a phytoalexin apparently present in the resistant species *P. tecunumanii* infected with *F. circinatum* (88). Additionally, in the proteome of the resistant species *P. pinea* it was found that most biological processes remained unchanged after being infected with *F. circinatum*, but there was a significant increase in proteins related to sulfur assimilation (68).

1.6.5. Growth-defense tradeoff

The constitutive expression of genes related to sulfur metabolism and flavonoid biosynthesis may allow resistant species to respond more rapidly to infection (87). In fact, the speed of response to *F. circinatum* is associated with resistant species, such as *P. pinea*, *P. tecunumanii* and *P. oocarpa*, while susceptible species, such as *P. radiata* and *P. patula*, present late activation of defenses (49,87,88). In addition, it is also observed in the resistant species *P. tecunumanii* and *P. oocarpa*, an early downregulation of growth-associated genes such as genes associated with cell and DNA replication, and photosynthesis (87,88). This fact coupled with the speed of response to the fungus observed in resistant species suggest a tradeoff between growth and defense (87). Additionally, in the susceptible species *P. patula*, there is a lack of defense induction, such as a downregulation of xyloglucan:xyloglucosyl transferase, an enzyme responsible for cell wall reinforcement, and an upregulation of growth-associated genes as those coding for expansins (88), corroborating the above-mentioned tradeoff. Considering that plants have limited resources, the allocation of these resources for growth and defense must occur in order to optimize the plant fitness (109).

Thus, in the *Pinus-F. circinatum* pathosystem, host resistance appears to be dependent on the plant's constitutive defenses and the rapid perception of infection and activation of PTI, increasing defense compounds such as hormones, PR proteins and metabolites of the phenylpropanoid pathway. Also, the tradeoff between defense and growth seems to be relevant for plant survival. On the other

hand, the fungus seems to present several mechanisms of subversion of the plant's defense responses, mainly against hormones, but also subvert HR in its favor.

1.7. PPC and drought stress

1.7.1. Pine's drought-like response to *F. circinatum*

As *F. circinatum* infection progresses, the PPC characteristic visual symptoms emerge. In the final stages, drought-like symptoms such as wilting of succulent tissues and tissue desiccation are evident. Also at the molecular level, Morse et al. (110) reported the induction of the pi307a sequence, possibly coding for a boiling-stable protein implicated in the response to water stress, in the susceptible species *Pinus elliottii* when infected with *F. circinatum*. These authors define the induction of this gene as a molecular symptom of PPC, claiming that due to the destruction or obstruction of the plant's vascular system by the fungus, drought-like symptoms arise in the upper parts of the plant (110). This hypothesis is in line with the description made in the 1.5. section, regarding the colonization of plant tissues by the fungus which ultimately causes water transport collapse by obstructing the conducting vessels (66).

When plants face a drought situation, transcriptional and metabolic changes occur that aim to protect it from possible cellular damage (111), such as osmotic adjustment and ABA accumulation. The osmotic adjustment consists of osmolyte accumulation, such as proline, glycine-betaine, and soluble sugars, leading to the decrease in the water potential of the cells, maintaining the cellular turgor without interfering with the metabolism (112). In the *Pinus-F. circinatum* pathosystem, there is also an accumulation of proline and soluble sugars in drought-like symptomatic *P. pinaster* and *P. radiata* (101,113,114).

The accumulation of ABA that occurs during a drought episode is essential in the response and resistance to water stress, by regulating stomatal closure and stress responsive genes expression (112). In the absence of stress, type 2C protein phosphatases (PP2C) inhibit ABA signaling, keeping the stomata open. The drought episode triggers in the plant signals that will induce the accumulation of ABA and ROS, as hydrogen peroxide, increasing the Ca^{2+} concentration in the cytoplasm. Increased ABA inhibits PP2C activity and induces Ca^{2+}-independent phosphorylation of Snf1-related protein kinases (SnRKs) and Ca^{2+}-dependent phosphorylation of calcineurin B-like proteins (CBL), CBL-interacting protein kinases (CIPK), and CDPK, resulting in transcriptional changes. SnRK2.6 phosphorylates anion channels and inhibits the activity of K^+ channels, which decreases turgor pressure, resulting in stomata closure and consequently a decrease in water loss by transpiration (115). In *P. radiata* and *P. pinaster* with tip-wilting resulting from *F. circinatum* infection there is also a decrease in water potential and relative water content (RWC) and the consequent accumulation of ABA, closure of the stomata and decreased transpiration and photosynthetic rate (89,101,113,114). Moreover, in *P. pinaster* there is an upregulation of SnRK2.6 (101).

On the other hand, the resistant species *P. pinea* did not show significant differences in the amount of ABA at any time, but interestingly it showed an increase in stomatal conductance and transpiration rate after infection with *F. circinatum* (89). Another study also observed the same pattern and reported a decrease in SnRK2.6 (101). However, Zamora-Ballesteros et al. (49) contradicted this by having reported an apparent ABA biosynthesis and upregulation of genes associated with ABA signaling, after having analyzed the transcriptome of *P. pinea* infected with *F. circinatum*.

Also, in this resistant species there was a significant increase of phaseic acid (PA) in all timepoints (89). PA is a product with ABA-like activity resulting from the catabolism of ABA by ABA 8'-hydroxylase (116). On the other hand, the susceptible species *P. radiata* does not present significant

differences in the amount of PA, while it presents an increase of dihydrophaseic acid (DPA) (89). DPA is another metabolite resulting from the catabolism of ABA, resulting from the reduction of PA which, unlike this one, has no detectable ABA-like activity (116). Due to these results, Amaral et al. (89) proposed that PA accumulation was related to *F. circinatum* resistance. In fact, Visser et al. (88) reported that, in the resistant species *P. tecunumanii*, there seems to be a decrease in ABA signaling, but simultaneously ABA synthesis, upregulation of its receptors and downregulation of ABA 8'-hydroxylase. The suppression of ABA signaling may be due to ET which, as referred to in the 1.6.2. section, has its synthesis upregulated in these same conditions (88). In the moderately susceptible *P. pinaster*, Hernandez-Escribano et al. (76) observed that the genes for ABA 8'-hydroxylase were upregulated, hence the conversion of ABA to PA seems to occur in this species. In addition, the genes coding for PP2C, responsible for inhibiting ABA signal transduction, were upregulated (76), indicating ABA signaling suppression. Nevertheless, the transcriptomic data seem to intuit for ABA biosynthesis in all timepoints analyzed after infection (76). Similarly, there is an upregulation of ABA 8'-hydroxylase in the susceptible species *P. radiata* and *P. patula* (49,88) and in *P. radiata* there seems to be ABA biosynthesis (49). In *P. patula*, there seems to be a decrease in ABA synthesis, but an apparent increase in ABA signaling (88).

In addition, Elvira-Recuenco et al. (52) reported in *P. pinaster* a high positive correlation between resistance to *F. circinatum* and to drought, evidencing once again the connection that exists between the two stresses. In the resistant species *Pinus taeda*, a SNP was identified in a gene coding for a dehydration-responsive protein RD22-like that is apparently associated with resistance to *F. circinatum* in this species (117). This gene is associated with water stress and can be activated by ABA (118), and in *A. thaliana* its knock-out increases tolerance to water stress (119). Thus, the discovery of Lu et al. (117) suggests that resistance to water stress may be induced during the defense mechanism against PPC. Therefore, it is indispensable to understand the typical response of *P. pinaster* to drought stress.

1.7.2. *P. pinaster*'s response to drought

As previously mentioned in 1.1.3. section, drought is the main limiting factor for *P. pinaster* growth in Mediterranean climate regions (17) hence, unsurprisingly, this species presents some adaptations to drought scenarios. In fact, as an isohydric species (20), *P. pinaster* keeps a relatively constant midday leaf water potential through reducing stomatal conductance in order to limit water loss via transpiration once water availability drops (21). This strategy comes with the cost of reducing photosynthesis through CO_2 restriction (120), leading to a decrease in total biomass (121). Thus, *P. pinaster* growth relies on the precipitation period, between autumn and early spring, that precedes the growing season (122). In response to drought, this species also increases the concentration of cyclitols, alpha-tocopherol and ascorbate cycle intermediaries due to their ROS scavenging properties. Additionally, there is a general increase in sterol concentration to reduce membrane permeability which prevents further water loss, as well as a rise in amino acids content (123).

Despite the conserved isohydric adjustment (124), *P. pinaster* populations exhibit some variation in their response to drought depending on their origin site, whether it is a predominantly mesic or xeric Mediterranean climate. Pines from more xeric environments can delay stomatal closure during drought since they typically present a greater osmotic adjustment capacity than mesic pines (125), which rely more on stomatal sensibility to maintain water homeostasis (126). Moreover, *P. pinaster* from xeric regions also present a higher concentration of non-structural carbohydrates, such as soluble sugars and starch (127), as well as a more conservative use of water (128) comparing to mesic populations that present a more reckless growth strategy (124).

During drought, *P. pinaster* plants also undergo organ-specific metabolic changes, especially in the roots, where the stress is first perceived and signalized to other parts of the plant. In this organ, there is an increase in osmolyte concentration to maintain water absorption as well as alterations in the energetic metabolism due to the additional energetic needs (123). On the other hand, the accumulation of flavonoids characterizes the drought-induced changes on the aerial organs of *P. pinaster* as these molecules function as key antioxidants during drought and light stress interaction (123,129).

1.7.3. Aim of this thesis

Although the morphological and molecular symptoms resulting from *F. circinatum* infection present similarities with those resulting from water stress, as far as has been ascertained, there has not yet been a direct comparison between *P. pinaster* plants exposed to drought stress and plants infected with *F. circinatum* in order to detect the similarities and divergences of the two conditions for the plant. Amaral et al. (83) proposed that the convergence between the two response mechanisms should be explored, as the direct role of the fungus in the drought-like symptoms and plant death cannot yet be ruled out. Thus, the aim of this work is to evaluate the similarities and divergences between the mechanisms of response to drought and *F. circinatum* in *P. pinaster*. The knowledge obtained in this work may contribute to the creation of the necessary foundations to be able to distinguish more quickly between drought and *F. circinatum* infection in precision forestry.

Chapter 2

Material and methods

2.1. Plant material

Nine-month-old *P. pinaster* plants (plant height 14.6 ± 0.2 cm) from the PNB-RP IV Ovar provenance were obtained from Melo & Cancela Lda. (Anadia, Portugal). Plants were placed in 300 cm^3 pots filled with a 3:2 (v/v) peat:perlite mixture and kept in a climate chamber (Fitoclima D1200, Aralab, Sintra, Portugal) under the following day/night conditions: 16/8h photoperiod, 25/20°C temperature, 60/65% relative humidity, and 500 μmol m^{-2} s^{-1} photon flux density. Plants were acclimatized for two weeks, watered daily to maintain 100% field capacity, and fertilized weekly (5 mL L^{-1}, Nutriquisa, Agrototal, Lisbon, Portugal).

2.2. Spore solution preparation

The FcCa6 *F. circinatum* isolate was obtained from the Forest Entomology and Pathology Lab at the University of Valladolid (130). It was grown on potato dextrose agar (PDA; VWR Chemicals, Leuven, Belgium) at 25°C. Five pieces of mycelium were grown under agitation on potato dextrose broth (PDB; VWR Chemicals, Leuven, Belgium) for 72h. Spores were counted using a hemocytometer and the concentration was adjusted at $1x10^6$ spores mL^{-1}.

2.3. Experimental design and sampling criteria

The experiment was run as a completely randomized design. After a 2-week-acclimatation period, the seedlings were divided into 2 treatments and respective controls with 20 plants each: mock-inoculated control (C), *F. circinatum*-inoculated plants (F), well-watered control (WW) and water-stressed plants (WS). To inoculate the F group, the stem surface was wounded using a sterile scalpel and 10 μL of the spore solution was placed in the wound, resulting in a total of 10^3 spores per plant. C group was equally wounded and charged with an equal volume of sterile PDB, to function as the control for F group. Wounds were sealed with Parafilm®. Simultaneously, the WS group was left without any kind of watering until the end of the experiment. All the other groups were maintained at the acclimatation period conditions without fertilization.

F and WS groups' visual symptoms were evaluated daily. The sampling criteria consisted of collecting the symptomatic plant and the respective control as it presented symptoms, until reaching 50% of collected plants in each group. Tip-wilting was the expected sampling-criteria symptom for both F and WS groups. All plants from the control groups, i.e., C and WW groups, were sampled with no symptoms.

2.4. Sample collection, morphological assessment, and Koch's postulates

At each sampling day, physiological measurements were performed on symptomatic plants and respective controls, and the plant material was harvested for further analysis. Physiological measurements comprised needle gas-exchange related parameters and water potential. Transversal stem sections at roughly 0.5 cm above the inoculation point were fixed in 70% ethanol to perform histological analysis of transversal stem sections. The collected fresh needles were frozen in liquid nitrogen and stored at −80°C for biochemical and hormonal analysis.

The plant's height increment was calculated through measuring the height of the plants in the beginning of the experiment and at the sampling day. The soil water content (SWC) of WS group

harvested plants was determined through a gravimetric method. The relative internal necrosis (RIN) of F group harvested plants was determined by measuring internal stem lesion length and calculated as a proportion of the total stem length. The longitudinal stem cuts images of all sampled plants were obtained using a zoom stereomicroscope (SMZ1500, Nikon Instruments Europe B.V., Amstelveen, Netherlands) coupled to a high-resolution digital microscope camera (DS-Fi1, Nikon Instruments Europe B.V.) and respective controller (DS-U3, Nikon Instruments Europe B.V.), and the image acquisition was performed using the NIS-Elements Documentation imaging software (v. 64bit 3.22.15, Nikon Instruments Europe B.V.). Koch's postulates were guaranteed by incubating stem cuttings in PDA.

2.5. Physiological parameters assessment

For needle gas exchange-related parameters, all plants that validated the sampling criteria and respective controls were measured using a gas exchange system (LCpro-SD, ADC BioScientific Limited, Hoddesdon, United Kingdom) coupled to a conifer-type chamber. Net CO_2 assimilation rate (A), sub-stomatal CO_2 concentration (Ci), stomatal conductance (gs), transpiration rate (E), intrinsic water-use efficiency (iWUE), and instantaneous water-use efficiency (WUE) were the needle gas exchange-related parameters recorded. Measurements were performed at 1500 μmol m^{-2} s^{-1} and 25°C. The photon flux density was obtained through a light response curve. Data were recorded when parameters remained stable, approximately six minutes.

Midday stem water potential (Ψmd) of all harvested plants was measured using a Scholander-type pressure chamber (PMS Instrument Co., Albany, OR, United States).

2.6. Histological stem section analysis

Histological analysis of transversal stem sections was performed according to Lopes et al. (131). Briefly, 70% ethanol-fixed transversal stem sections of three plants per treatment were embedded in paraffin and sectioned at 40 μm thickness with a sliding microtome (Jung AG Heidelberg, Germany). Next, 40-μm-thickness sections were cleared with Javel water, then neutralized in 1% acetic water (v/v) and washed with distilled water. For the histological staining, iodine green and alum carmine were used. Lastly, the stem sections were dehydrated using a sequence of ethanol-based solutions followed by 1:1 ethanol: Q Path® Safesolv (VWR International, Leuven, Belgium) solution (v/v) and 100% Q Path® Safesolv solution. The permanent preparations were generated by mounting the stem sections in glass microscope slides using Entellan™ new (Merck Millipore, Billerica, MA, USA).

The transversal stem section images were obtained using an optical microscope (ECLIPSE 80i, Nikon Instruments Europe B.V., Netherlands) coupled to a high-resolution digital microscope camera (DS-Ri1, Nikon Instruments Europe B.V., Netherlands) and respective controller (DS-U3, Nikon Instruments Europe B.V.), and the image acquisition was performed using the NIS-Elements Documentation imaging software (v. 64bit 3.22.15, Nikon Instruments Europe B.V.).

2.7. Biochemical analysis

Biochemical analysis was performed according to López-Hidalgo et al. (132). Briefly, the extraction process was performed by adding cold ethanol 80% (v/v) to 70 mg of homogenized material. The solution was then centrifuged for 10 min, at 10 000 g, and 4°C. The supernatant was used to quantify photosynthetic pigments (PHP), total soluble sugars content (TSS), total phenolic compounds (TPC), total flavonoids content (TFL), free amino acids content (FAA) and malondialdehyde (MDA), while the resulting pellet was hydrolyzed using perchloric acid to quantify starch content (STA).

To quantify PHP, the supernatant absorbance was read at 470, 649, and 664 nm and chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*), total chlorophyll content, ratio chlorophyll *a*/*b* and carotenoids values were calculated. Both TSS and STA were quantified using the anthrone reagent method with a D-glucose standard curve. TPC was analyzed using Folin-Ciocalteu reagent with a gallic acid standard curve, while TFL quantification was performed using the aluminum chloride method with quercetin standard curve. To measure FAA content, a ninhydrin reagent was used and a 1:1 L-proline and L-glycine standard curve was performed. The absorbance was read at 440 nm. The MDA quantification was carried out using the trichloroacetic acid/2-thiobarbituric acid test. The absorbances were read at 440, 532, and 600 nm and MDA levels were calculated. For each biochemical quantification, six biological replicates per group were used.

2.8. Hormone analysis

The hormone analysis was performed based on Sánchez-Bel et al. (133) and Pastor et al. (134) protocols with some modifications. The -80°C-stored material from six plants per treatment were freeze-dried, and approximately 25 mg of the resulting material were used to extract and quantify the hormones ABA, indol-3-acetic acid (IAA), SA and its non-active derivative salicylic acid glucoside (SAG), oxo-phytodienoic acid (ODPA), JA and its active form jasmonic acid-isoleucine (JA-Ile). The extraction was performed by adding 1 mL of extraction mixture to the biological material. The extraction mixture contained 10% MeOH in ultrapure water, stabilized with 0.01% formic acid and 100 ng mL^{-1} of an internal standard mixture with deuterated and dehydrogenated hormones. Subsequently, the samples were homogenized in a TissueLyser II (Qiagen) device for 30 s, at 30 Hz, using glass beads (2 mm ø), incubated on ice for 30 min and centrifuged 15 min, at 13 000 rpm and 4°C. The supernatant was collected, filtrated through 0.2 µm cellulose filters (Teknokroma) and diluted (1:3). The diluted samples were injected into an Acquity Ultra Performance Liquid Chromatography system (Waters, Mildford, MA, USA) carrying a Kinetex C18 analytical column (Phenomenex) and connected to a triple quadrupole mass spectrometer (Waters, Manchester, UK). The chromatography and mass spectrometry were executed based on Gamir et al. (135). For hormone quantification, external calibration curves were performed.

2.9. Data analysis

The significant differences among means between each treatment (F and WS) and the respective control (C and WW) were tested using two-sample t-test. The required assumptions were ensured using Shapiro-Wilk test to verify the normality of each group and F test to compare the homogeneity of two variances. Welch's two-sample t-test and Wilcoxon rank-sum test were used when data did not follow homoscedasticity or normality assumptions, respectively. Data was presented as mean ± SE (standard error). Outliers were removed using the interquartile range method.

The percentage of variation between the treatment and its respective control (Treatment-Induced Shift) was calculated using Rehschuh et al. (136) equation with Dorado et al. (137) modifications (2.1), where x is the individual value obtained in a particular treated plant for a given parameter and $mean_{control}$ is the mean of the respective control for the same parameter. The Treatment-Induced Shift was only calculated for the parameters whose treatments statistically differed from the respective controls. For the same parameter, F and WS Treatment-Induced Shift were compared using two-sample t-test.

$$\text{Treatment Induced Shift (\%)} = \frac{x - \text{mean}_{\text{Control}}}{\text{mean}_{\text{Control}}} \times 100 \quad (2.1)$$

Principal Component Analysis (PCA) was carried out using the R software package "FactoMineR" to identify variation patterns in all parameters. The data were centered and standardized using "missMDA" R software package to reduce scale effects and PCA biplot was carried out using the "factoextra" R software package.

For all statistical tests the significant p-value was < 0.05. All statistical analyses were performed using R software environment version 4.2.3 (R Foundation for Statistical Computing).

Chapter 3

Results

3.1. Symptoms evaluation and progression

F. circinatum-inoculated *P. pinaster* seedlings began to show symptoms 10 dpi and surpassed the 50% milestone two days later, whereas water-stressed *P. pinaster* seedlings took 17 days for the first symptomatic appearance and 21 days to reach 50% symptomatic plants (Figure 3.a). Every plant from F and WS groups showed the expected tip-wilting (Figure 3.b), taking no more than 25 and 39 days to show symptoms, respectively. Drought-like symptoms were not detected neither in the wounded control nor in the well-watered group, as expected (Figure 3.a, b). F group was the only group to show internal necrosis (Figure 3.c), with a RIN around 15.6 ± 6.3% and Koch's postulates validated the presence of *F. circinatum* only in this group. Harvested plants from WS group showed tip-wilting with a SWC around 11.7 ± 2.1%.

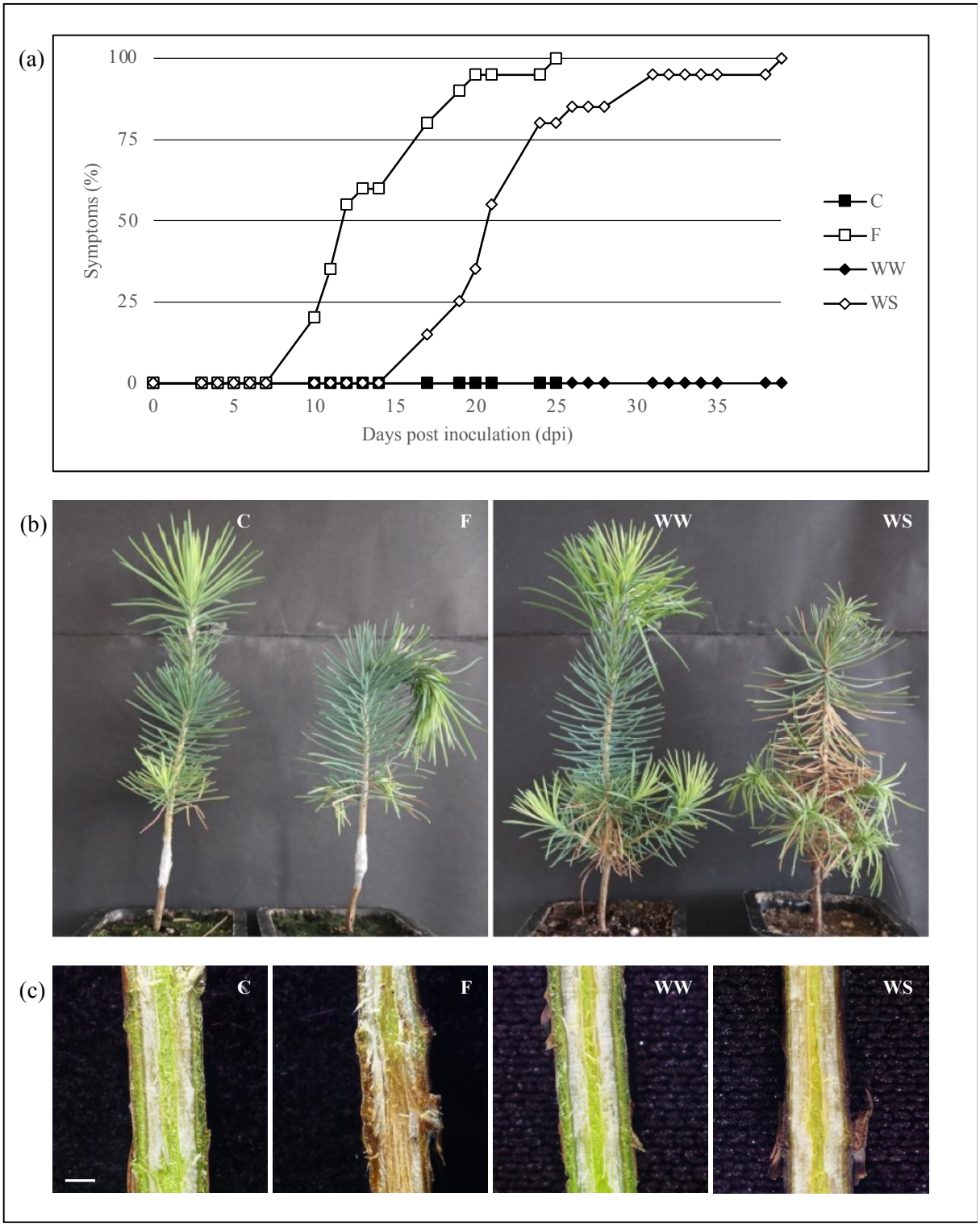

Figure 3.1 | Progression of drought-like symptoms in *P. pinaster*. (C) mock-inoculated control; (F) *F. circinatum*-inoculated plants; (WW) well-watered control; (WS) water-stressed plants. (a) Time course of the percentage of plants showing drought-like symptoms for each group. (b) Images representative of the symptoms for each group. (c) Representative longitudinal stem cuts for each group observed using a zoom stereomicroscope when stress treatments (F and WS) showed drought-like symptoms. Scale bar = 1 mm.

3.2. Histological analysis of *P. pinaster* transversal stem sections

The transversal stem sections allowed to see several differences between the *F. circinatum* inoculation and water deficit treatments. All the *F. circinatum*-inoculated plants assessed had tissue

breakdown in the pith and in the vascular cambium with a particular cortical parenchyma destruction. Also, it seems to have some resin ducts damaged (Figure 3.). No visible differences were observed between C, WW and WS groups.

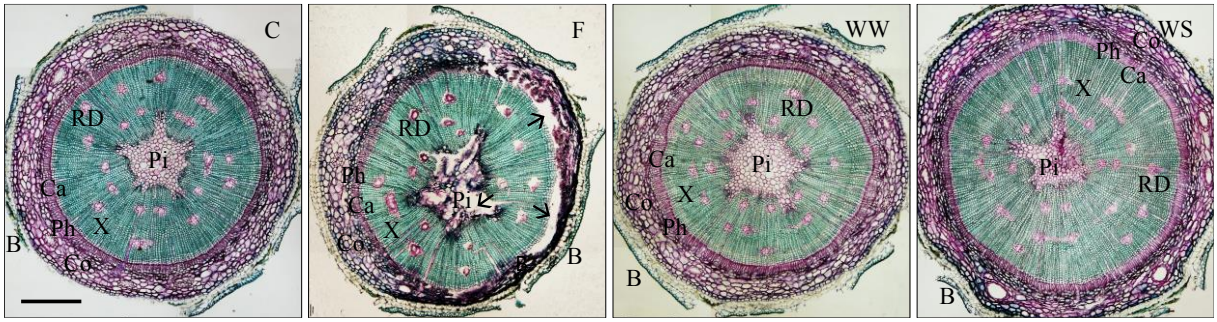

Figure 3.2 | *P. pinaster* transversal stem sections. (C) mock-inoculated control; (F) *F. circinatum*-inoculated plants; (WW) well-watered control; (WS) water-stressed plants. Stem structures are indicated by (B) bark, (Co) cortex, (Ph) phloem, (Ca) cambium, (X) xylem, (RD) resin duct, (Pi) pith. Arrows indicate tissue breakdown. Scale bar = 500 μm.

3.3. Morphological and physiological status of *P. pinaster* plants

At the drought-like symptoms appearance point, both *F. circinatum* infection and water deprivation resulted in a significatively lower height increment (Figure 3.a) and a significantly reduced Ψmd (Figure 3.b) comparing with the respective controls.

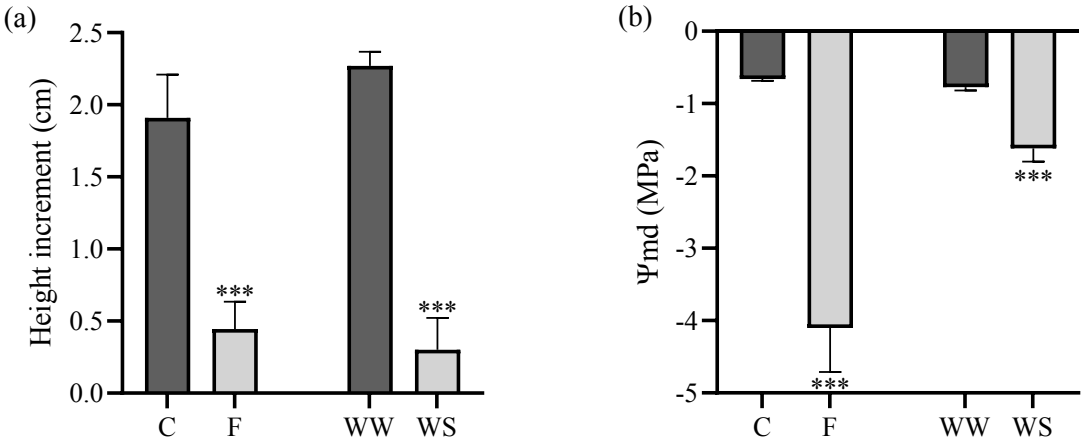

Figure 3.3 | Height increment (a) and midday water potential (b) at the day of drought-like symptoms appearance. (C) mock-inoculated control; (F) *F. circinatum*-inoculated plants; (WW) well-watered control; (WS) water-stressed plants. Data are presented as mean ± SE. Significance codes '*' ($P < 0.05$), '**' ($P < 0.01$), '***' ($P < 0.001$) on light grey bars indicate significant differences between each stress (F and WS) and the respective control (C and WW) while 'ns' is used for non-significant differences.

At the drought-like symptoms appearance point, *F. circinatum* infection and water stress seemed to generate the same changes in the needle gas exchange-related parameters (Figure 3.). Both *F. circinatum* infection and drought treatments result in a negative net CO_2 assimilation rate (Figure 3.a), a significant increase in sub-stomatal CO_2 concentration (Figure 3.b) and a significant decrease in stomatal conductance (Figure 3.c) and transpiration rate (Figure 3.d). Also, both intrinsic and instantaneous water-use efficiency (Figure 3.e, f) were negative at the drought-like symptoms appearance point.

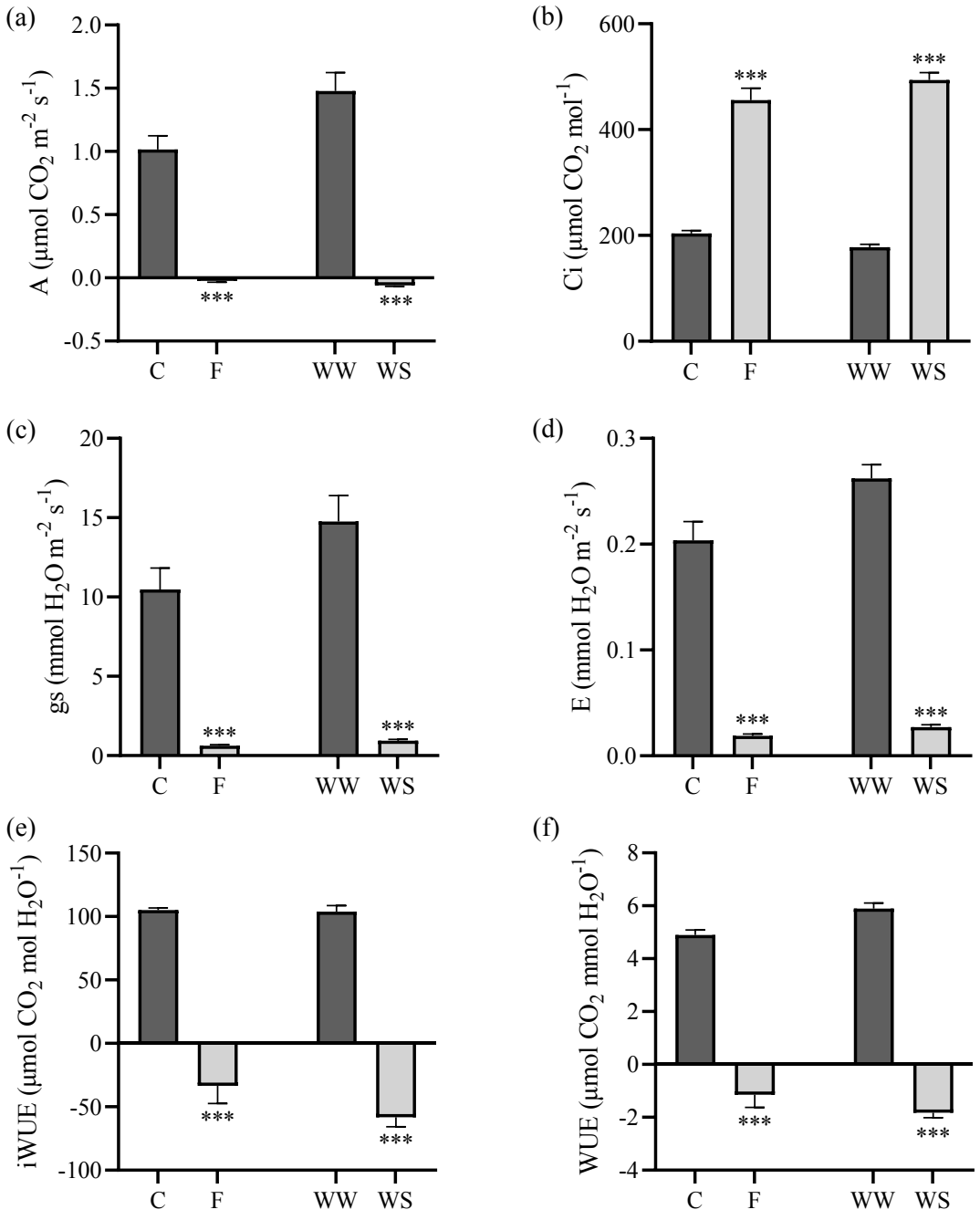

Figure 3.4 | Needle gas exchange-related parameters at the day of drought-like symptoms appearance. (a) Net CO_2 assimilation rate; (b) Sub-stomatal CO_2 concentration; (c) Stomatal conductance; (d) Transpiration rate; (e) Intrinsic water-use efficiency; (f) Instantaneous water-use efficiency. (C) mock-inoculated control; (F) *F. circinatum*-inoculated plants; (WW) well-watered control; (WS) water-stressed plants. Data are presented as mean ± SE. Significance codes '*' ($P < 0.05$), '**' ($P < 0.01$), '***' ($P < 0.001$) on light grey bars indicate significant differences between each stress (F and WS) and the respective control (C and WW) while 'ns' is used for non-significant differences.

3.4. Biochemical response of *P. pinaster* to *F. circinatum* infection and drought

Concerning the photosynthetic pigments, only the water stress treatment caused a significant increase in chlorophyll *a* (Figure 3.a) and chlorophyll *b* (Figure 3.b), and a significant increase in total chlorophyll (Figure 3.c), as a result. Neither *F. circinatum* infection nor drought seemed to change the

ratio between chlorophyll *a* and *b* (Figure 3.d). Both *F. circinatum*-infected and water-stressed plants had carotenoids content significantly increased (Figure 3.e).

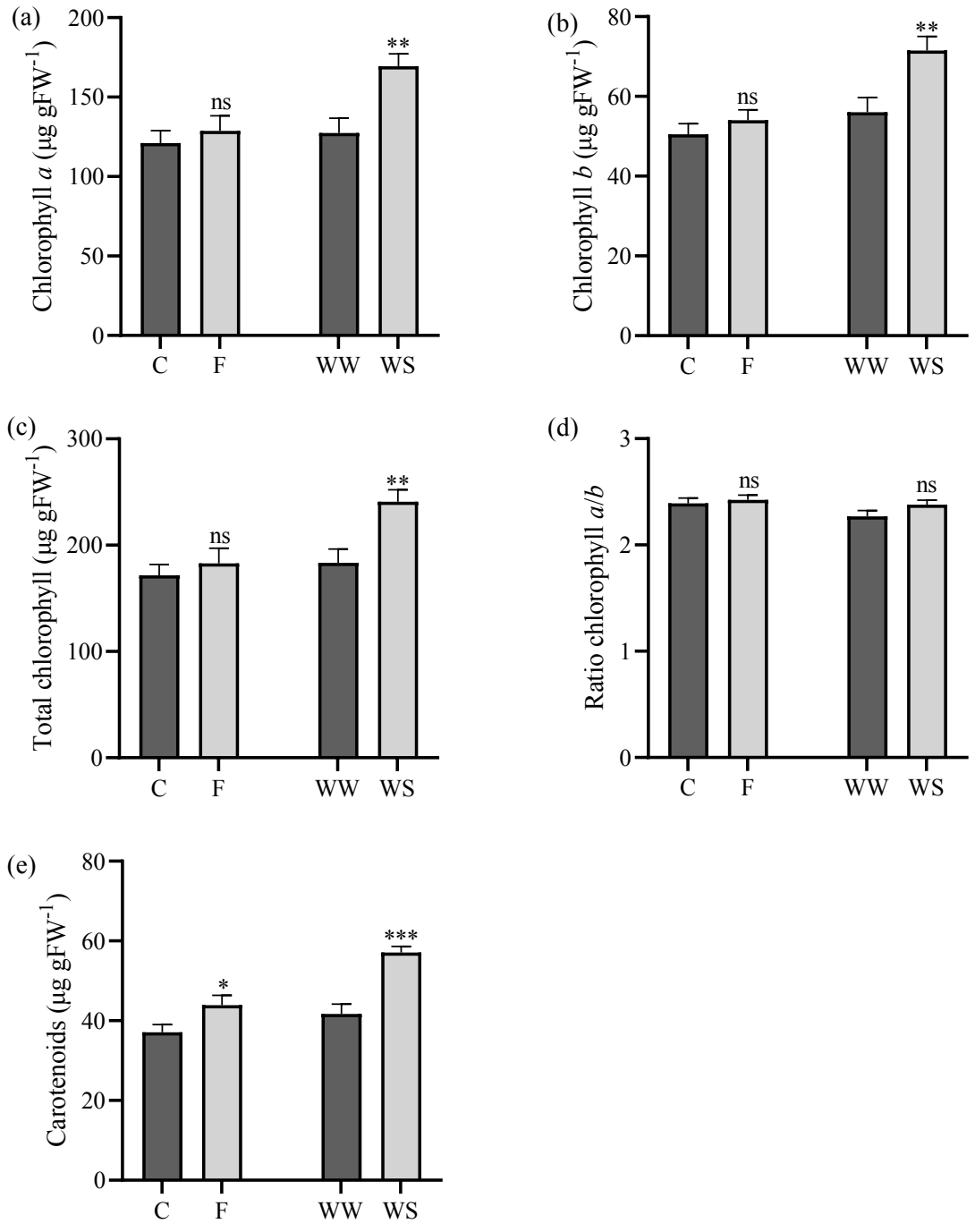

Figure 3.5 | Photosynthetic pigments (PHP) and related parameters at the day of drought-like symptoms appearance. (a) Chlorophyll *a*; (b) Chlorophyll *b*; (c) Total chlorophyll; (d) Ratio chlorophyll *a*/*b*; (e) Carotenoids. (C) mock-inoculated control; (F) *F. circinatum*-inoculated plants; (WW) well-watered control; (WS) water-stressed plants. Data are presented as mean ± SE. Significance codes '*' ($P < 0.05$), '**' ($P < 0.01$), '***' ($P < 0.001$) on light grey bars indicate significant differences between each stress (F and WS) and the respective control (C and WW) while 'ns' is used for non-significant differences.

The *F. circinatum* infection and the drought treatment significantly increased starch content (Figure 3.a), total soluble sugars (Figure 3.b), and free amino acids content (Figure 3.e). WS plants had total phenolic compounds (Figure 3.c) and total flavonoids contents (Figure 3.d) increased significantly

as well as MDA levels (Figure 3.f), whereas the F group did not show significative differences in these parameters.

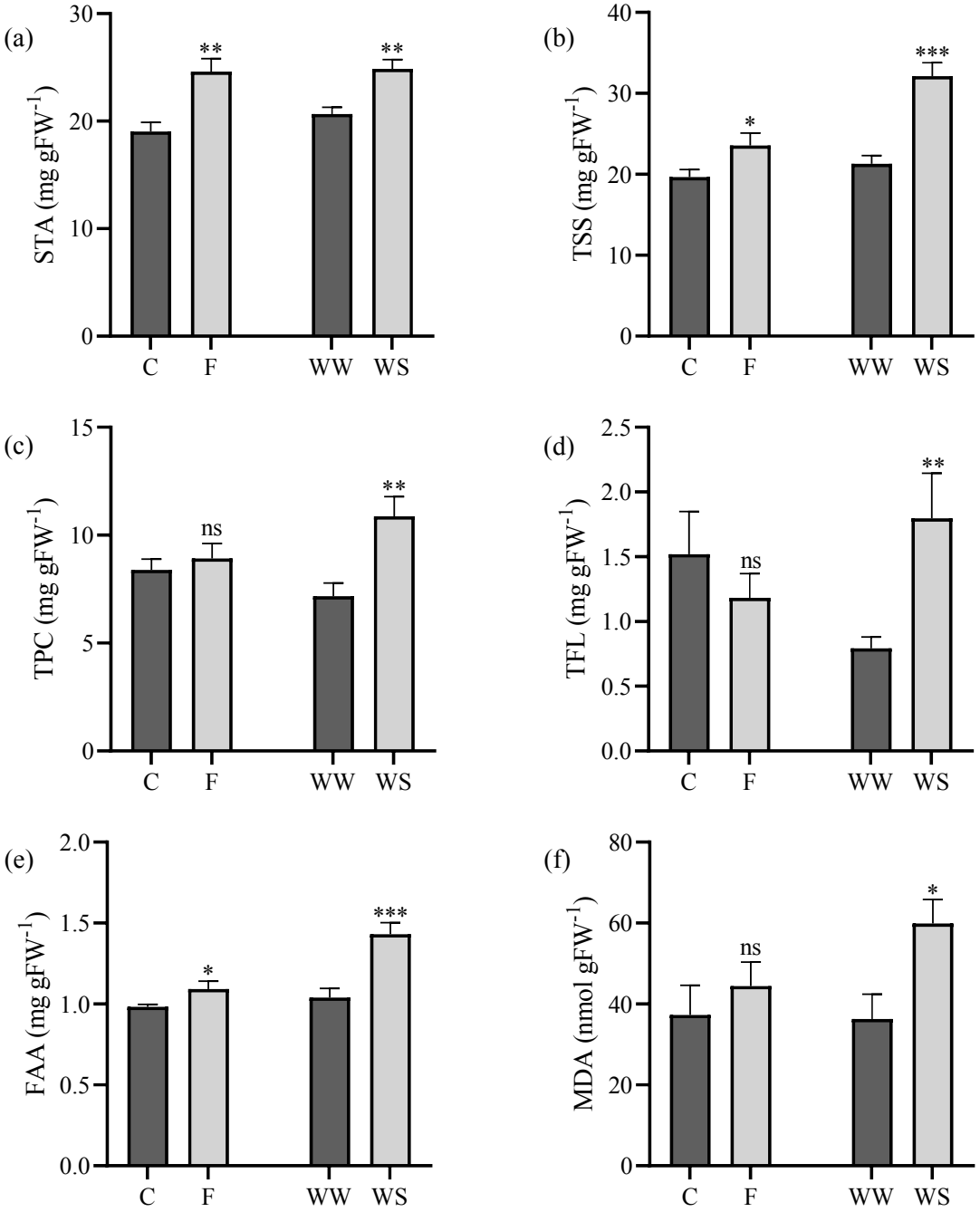

Figure 3.6 | Biochemical parameters at the day of drought-like symptoms appearance. (a) Starch content; (b) Total soluble sugars content; (c) Total phenolic compounds; (d) Total flavonoids content; (e) Free amino acids content; (f) Malondialdehyde. (C) mock-inoculated control; (F) *F. circinatum*-inoculated plants; (WW) well-watered control; (WS) water-stressed plants. Data are presented as mean ± SE. Significance codes '*' ($P < 0.05$), '**' ($P < 0.01$), '***' ($P < 0.001$) on light grey bars indicate significant differences between each stress (F and WS) and the respective control (C and WW) while 'ns' is used for non-significant differences.

3.5. *P. pinaster* hormonal profile in response to *F. circinatum* infection and drought

Concerning the hormonal profile, ABA significantly increased in both *F. circinatum* infection and the water stress treatment (Figure 3.a). No differences were reported in IAA, SAG, SA and OPDA (Figure 3.b, c, d, e). JA only increased significantly in the F group (Figure 3.f) and JA-Ile increased significantly in both treatments (Figure 3.g).

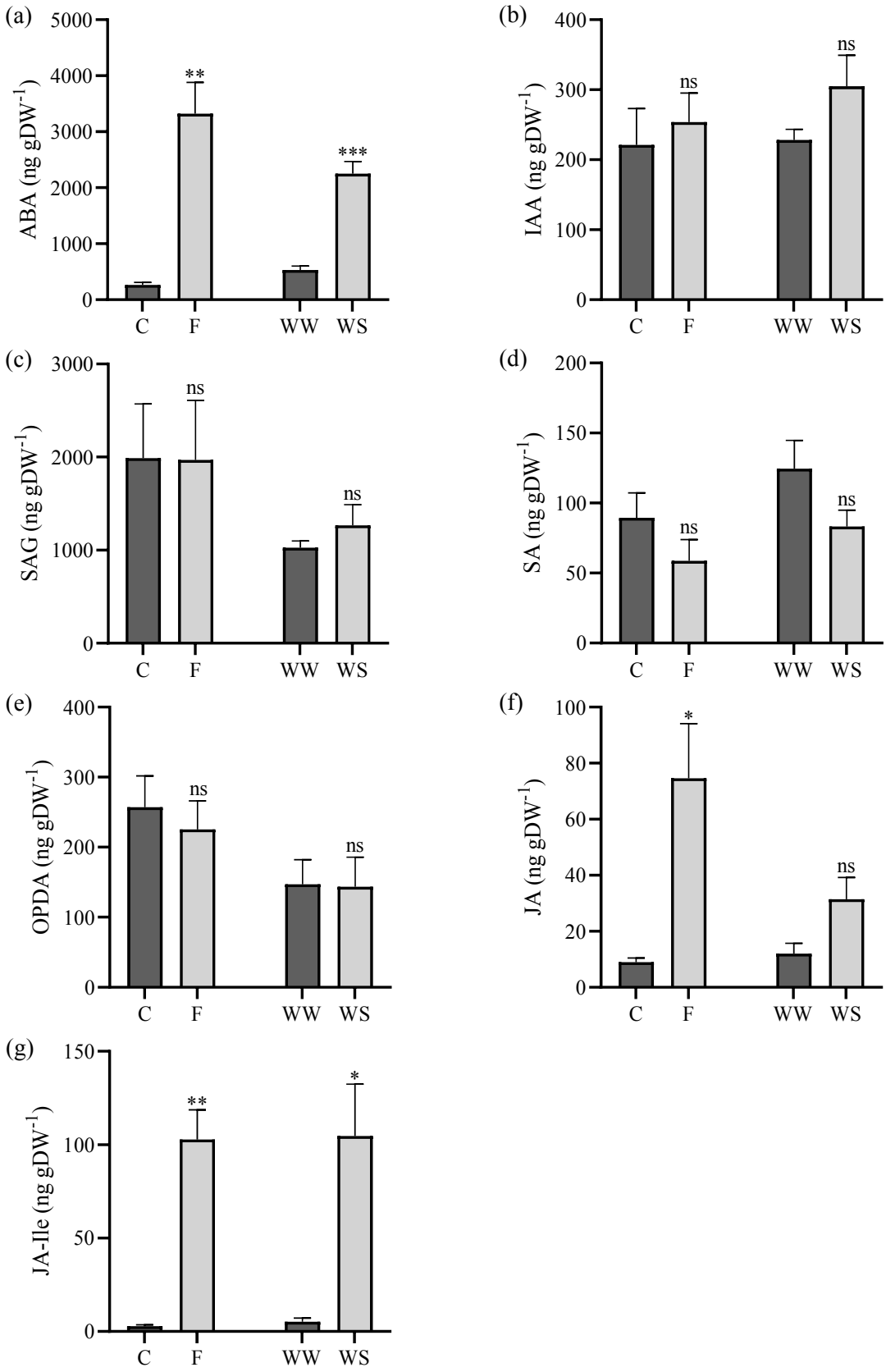

Figure 3.7 | Hormones content at the day of drought-like symptoms appearance. (a) Abscisic acid; (b) Indole-3-acetic acid; (c) Salicylic acid glucoside; (d) Salicylic acid; (e) Oxo-phytodienoic acid; (f) Jasmonic acid; (g) Jasmonic acid-isoleucine. (C) mock-inoculated control, (F) *F. circinatum*-inoculated plants, (WW) well-watered control, (WS) water-stressed plants. Data are presented as mean ± SE. Significance codes '*' ($P < 0.05$), '**' ($P < 0.01$), '***' ($P < 0.001$) on light grey bars indicate significant differences between each stress (F and WS) and the respective control (C and WW) while 'ns' is used for non-significant differences.

3.6. Differences between *F. circinatum* infection and drought in *P. pinaster*

Direct comparison between the Treatment-Induced Shift of both treatments, i.e., *F. circinatum* infection and drought, found no statistical differences in height increment, A, gs, E, iWUE, WUE, STA and JA-Ile. The increase in ABA and the decrease in Ψmd were more pronounced after *F. circinatum* infection than in the drought subjected pines. On the other hand, Ci, carotenoids, TSS and FAA presented greater shifts in the WS group in comparison to the F group (Table 3.1).

Table 3.1 | Treatment-Induced Shift. (Ψmd) Midday water potential; (A) Net CO_2 assimilation rate; (Ci) Sub-stomatal CO_2 concentration; (gs) Stomatal conductance; (E) Transpiration rate; (iWUE) Intrinsic water-use efficiency; (WUE) Instantaneous water-use efficiency; (STA) Starch content; (TSS) Total soluble sugars content; (TPC) Total phenolic compounds; (TFL) Total flavonoids content; (FAA) Free amino acids content; (MDA) Malondialdehyde; (ABA) Abscisic acid; (JA) Jasmonic acid; (JA-Ile) Jasmonic acid-isoleucine. Significance codes '*' ($P < 0.05$), '**' ($P < 0.01$), '***' ($P < 0.001$) indicate significant differences between the two Treatment-Induced Shifts while 'ns' is used for non-significant differences. The '–' is used when Treatment-Induced Shift is not applied.

Parameter	Treatment	Treatment-Induced Shift (%)	Significance	
Height increment	*F. circinatum* infection	-76.7 ± 9.9	P = 0.4759	ns
	Drought	-86.8 ± 9.8		
Ψmd	*F. circinatum* infection	522.1 ± 92.6	P = 0.0012	**
	Drought	109.8 ± 23.5		
A	*F. circinatum* infection	-102.3 ± 1.1	P = 0.2139	ns
	Drought	-104.0 ± 0.7		
Ci	*F. circinatum* infection	123.6 ± 11.0	P = 0.0009	***
	Drought	177.4 ± 8.1		
gs	*F. circinatum* infection	-94.0 ± 0.5	P = 0.6368	ns
	Drought	-93.6 ± 0.6		
E	*F. circinatum* infection	-90.8 ± 0.9	P = 0.3596	ns
	Drought	-89.6 ± 0.9		
iWUE	*F. circinatum* infection	-131.9 ± 13.3	P = 0.1397	ns
	Drought	-156.1 ± 7.3		
WUE	*F. circinatum* infection	-123.3 ± 9.9	P = 0.4775	ns
	Drought	-130.9 ± 3.2		
Chlorophyll *a*	*F. circinatum* infection	–	–	
	Drought	33.0 ± 6.1		
Chlorophyll *b*	*F. circinatum* infection	–	–	
	Drought	27.6 ± 6.3		
Total chlorophyll	*F. circinatum* infection	–	–	
	Drought	31.3 ± 6.1		
Carotenoids	*F. circinatum* infection	18.3 ± 6.6	P = 0.019	*
	Drought	36.9 ± 3.7		
STA	*F. circinatum* infection	29.3 ± 6.2	P = 0.2598	ns
	Drought	20.3 ± 4.2		
TSS	*F. circinatum* infection	19.9 ± 7.7	P = 0.011	*
	Drought	50.9 ± 7.8		
TPC	*F. circinatum* infection	–	–	
	Drought	51.5 ± 12.8		
TFL	*F. circinatum* infection	–	–	
	Drought	126.6 ± 43.7		
FAA	*F. circinatum* infection	11.3 ± 4.9	P = 0.0048	**
	Drought	37.7 ± 6.8		
MDA	*F. circinatum* infection	–	–	
	Drought	65,0 ± 16.3		
ABA	*F. circinatum* infection	1165.1 ± 212.4	P = 0.01	*
	Drought	323.2 ± 40.4		
JA	*F. circinatum* infection	736.9 ± 218.5	–	
	Drought	–		
JA-Ile	*F. circinatum* infection	3622.9 ± 572.4	P = 0.0586	ns
	Drought	1902.9 ± 530.4		

3.7. PCA overview

PCA shows clear differences between control and treated groups (Figure 3.). An overlap between WS and F group is evident. Nevertheless, the treated groups seem to separate in photosynthetic pigment content, secondary metabolites (TFL and TPC) and JA content. The two principal components explain 56.6% of the total variability.

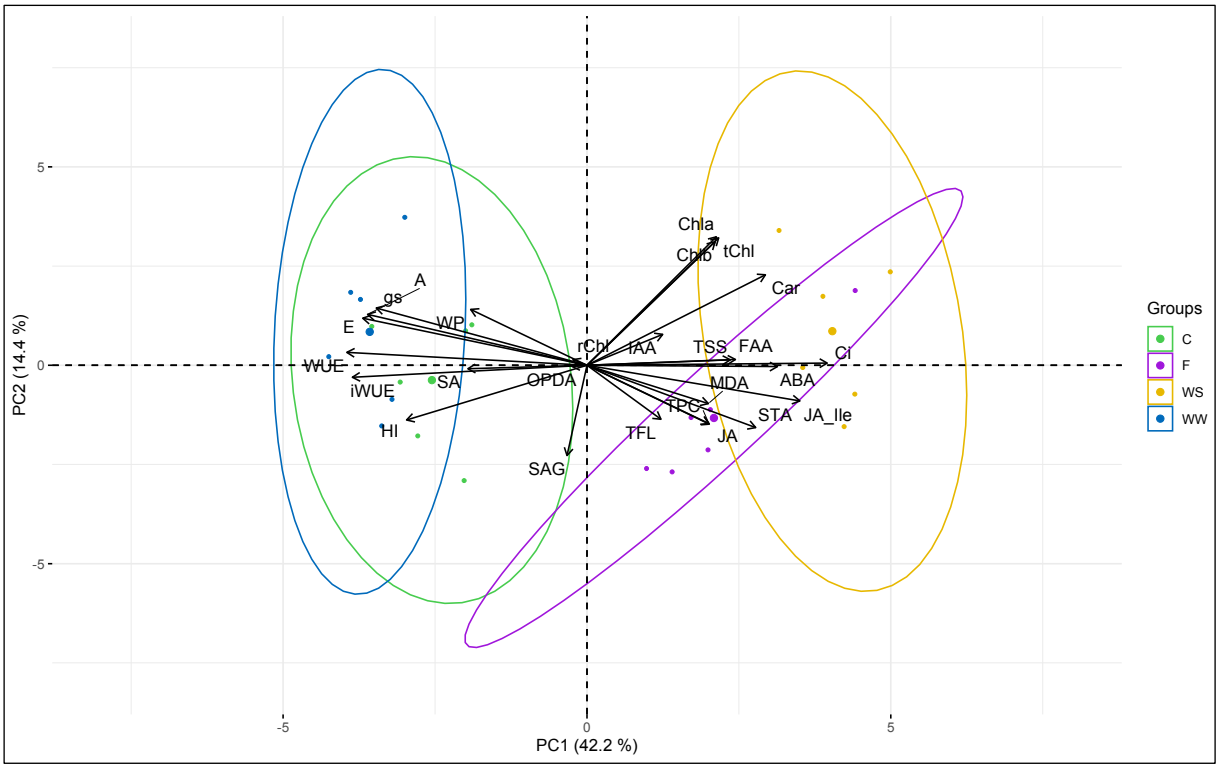

Figure 3.8 | PCA biplot. This plot shows the ordination of (C) mock-inoculated control, (F) *F. circinatum*-inoculated plants, (WW) well-watered control, (WS) water-stressed plants in the two first principal components. Explained variance by each axis is shown in parenthesis. (HI) Height increment; (WP) Midday water potential; (A) Net CO_2 assimilation rate; (Ci) Sub-stomatal CO_2 concentration; (gs) Stomatal conductance; (E) Transpiration rate; (iWUE) Intrinsic water-use efficiency; (WUE) Instantaneous water-use efficiency; (Chla) Chlorophyll *a*; (Chlb) Chlorophyll *b*; (tChl) Total chlorophyll; (rChl) Ratio chlorophyll *a*/*b*; (Car) Carotenoids; (STA) Starch content; (TSS) Total soluble sugars content; (TPC) Total phenolic compounds; (TFL) Total flavonoids content; (FAA) Free amino acids content; (MDA) Malondialdehyde; (ABA) Abscisic acid; (IAA) Indole-3-acetic acid; (SAG) Salicylic acid glucoside; (SA) Salicylic acid; (OPDA) Oxo-phytodienoic acid; (JA) Jasmonic acid; (JA_Ile) Jasmonic acid-isoleucine. Ellipse confidence level = 0.95.

Chapter 4

Discussion

As far as we know, this was the first time that *F. circinatum*-infected plants were directly compared with water-stressed plants aiming to discuss the similarities and divergences of the two conditions for the plant. The overall results indicate that the two treatments, although showing some similarities between them, generate slightly different metabolic shifts in *P. pinaster* plants.

4.1. *F. circinatum* infection leads to a severe drought scenario in *P. pinaster*

According to the current literature, *P. pinaster* is a drought-avoidant species (138) that exhibits isohydric behavior (20), since it maintains the midday water potential relatively constant, except in near death circumstances (139). This is achieved by reducing stomatal conductance through the increase of ABA concentration (140) to limit transpiration as water availability drops (21,141). The stomatal closure restricts CO_2 intake, leading to a decrease in sub-stomatal CO_2 concentration and, subsequently, a decline in the photosynthetic rate (138). As the stomatal conductance reduction is more accentuated than the decline in the photosynthetic rate, the iWUE typically increases (141).

In this study, the pines treated with both *F. circinatum* infection and drought presented similarities with the previously described *P. pinaster* isohydric response, at the appearance of the drought-like symptoms. The ABA content increased, as expected (142), and there was a decrease in stomatal conductance and transpiration rate. Nevertheless, not all our findings were in agreement with the typical isohydric response. Despite the decrease in the net CO_2 assimilation rate, the values were negative, which implies that the plant is releasing more CO_2 than it is assimilating (143). Furthermore, both treatments decreased water potential, increased sub-stomatal CO_2 concentration, and decreased both intrinsic and instantaneous WUE. Similar findings were reported in other studies with *F. circinatum*-infected *P. pinaster* plants (101,114).

As the sub-stomatal CO_2 concentration is greater in the treated plants but the net CO_2 assimilation rate is negative in those conditions, it is possible that a non-stomatal inhibition of photosynthesis is occurring (138,144,145). This non-stomatal inhibition is common as drought severity escalates, leading to the accumulation of CO_2 and a drop in iWUE (144,146), as observed in our study. In fact, Flexas and Medrano (145) have reported that although stomatal closure is the main photosynthesis limitation in moderate drought scenarios, the decrease in ribulose bisphosphate content becomes the greater limitation to photosynthesis during severe drought. Even though our study does not evaluate the non-stomatal photosynthesis limitations, it seems that both treatments caused a severe drought-like response, including the significant and apparently unexpected decrease in water potential in this isohydric plant, resembling a near death situation (139).

In the moment of the drought-like symptoms appearance, the plants subjected to both treatments presented similar water status, suggesting that the wilting of succulent tissues and tissue desiccation seen in *F. circinatum*-infected plants could be due to a *F. circinatum*-caused water deprivation. Martín-Rodrigues et al. (66) have previously proposed that the colonization of plant tissues by the fungus would lead to water collapse by tissue breakdown. In fact, cavities in stem tissues caused by *F. circinatum* colonization were also reported in our study, which could be the reason for the water potential decline observed in the *F. circinatum*-infected plants. Moreover, Martín et al. (147) reported that *F. circinatum* did not cause any destruction cavities in *P. pinea*, a resistant pine that typically does not present drought-like symptoms, which may support the hypothesis that those symptoms are directly caused by tissue breakdown.

In general, *P. pinaster* plants subjected to both treatments tended to adjust the water status-related parameters in similar ways. Nevertheless, there are clear differences on the severity of those adjustments between the two groups. *F. circinatum*-infected plants had a significantly more pronounced decrease in water potential as well as a higher increase in ABA content comparing with the drought-subjected plants, which suggests that *F. circinatum*-infected plants endured a worse water status in the upper inoculation-site tissues. In addition, Amaral et al. (89) reported that the needle gas exchange-related parameters of *P. radiata* plants infected with *F. circinatum* did not differ from control plants on the first days of infection but changed abruptly when the symptoms appeared. In contrast, drought-subjected pines would have most presumably activated responses against water stress, such as the decrease in stomatal conductance, as soon as the water availability dropped, as a consequence of *P. pinaster*'s isohydric behavior (20). The worse water status reported in our study coupled with the available bibliography seem to point towards the hypothesis that the fungus infection causes a more abrupt deterioration in the plant water status possibly due to the way it disrupts the water flux.

4.2. Changes in the carbohydrate metabolism suggest similar osmotic adjustment between both treatments and severe drought-caused phloem impairment

It seems that both treatments affect the carbon metabolism, since there is a significant increase in non-structural carbohydrates i.e., total soluble sugars and starch content, in the needles. In normal physiological conditions, most photosynthetic-derived carbohydrates are used for metabolism and structural biomass and only a small fraction is allocated to non-structural carbohydrates (148). Nevertheless, the non-structural carbohydrates concentration can increase during drought conditions because the plants growth rate declines faster than photosynthesis, increasing carbon storage (149). In fact, in both *F. circinatum* infection and drought subjected plants, it was registered a decrease in height increment.

The increase in total soluble sugars content observed in the needles of pines subjected to both treatments is likely due to their osmolytic properties, as a response to the water potential decline (112). The rise in free amino acids content under the same conditions may also reflect the need to maintain cellular turgor, because in the FAA quantification method, the absorbance was read at the maximum absorption wavelength of imidic acids, which comprise proline, a well-known osmolyte (132,150). In other studies, it was also reported an accumulation of fructose, glucose, and some amino acids, such as proline in *P. pinaster* infected with *F. circirnatum* (101,114). Despite similar shift in the concentration of these osmolytes in both imposed treatments, drought caused a greater increase in these molecules than the fungus infection. Once again, it may suggest that the water-stressed plants were able to adjust their metabolism as an attempt to maintain the water homeostasis while the *F. circinatum*-infected plants suffered an abrupt disruption in the water flux that could not be as well managed. As these molecules protect against water potential decline (112), their greater increase in drought subjected pines is in scope with the fact that in this group the water potential did not decrease as much as in the infected plants.

Unlike soluble sugars, starch is considered an inert molecule that functions as an energy reservoir and is typically degraded during mild drought (148,151). Nevertheless, there has been discovered plasticity in the starch metabolism which suggest that there may be many more unknown roles to this molecule (152). In our study, starch content increased in both treatments, despite the expected decrease. As starch is not transportable through the plant (153), the starch increase in the leaves must be a consequence of local polymerization, suggesting that starch is being actively synthesized in treated plants. Hartmann et al. (154) reported similar results in *Picea abies* exposed to severe drought, as starch and sucrose concentration increased in above-ground tissues. These authors suggested that the trees were not able to redistribute the available carbon from source to sink organs due to the collapse of

phloem functioning (154). In fact, during severe drought there is an increase in fluid viscosity that may block the phloem (155). This hypothesis is in accordance with our previous observations that *F. circinatum*-infected and drought-subjected plants have severe drought responses, even though from different origins.

4.3. *F. circinatum* infection and drought cause distinct shifts in secondary metabolism, photosynthetic pigments and redox homeostasis

The secondary metabolism was mainly impacted by water scarcity. The total phenolic compounds and total flavonoid content did not differ in *F. circinatum*-infected plants, but significantly increased in water-stressed *P. pinaster*. As these compounds act as antioxidants, mitigating damage from ROS (156), it is possible that they prevent further stress during drought (129). Their increase in the needles of *P. pinaster* during drought has been previously reported (123) as well as an upregulation of flavonoid and phenylpropanoid biosynthesis pathways related enzymes (157), hence their increase in drought conditions was expected. Although *F. circinatum* infection did not change these levels, it has been suggested before that the expression of certain phenolic compounds (100) and flavonoids (49,87) may be associated with pine resistance to the fungus. However, Leitão et al. (158) reported that neither *P. radiata* nor *P. pinea* changed the content in these metabolites after infection with *F. circinatum*, which questions the real role of these molecules in this pathosystem.

In addition, drought subjected plants had a higher increase in carotenoids than the group infected with *F. circinatum*. Carotenoids are terpenes involved in various processes including photosynthesis, photoprotection and ROS scavenging (159), that can increase as part of the plant's defense mechanism. Despite their increase in *F. circinatum* infected pines, contradictory results have been published showing no significant differences in carotenoid content in *P. pinaster* under the same conditions (114). In general, our results indicate that there are clear differences in the secondary metabolism response to both treatments since drought induced the production of more antioxidant molecules than the fungus infection that need to be more explored.

Moreover, each group of treated plants seem to have different oxidative stress status as indicated by the significantly higher concentration of MDA, a lipid peroxidation product (160), in drought subjected plants but unchanged levels in *F. circinatum*-inoculated pines. Other studies show variability in the oxidative damage that the fungus causes in the trees. In *P. pinaster*, it has been reported both an increase (114) and no differences (101) in electrolyte leakage while in *P. radiata* no differences in electrolyte leakage were observed but there was a decrease in MDA concentration (113). These findings together with the secondary metabolism results suggest that there is great variability in the oxidative status of *F. circinatum*-infected plants.

The content in chlorophylls only changed in drought-subjected plants, in which chlorophyll *a* and *b* as well as total chlorophyll content were significantly higher. Neither treatment affected the chlorophyll ratio indicating that both chlorophyll *a* and *b* must have been synthesized in the same proportion, if there was chlorophyll synthesis in the drought scenario. As water-stressed plants also increased carotenoids to a greater extent than the plants infected with *F. circinatum*, as mentioned above, it seems that drought had a larger influence on the photosynthetic pigments' concentration than the fungus. Nonetheless, the non-alteration of chlorophyll concentration in *F. circinatum* infected *P. pinaster* was not necessarily expected as different studies have reported an increase in total chlorophyll content under the same conditions (101,114), which suggests variability in this parameter.

4.4. Treatment-specific triggers lead to similar hormonal profile

P. pinaster plants infected with *F. circinatum* increased both JA and its active form, JA-Ile, but not their precursor, OPDA. These results were expected since JA and JA-Ile are important defense mechanisms against this pathogen (76) and there are reports of JA increase in *P. pinaster* infected with *F. circinatum* (101,114). It has also been reported that resistance to *F. circinatum* is associated with changes in auxin biosynthesis as well as SA activation (49,88), but in our study the biotic treatment did not induce significant differences in IAA, SAG, and SA levels. This may be due to *P. pinaster* not being a resistant species but a moderately susceptible pine (101). In fact, there seems to be some variation in SA adjustment during *F. circinatum* infection in *P. pinaster*. Similarly to our study, no alterations in SA content of infected *P. pinaster* have previously been reported (101). Nevertheless, increases in this hormone have also been detected (114). These observed differences may be due to *P. pinaster* genotypic variability leading to different timings in hormone action (87).

There were also changes in the hormonal profile of water-stressed plants. The increase in ABA content is a typical response to drought and has been previously analyzed on the 4.1. section. However, the raise in JA-Ile concentration has not yet been discussed. Despite similar to the increase observed in this hormone in *F. circinatum*-infected plants, the motive underlying this shift is most likely different. Jasmonates mediate several biological processes such as responses to biotic and abiotic stresses, including drought (161). In fact, it has been suggested that JA and JA-Ile may have an important role in tolerance to water stress since jasmonates seem to have ABA-like properties during drought (162). Moreover, the activation of genes involved in JA-related pathways during drought has already been reported in *Pinus* sp. (163). Thus, the increase in JA-Ile in drought subjected plants is most likely to be due to water scarcity while in *F. circinatum* infected plants it is possibly associated to a response to the fungus. Nevertheless, as *F. circinatum* abruptly disrupts the water flux, it is possible that the JA and JA-Ile accumulation continues as a response to the drought scenario that follows infection, hence new studies are needed to distinguish between biotic or abiotic induction of jasmonates.

Chapter 5

Conclusion

This was the first study to directly compare the response of *P. pinaster* to drought and to *F. circinatum* infection, in order to detect similarities and divergences between the two conditions for the plant. Both treatments caused a severe drought-like response since the pines seemed to undergo a non-stomatal photosynthesis impairment, a decrease in water potential and an accumulation of starch in the needles that may reflect phloem disruption. However, the fungus apparently disrupts the water allocation more abruptly than drought because *F. circinatum*-subjected *P. pinaster* presents a greater decrease in water potential and a less prolongated stomatal closure while drought-subjected pines present greater accumulation of osmolytes and antioxidant metabolites. This greater metabolic shift displayed in water-stressed plants suggests that they perceived the drought scenario earlier than the *F. circinatum*-infected plants. These results are in accordance with the idea that the drought-like symptoms that characterize PPC result from the fungus-caused tissue breakdown. This study emerged in a context of great scientific speculation about the possible connections between the two conditions, to cope with the lack of information about this topic. Even though it provided some insight, there were some limitations to our study such as the different sampling timepoints caused by the sampling criteria based on symptoms appearance and possible genotypic variation. After this study, some questions remain to be answered hence we suggest that the progression of the responses to both treatments should be detailed as well as the metabolic changes in the root given its major role during drought stress. Moreover, our study does not explain why there is a positive correlation between tolerance to *F. circinatum* infection and to drought, thus a study that evaluates the response of different characterized genotypes to both treatments should be done. Overall, this thesis sheds some light on the possible overlap between the response of *P. pinaster* to *F. circinatum* and to drought, therefore expanding the current knowledge on PPC disease.

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