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UNDERSTANDING THE ROLE OF METHYL JASMONATE AND ITS INHIBITORS ON PINE DEFENSE AGAINST THE PINEWOOD NEMATODE

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UNDERSTANDING THE ROLE OF METHYL JASMONATE AND ITS INHIBITORS ON PINE DEFENSE AGAINST THE PINEWOOD NEMATODE

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by

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Resumo

Nos últimos anos, a colonização de novas áreas florestais por espécies invasoras tem vindo a acontecer com maior frequência. Uma das maiores ameaças, trata-se do nemátode *Bursaphelenchus xylophilus,* comummente conhecido como o nemátode da madeira do pinheiro (NMP).

Avaliações preliminares demonstraram que a aplicação exógena de metil jasmonato (MeJa), um análogo sintético do ácido jasmónico (AJ), em plantas de pinheiro bravo (*Pinus pinaster*) pode induzir a alteração fenotípica desta espécie de uma condição altamente suscetível ao NMP para um fenótipo moderadamente tolerante. Assim, hipotetiza-se que o possível motivo para a elevada suscetibilidade de *P. pinaster* ao NMP passe pela ineficiente ativação da via do AJ ou outra via de defesa a ela associada.

Com o objetivo de testar esta hipótese, propuseram-se dois trabalhos experimentais: 1) compreender qual a influência da aplicação exógena de MeJa e de dois dos seus inibidores, o ácido dietilditiocarbamico (DIECA) e o n-propil galato (nPG) na produção de metabolitos de defesa (clorofilas, carotenoides, polifenóis, flavonoides, antocianinas, lenhina, saponinas, malondialdeído), e na expressão genética (*SAMS1*, *csAPX*, *ACO*, *PAL*) de plantas saudáveis de *P. pinaster* e *P. pinea*; 2) avaliar da capacidade de infeção do NMP em plantas de *Pinus spp*. com suscetibilidades distintas à doença, após o tratamento com MeJa, DIECA e nPG.

Verificou-se que, após tratamento com 50 mM de MeJa, não só a concentração em polifenóis solúveis foi mais elevada em folhas de *P. pinaster* (ca. 61%), como também a concentração em lenhina nas suas folhas, caules e raízes (pelo menos 50%), comparativamente com as plantas controlo (tratadas com água). Em *P. pinea,* o tratamento com MeJa promoveu um aumento da concentração em lenhina e saponinas de 70%, comparativamente com as plantas controlo. Pelo contrário, a aplicação de DIECA e nPG induziu uma menor acumulação de flavonóides em folhas de *P. pinaster* e em raízes de *P. pinea* (em pelo menos 20%), na concentração de antocianinas em folhas de *P. pinaster* (superior a 38%), e também na concentração em lenhina nas folhas de *P. pinea* (35%).

Independentemente do tratamento, a expressão relativa do gene aminociclopropano carboxilato (ACO) foi a mais afetada após o período experimental. O tratamento com MeJa promoveu um aumento da sua expressão em folhas de *P. pinaster* (2,4 vezes), e em folhas, caules e raízes de *P. pinea* (2,7, 1 e 1,5 vezes, respetivamente), enquanto que o nPG aumento a expressão da *ACO*, em caules e raízes de *P. pinea* (em 2,8 e 0,4 vezes, respetivamente).

Comparativamente a *P. pinea*, *P. pinaster* demonstrou uma maior suscetibilidade ao patogénio, com uma população de nemátodes de 164 ± 62 por grama de tecido de caule, sendo 585% superior a *P. pinea*. Os tratamentos com MeJa, DIECA e nPG não induziram nenhuma alteração significativa.

Este estudo permitiu demonstrar que a aplicação exógena de MeJa, DIECA e nPG pode vir a ser uma ferramenta útil na manipulação dos mecanismos de defesa das plantas, nomeadamente no que diz respeito à síntese de polifenóis e lenhina. Porém, não foi possível correlacionar uma maior tolerância ao NMP nas plantas de *P. pinaster* com a elicitação da via do AJ. Este trabalho vem ainda acrescentar novas informações no que diz respeito à influência da via do AJ na síntese de metabolitos de defesa e na expressão genética de genes relacionados com a defesa das plantas.

Abstract

In the past years, the emergence of novel invasive species has become one of the most important threats to forest ecosystems. One of the major pests that has been introduced in the European conifer forest is the pinewood nematode *Bursaphelenchus xylophilus* (NMP).

Recent studies have demonstrated that the jasmonic acid (JA) pathway seems to play a very important role in pine trees defence against *B. xylophilus*. In fact, it was suggested that exogenous application of methyl jasmonate (MeJa), a jasmonic acid (JA) analogue, to *P. pinaster* plants changes the plant phenotype from highly susceptible to *B. xylophilus* to moderately tolerant. Perhaps in this plant species the low tolerance to *B. xylophilus* may be due to an inefficient activation of the JA pathways.

To test this hypothesis, two experimental trial were designed: 1) understand how exogenous application of MeJa and its inhibitors diethyldithiocarbamic acid (DIECA) and *n*-propyl gallate (nPG) impacted the production of defence-related metabolites (chlorophylls, carotenoids, polyphenols, flavonoids, anthocyanins, lignin, saponins, malondialdehyde) and gene expression (*SAMS1*, *csAPX*, *ACO, PAL*), in healthy *P. pinaster* and *P. pinea* plants; 2) evaluate the performance of PWN in *Pinus spp.* plants with reported distinct susceptibility to the disease, after treatment with MeJa, DIECA or nPG.

In general, *P. pinaster* plants treated with 50 mM MeJa had higher total soluble polyphenols concentration in leaves (*ca*. 61%) and lignin content in leaves, stems and roots (by at least 50%), compared with control plants (treated with water). In *P. pinea* MeJa treated plants lignin concentration was also 70% higher than in control plants. Additionally, in this plant species saponins content was 67% higher in MeJa treated plants, compared with controls. Contrastingly, DIECA and nPG application decreased flavonoids concentration in *P. pinaster* leaves and *P. pinea* roots (by at least 20%), anthocyanins concentration in *P. pinaster* leaves (up to 38%) and lignin concentration in *P. pinea* leaves (by 35%).

The relative expression of aminocyclopropanecarboxylate gene (ACO) was the most affected after plant treatment. MeJa treatment increased *ACO* expression in *P. pinaster* leaves (by 2.4-fold) and *P. pinea* leaves, stems and root (by 2,7-, 1- and 1,5-fold, respectively), whereas nPG increased ACO relative expression in *P. pinea* stems and root (by 2.8- and 0.4-fold respectively).

Regarding the second part of this work, where the role of JA pathway in *P. pinaster* and *P. pinea*'s susceptibility to *B. xylophilus* was evaluated, *P. pinaster* showed higher susceptibility than *P. pinea* to the pathogen, attaining 164 ± 62 nematodes per gram of stem tissue at the end of experimental period, more 585% than *P. pinea*. Plant treatment with MeJa or its inhibitors did not induce significant alterations in nematode population during the experimental period.

These results show that exogenous application of MeJa, DIECA and nPG could allow the manipulation of plant defence mechanisms, namely regarding the synthesis of polyphenols and lignin. Nevertheless, it was not possible to evaluate if the JA pathway is the key regulator of *P. pinea* defence mechanisms against *B. xylophilus*. The present work still provides novel information on how JA pathway influences the synthesis of defence-related metabolites and gene expression in plants.

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List of abbreviations and symbols

APX - Ascorbate peroxidase

CAT - Catalase

DDT - Dichlorodiphenyltrichloroethane

DIECA - Diethyldithiocarbamic acid

DNA - Deoxyribonucleic Acid

DNB - Dothistroma needle blight

DPP - Dimethylally pyrophophat

EDTA - Ethylenediaminetetraacetic Acid

EPPO - European and Mediterranean Plant Protection Organization

GPX - Glutathione peroxidase

HR - Hypersensitive response

H_2O_2 - hydrogen peroxide

IPP - Isopentenyl pyrophosphate

JA – Jasmonic Acid

JMT - Jasmonate carboxyl methyltransferase

LOX - Lipoxygenase

MDA - Malondialdehyde

MeJA - Methyl jasmonate

nPG - n-propil galate

O_{2-} - superoxide

PAL - Phenylalanine ammonia-lyase

PWD - Pine Wood Disease

PWN - Pine Wood Nematode

RNA - Ribosomal Ribonucleic Acid

ROS - Reactive oxygen species

SA - Salicylic acid

SAR - Systemic acquired resistance

SOD - Superoxide dismutase

1. Introduction

1.1 European Forest

The forest sector has an important impact all over the world, not only because of its ecological importance, but also in local and global economy, with pine trees (*Pinus spp.*) being one of the major resources for timber industries worldwide (UN, 2011). Nowadays, European forests face several challenges, such as disforestation (Branco *et al.*, 2014; Fernandes, 2014), climate change, invasive species (Vicente *et al.*, 2012) and habitat fragmentation, which call for enhanced protection (European Commission, 2013).

In Europe, forest and wood land total area represents around 210.9 million hectares, which comprises 40% of the total European territory. From that area, only 32 million ha have been targeted as protected areas to landscape and biodiversity. Therefore, the development and implementation of novel forest management methodologies that allow forests to be healthier and less susceptive to invasive pests, without compromising their economic value, is of great importance (UN, 2011; European Commission, 2013).

In Portugal, Maritime pine (*Pinus pinaster*) forests are widely distributed throughout the country and represent a total area of 715 000 ha. This pine species has a very important impact in the Portuguese economy, having generated, in 2015, a turnover of 3 606 million euros, representing 37% of the exportations in the forest sector (Centro *Pinus*, 2016). In Portugal, past forest management policies have been the major driver for *P. pinaster* dispersion throughout the country, as it was frequently used in national reforestation practices and in coastal areas as physical containment for maritime dunes (Silva, 2007).

Stone pine (*P. pinea*), also has a very important impact in the Portuguese economy due to pine nuts production, especially in Setubal district, where 68% of the 78 000 hectares of Portuguese *P. pinea* area is located (OMAIAA, 2011). This activity is very profitable, as about 95% of production goes to exportation, with pine nut price on large retail outlets being marketed around 90 euros per kilo in 2014 (OMAIAA, 2011; Dores, 2014).

Impact minimization in these forest ecosystems is of great concern. Not only abiotic factors, such as fires and climate change, deserve our attention, but also biotic stresses, induced by several pathogens and herbivores that spread around the world due to ineffective pest management, and increasing resistance to pesticides.

1.2. Forest pests in European countries

European forests are currently under various threats. The biotic challenges that require more attention are the large pine weevil, pitch canker, the red band needle blight, and the pine wilt disease (PWD).

Hylobius abietis Linnaeus (1761), commonly known as the large pine weevil, develops in stumps and roots of dying and dead conifer trees, and when adult weevils emerge they feed on stems, from root collar upwards, which can result in complete girdling, causing plant death (Långström and Day, 2004; Dillon *et al*., 2006). Pine weevil attacks are closely related to the practice of clearfelling and planting, which is the reason why the problem becomes more evident when forest resources are exploited more intensively (Långström and Day, 2004).

Until the late 1970s, dichlorodiphenyltrichloroethane (DDT) was used extensively in European countries to control pine weevil damages. However, during the 1980s, with the prohibition of DDT use in forest management, permethrin, and later other synthetic pyrethroids, replaced the chlorinated hydrocarbons for *H. abietis* control (Petersson and Örlander, 2003; Långström and Day, 2004; Dillon *et al.*, 2006). Since then, the research for more effective treatment intensified and in, later years, Zas *et al.* (2014) reported that exogenous application of methyl jasmonate in nursery seedlings before planting was effective in reducing weevil damage in *P. radiata*, *P. pinaster*, and *P. sylvestris* under field conditions. Moreover, this protection was long lasting, at least up to two seasons after planting, having the potential to become an environmentally-friendly and cost-effective alternative to fight this forest threat.

Another important forest threat, *Fusarium spp.*, has been recognized as the causal agent of several diseases in coniferous trees, some of which have significant economic and ecological impact. Coniferous trees can suffer significant damage from seedling disease caused by *Fusarium* spp., and from pitch canker, in particular the one caused by *F. circinatum* Nirenberg & O´Donnell. From the many fungal species that can cause seedling disease, such as *F. commune* and *F. circinatum, F. oxysporum* is the species most commonly identified, and has been confirmed as the cause of damping-off as well as root- and hypocotyl-rot in pine trees. This fungus is especially problematic in bare-root nurseries and in container cultures (Dick and Simpson, 2003; Gordon, 2005). Dick and Simpson (2003), reported that, in *P. pinaster* nursery plants, *F. circinatum* causes 50% loss of seedling emergence, and in some cases 100% of seedling mortality was observed just 35 days after emergence.

Pitch canker, on the other hand, is caused by *F. circinatum*, and was first recognized as a disease in the United States of America in 1945 (Hepting and Roth, 1946). In Portugal, the

first records of the disease date to 2009 (Bragança *et al.*, 2009). In mature trees, the symptom most typically associated with the infection is the formation of large resinous cankers on main trunks and lateral branches, which lead to branch death (Gordon *et al*., 2015). As such, in 2002 the European and Mediterranean Plant Protection Organization (EPPO) included *F. circinatum* in the A2 Action List of Pests, (Gordon, *et al*., 2015; EPPO, 2017; Forestry Commission, 2017).

Although the application of methyl jasmonate was used against *Fusarium* spp. in tomato plants with some degree of success (Król *et al.*, 2015; Zehra *et al.*, 2017), efficient and sustainable control methodologies against the dispersion and damage of this disease are still to be developed.

Dothistroma needle blight (DNB) is caused by *Dothistroma septosporum* or *D. pini* and is a foliage disease particularly affecting *Pinus spp.* (Barnes *et al.*, 2004, 2016). The most characteristic symptom of DNB is the appearance of distinct red bands (1-9 mm wide) in plant leaves, that can appear within weeks after the infection. The red colour of the bands is due to the production, by the fungus, and accumulation, in the leaves, of the mycotoxin dothistromin (Brown *et al.*, 2003; Bradshaw, 2004; Schwelm and Bradshaw, 2010). The most serious impacts of DNB are growth retardation and, consequently, wood yield loss due to defoliation (Brown *et al.*, 2003; Bradshaw, 2004). Nowadays, the most commonly used control methods are chemical control by fungicide spraying and breeding of resistant hybrids. Cooper fungicides have been used to control DNB since the 1970s, owing their effectiveness to the prevention of spore germination and their popularity to its relatively low cost (Bradshaw, 2004).

Regardless the ecological importance of these forest pests, in Portugal, the most destructive pine tree disease is caused by *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) Nickle 1970, a facultative endoparasite microscopic nematode responsible for the pine wilt disease (PWD). This nematode is native to North America where it is not harmful to the autochthonous forest, but in the early 19th century it started its expansion to the Orient (Japan, Taiwan, China and Korea), where it became a threat to coniferous forests (Dwinell, 1997). The inefficient control of infected commercial wood trades has caused it to spread worldwide (Zhao *et al.*, 2008).

In Portugal, the PWD was first detected in 1999 (Mota *et al.*, 1999) in Setúbal, and in 2008 the entire continental Portugal was declared as PWD-infected. In 2010, the first case of infection was described in Madeira island (Fonseca *et al.*, 2012) and in 2011 the pathogen was reported in Spain (Abelleira *et al.*, 2015). PWD has become a major threat to European forests, with an estimated mortality risk of 50% of *P. pinaster* plants (Zhao *et al.*, 2008).

The pinewood nematode (PWN) life cycle and regional dissemination is closely related to an insect vector, the longhorn beetle *Monochamus galloprovincialis* (Sousa *et al.*, 2001, 2002). In Portugal, before the arrival of the PWN, *M. galloprovincialis* had a stable interaction with pine trees, feeding on branches and colonizing the decadent plants (Berryman, 1986; Cabral, 1995). After the introduction of the PWN, *M. galloprovincialis* has become the primary cause of disease dissemination. As the PWN is present on the trachea of the insect vector, transmission occurs directly from de insect to a healthy host (Begon *et al.* 1986) through the feeding wounds made in young branches as the insect feeds (Naves *et al.*, 2007). A secondary way of PWD dissemination is associated with insect vector oviposition in infested dead trees, in which *B. xylophilus* feeds on the fungi that colonize the decadent trees (Oh *et al.*, 2009). When the insect larvae emerge from the eggs, they are infected by the entry of nematodes through the spiracle, being able to transmit the pathogen as they feed on healthy trees (Wingfield, 1983).

Once inside the host, PWN feeds on parenchymal cells (Jones *et al.*, 2008), moving through xylem and cortex resin canals and through the cambium cells, where it nourishes and reproduces (Ichihara *et al.*, 2000). This process blocks the vascular system of the infected plant due to the appearance of secondary resin as a result of the damage induced by the pathogen to the radial parenchyma cells. Moreover, water transport to the shoots is negatively affected due to cavitation phenomena (Jones *et al.*, 2008), which leads to rapid leaf discoloration. The appearance of wilting symptoms suggests a successful nematode infection, but symptom severity depends on host species and the season in which infection occurs. During the summer, there is a rapid progression of symptoms, and the temporal space between infection and tree death is quick, whereas in winter symptoms can be omitted for a long period of time, due to the decreased activity of the pathogen at lower temperatures (Kiyohara and Tokushige, 1971; Jones *et al.*, 2008). Therefore, the predicted future climate changes, such as water shortage and higher temperatures, increase the concerns about this disease worldwide (Mota and Vieira, 2008).

In Portugal, the most susceptible host to PWN is *P. pinaster*, which represents 23 % of the national forest territory (ICNF, 2013), whereas in *P. pinea*, also very prevalent in national territory, the disease develops more slowly (Mota and Vieira, 2008; Nunes da Silva *et al.*, 2015). Until now, the efforts to control the disease have taken a large amount of European and National funding. Between 2007 and 2012, over than 32 million euros were spend by the Permanent Forest Fund in order to support control, eradication and research programs concerning the PWN (Tribunal de Contas, 2016).

So far, no effective and sustainable control methods are available and these and many other diseases remain uncontrolled, posing a great threat to forest ecosystems. Most of the currently employed control strategies rely on insecticides and other toxic and non-ecological substances, which, apart from being expensive, represent a threat to the environment. Therefore, it is imperative to keep the scientific effort focused on the research of new alternative methods, with high efficiency and less impact on the forest ecosystem, to control these pests and improve forests health and productivity.

In European countries chemical control is currently being discouraged, and the rules have become tightened over the years for forest management practices. The implementation of the Forest Stewardship Council (FSC) certification to forest products led to the non-renewal of pesticide licenses in several cases (Långström and Day, 2004). Consequently, the identification of cost-effective and environmentally friendly methods to minimize the use of noxious chemicals is greatly encouraged. However, before new control strategies can be devised, it is important to understand the defence mechanisms being triggered by the host plant after infection, and how the potential biocontrol strategies modulate those mechanisms.

1.3. Plant defence signalling

Since plants cannot move to escape environmental challengers, they have evolved sophisticated mechanisms to perceive biotic attacks and abiotic stresses. To protect themselves, plants take advantage of a vast array of defence mechanisms to effectively detect invading organisms and stop them before they are able to cause extensive damage: constitutive and induced defences. Pre-existing defence barriers, known as constitutive defences, include cell wall, waxy epidermal cuticles and bark, whereas inducible defences, which are activated after the detection of an invading pathogens, involve the production of toxic chemicals, pathogen-degrading enzymes, and programmed cell apoptosis (Gershenzon, 1998; Desikan *et al.*, 2005).

1.3.1. Antioxidant enzymes

After infection by a pathogen, plants deploy a broad spectrum of defences against the invaders. One of the most rapid defence reactions is the production of reactive oxygen species (ROS) via oxidase enzymes, in a process called oxidative burst (Apostol *et al.*, 1989; Baker and

Orlandi, 1995). Immediately after infection, ROS play a role in initiating the hypersensitive response (HR), which is a form of deliberated plant cell apoptosis surrounding the infection site, in order to suppress pathogen invasion (Desikan, Hancock and Neill, 2005). The most common ROS are superoxide (O_2^-) and hydrogen peroxide (H_2O_2), but also perhydroxyl radical (the protonated form of superoxide) and the hydroxyl radical (the most reactive one). As these molecules are toxic to plant cells, to deal with the oxidative damage cells have developed ROS scavenging mechanisms, which is an enzymatic process performed by superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). SOD is known as the first line of defence against oxidative damages, dismutating O_2^- to H_2O_2. Subsequently, APX, GPX and CAT are responsible to detoxify plant cells from H_2O_2, converting it to water and oxygen (Mittler *et al.*, 2004).

Peroxidation of the unsaturated lipids that support plant cells membranes is one of the noxious consequences of ROS accumulation, and is considered a biochemical indicator of oxidative damage (Apel and Hirt, 2004). Malondialdehyde (MDA), in particular, is a secondary product of this process and seems to increase as a result of cell necrosis, being widely used as an assessment of plant fitness (Yamada, 2008; Nunes da Silva *et al.*, 2015).

1.3.2. Secondary metabolites

A large variety of low molecular weight compounds are produced by plants. They are usually up-regulated by different pathways and are divided into two main groups: primary metabolites and secondary metabolites.

Primary metabolites, such as chlorophylls, amino acids, simple carbohydrates or membrane lipids, are substances produced by all plant cells and are essential for plant growth and development. On the other hand, secondary metabolites play critical roles in plant interaction with the environment, namely in plant protection against herbivorous and infections by pathogens, in plant-plant competition and as attractants for pollinator and seed-dispersing animals (Kieber, 1998). Secondary metabolites can be divided in three major chemical groups: nitrogen-containing secondary products, terpenes and phenolic compounds (Gershenzon, 1998; Olivoto *et al.*, 2017). The most abundant nitrogen compounds in plant tissues are alkaloids, glycosides and non-protein amino acids, which have a vast array of functions, ranging from plant development, defence and regulation (Gershenzon, 1998; Olivoto *et al.*, 2017). In general,

they all act as protective substances due to their toxicity being, therefore, important chemical barriers against feeding insects and herbivorous.

Terpenes, also known as isoprenoids, are the largest class of secondary metabolites and are synthesized from primary metabolites by two different pathways: the mevalonic acid pathway, which uses molecules of acetyl CoA to produce isopentenyl pyrophosphate (IPP), and the non-mevalonic acid pathway, where IPP can be formed from intermediates of glycolysis or from the photosynthetic carbon reduction cycle (Lichtenthaler *et al.*, 1997). IPP and its isomer, dimethylallyl pyrophosphate (DPP) are the basic units of terpene biosynthesis (Gershenzon, 1998). In plants, they can be found mixed with volatile compounds, conferring them specific odours, which act as between-plants communication signals or play important functions in plant growth and development, being involved in gibberellin biosynthesis, for example (Olivoto *et al.*, 2017). Terpenes also have vital roles in plant defence against herbivorous, such as insects (Veitch, Boyer and Ley, 2008) and nematodes (Soriano *et al.*, 2004). In some conifer species, the exudation of terpene-rich resin seems to be triggered as a defence mechanism against fungi and insects (Franceschi *et al.*, 2005; Olivoto *et al.*, 2017).

Phenolic compounds (PC) are characterized by a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group, and are subdivided into five groups: coumarins, lignins, flavonoids, phenolic acids and tannins. Lignin, in particular, is a very important compound in plant cells, providing cell wall rigidity and acting as a barrier to many aggressions (Gershenzon, 1998). Most of these PCs derive from phenylalanine, a product of the shikimic acid pathway, which is converted into cinnamic acid by phenylalanine ammonia-lyase (PAL) (Hahlbrock and Scheel, 1989; Heredia and Cisneros-Zevallos, 2009; De Jong *et al.*, 2015).

The activation of induced plant defences is mediated by a complex signalling network in which the plant hormones jasmonic acid (JA), salicylic acid (SA) and ethylene play a key role (Pieterse*et al.*, 2001). However, evidences show that compounds from SA, JA and Ethylene-dependent defence pathways can affect each other's signalling, either positively (synergetic) or negatively (antagonisticaly). They can act locally and systemically, providing an optimal defence against the invader due to is regulatory potential to activate multiple resistance mechanisms in varying combinations. This cross-talk can help the plant to prioritize the activation of one particular pathway over another (Farmer and Ryan, 1992; Pieterse *et al.*, 2001). The JA pathway is generally associated with responses against herbivorous feeding through the regulation of secondary metabolites that dissuade feeding or inhibit digestion, and with plant volatiles induction that can repel herbivorous or attract their natural enemies (Smith *et al.*, 2009). Contrastingly, SA mediated pathway is normally activated in response to

pathogens, triggering a hypersensitive response (HR) against the invader and conferring systemic acquired resistance (SAR).

1.3.3. Ethylene pathway

Ethylene is a plant hormone involved in a large number of developmental processes (Ecker, 1995). It is produced by almost all higher plants, although with different production rates depending on the type of tissue and developmental stage. It is present during seed germination, root hair development and nodulation, flower senescence, leaf abscission, as well as fruit ripening. In addition, plant damage resulting from wounding (e.g. pathogen attack) or physiological stress (e.g. chilling, flooding, disease, temperature or drought stress) can induce ethylene biosynthesis (Johnson and Ecker, 1998; Kieber, 1998). In 1984, Yang and Hoffman established that ethylene is synthesized from carbons C-3 and C-4 of methionine through a series of reactions, in which the first step is the conversion of S-adenosyl-L methionine (AdoMet) to 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is the immediate precursor of ethylene that can suffer two fates: inactivation by conjugation to form malonyl- or glutamyl-ACC, or oxidation to produce the bioactive hormone. The function of ethylene in plant resistance seems to be more ambiguous than SA or JA.

1.3.4. Salicylate-Mediated Responses

SA plays an important role in many aspects of plant development and growth, as well as in thermogenesis and disease resistance. So far, two different SA biosynthetic pathways have been recognised; the isochorismate (IC) pathway and the phenylalanine ammonia-lyase (PAL) pathway, which have the same precursor, chorismate (Dempsey *et al.*, 2011; Dempsey and Klessig, 2017).

SA is widely known to be activated almost exclusively in response to pathogen attack. It mediates the initiation of the HR, which is characterized by the production of phytoalexins and pathogen-related proteins that confer systemic acquired resistance (SAR). It is generally acknowledged that SAR induction mechanisms occur during the first days after infection and that if the plant is able to survive the attack a long-term immunity to subsequent attacks is established. SAR seems to result from increased levels of certain defence compounds, including chitinases and other hydrolytic enzymes (Pieterse *et al.*, 2001; Smith *et al.*, 2009).

1.3.5. Jasmonate-Mediated Responses

Jasmonates, including JA and its derivates, are oxylipins or oxygenated fatty acids produced in the octadecanoid pathway (Fig. 1), which has linolenic acid as precursor (Cheong and Choi, 2003; Smith*et al.*, 2009).

The free-acid JA and its methylated form methyl jasmonate (MeJa) are important signalling molecules in plants, activating the production of secondary metabolites and inducing the synthesis of plant volatiles (Wasternack and Parthier, 1997; Smith*et al.*, 2009). Jasmonates are also involved in several developmental processes like fruit ripening, senescence, reproduction (production of viable pollen, seed germination, flower and fruit development), photosynthesis and root grown, and in defence responses to wounding, especially against chewing insects and necrotrophic pathogens (Turner*et al.*, 2002; Devoto and Turner, 2003; Smith*et al.*, 2009). Notwithstanding their recognized importance in plant physiology, the understanding of jasmonates signalling is complicated because the responses mediated by them can be triggered by a series of diverse environmental or developmental signals, and by elicitor molecules that interact between them (Devoto and Turner, 2003).

MeJa is a plant volatile that accumulates in plant cells when JA is methylated by JA carboxyl methyltransferase (JMT) (Fig. 1) (Devoto and Turner, 2003), and has become the stronger candidate to act in response against wounding or pathogen attack. In fact, in recent decades exogenous application of MeJa has been used as a plant chemical elicitation strategy to induce chemical defensive responses and to improve resistance against pests (Seo *et al.*, 2001; Moreira *et al.*, 2009; Nunes da Silva *et al.*, 2013).

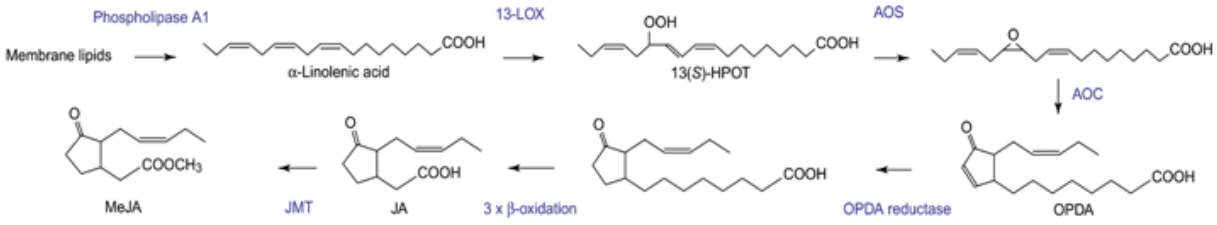

Figure 1 - Main pathway of jasmonate biosynthesis. A phospholipase A1 releases α-linolenic acid from membrane lipids. The α-linolenic acid is oxygenated by lipoxygenase (LOX) to 13(S)-hydroxy linolenic acid (13-HPOT), which is then convert to 12-oxo-phytodienoic acid (OPDA) by allene oxide synthetase (AOS) and allene oxide cyclase (AOC). Jasmonic acid (JA) is synthetized from OPDA through reduction and three steps of β-oxidation, and is further converted to methyl jasmonate (MeJA) by JA carboxyl methyltransferase (JMT). *Adapted from*: Cheong and Choi (2003).

1.4. The role of jasmonic acid pathway in plant defense

Chemical information plays an important role in plant-insect or plant-pathogen interaction. When a plant is damaged, a specific volatile or toxin can be produced to fight the aggression. In fact, recent finding show that jasmonates mediate the induction of volatile emission, increase toxin levels and upregulate defense-related gene expression, thus enhancing plant defense mechanisms against pathogens or herbivores (Bruinsma *et al.*, 2010). Therefore, elicitors and inhibitors of the JA pathway may be useful to modulate plant response against pathogens and herbivores and increase their resistance against certain diseases. The role of JA pathway in plant defence against important pest has been, in fact, under the attention of the scientific community. In the past decade, several works were developed in order to understand how inducing or inhibiting JA pathway interfered with plants defence ability against important pests.

MeJa exogenous applications by leaf spraying, for example, induces the same responses as insect wounds or pest attacks, probably because it is able to act as an intra-cellular regulator and signal transducer, mediating intra and interplant communications (Farmer and Ryan, 1992; Seo *et al.*, 2001). Moreira *et al.*, (2009), performed MeJa applications in one-year old *P. pinaster* plants and observed that after the experimental period the pine weevil *H. abietis* consumed 80% less phloem in treated plants compared to control, probably because MeJa application induced chemical defensive responses. In a similar manner, MeJa foliar spraying of *P. radiata* seedlings induced an increase in plant resistance against *Sphaeropsis sapinea* (Gould *et al.*, 2008).

Nunes da Silva *et al.* (2013) also evaluated how MeJa treated *P. pinaster* and *P. radiata* plants performed after artificial inoculation with PWN and observed a significative reduction in nematode population, suggesting that MeJa impairs PWN reproduction rate or enhances plant defences against the pathogen. Kepczyńska and Król (2012) demonstrated that fumigation of tomato plants with MeJa was efficient in inducing resistance against *Alternaria porri* f. sp. *solani*. A similar effect was reported by Seldal *et al.* (2017) that showed that among distinct treatments (physical and chemical) aimed to induce blueberry plants defence responses under natural field conditions, MeJa treatment produced better results than physical treatment, due to higher elicitation of plants defence mechanism.

Nevertheless, and in spite the promising results in the induction of plant defences, it is of great importance to choose the appropriate MeJa concentrations for exogenous foliar applications. Reports from recent studies showed that some species are less tolerant to higher

MeJa concentrations (Solla *et al.*, unpublished results; Nunes da Silva *et al.*, 2010; Zas *et al.*, 2014). Even so, MeJa appears to have a great potential as a biological plant helper against biotic stress (Miller *et al.*, 2005; Yu *et al.*, 2011; Nunes da Silva *et al.*, 2013; Zas *et al.*, 2014).

On the other hand, several compounds could interfere with the octadecanoid pathway blocking the production of JA by repressing the process at different steps. Two of these inhibitors are diethyldithiocarbamic acid (DIECA) and *n*-propyl gallate (nPG) (Bruinsma *et al.*, 2010; Cooper and Rieske, 2011; Rajendran *et al.*, 2014).

DIECA reduces 13-hydroperoxylinolenic acid to its corresponding alcohol 13-hydroxylinolenic acid, which is not a signalling intermediate of the pathway and cannot be converted into JA, whereas nPG is less specific, inhibiting both lipoxygenase (LOX) and allene oxide cyclase (Fig. 3) (Bruinsma *et al.*, 2010). These effects ultimately interfere in plant defence capacity because LOX is responsible for oxygenating α-linolenic acid to 13(S)-hydroxy linolenic acid and allene oxide cyclase is an enzyme that catalyses the step to 12-oxo-phytodienoic acid, thus impairing the synthesis of JA (Bruinsma *et al.*, 2010).

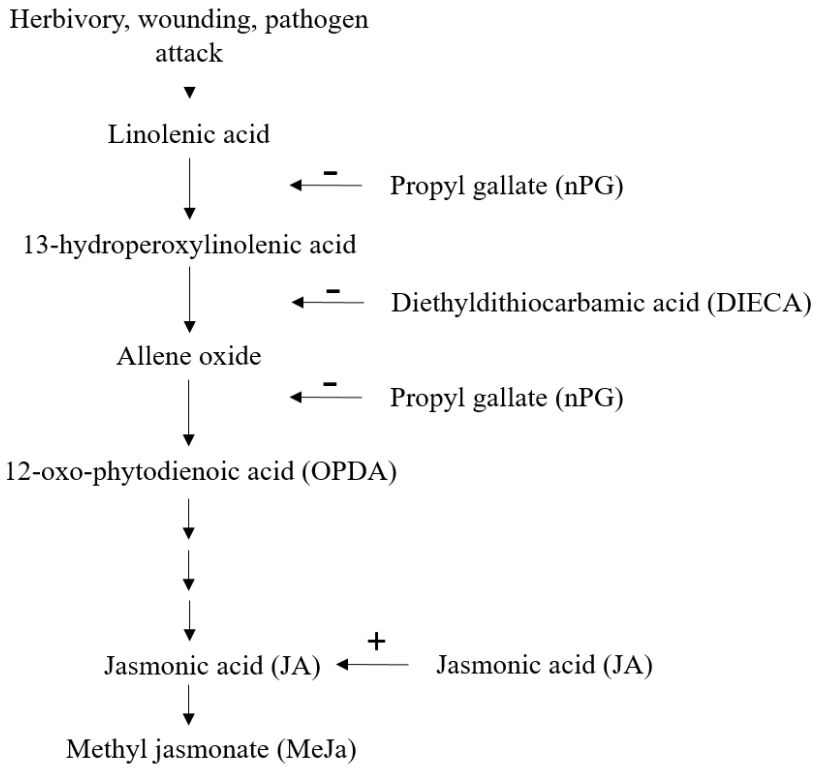

Figure 2 - Schematic representation of the octadecanoid pathway with indication of which step of the signal-transduction pathway is affected by the different elicitors (+) and inhibitors (-). *Adapted from*: Bruinsma *et al*. (2010).

Cooper and Rieske (2011) analysed the influence of JA treatment in the development and community interactions of galls produced by the Asian chestnut gall wasp, *Dryocosmus kuriphilus* in two chestnut species. While DIECA application seemed to decrease the number of galls and induce alterations in galls characteristics and defence capacity, JA application had an opposite effect in galls prevalence in American chestnut (*Castanea dentata*). An identical trend was observed by Bruinsma *et al.* (2010) that evaluated the effect of three inhibitors of the JA pathway (DIECA, phenidone, and nPG) on the interaction between the white butterfly *Cotesia glomerate* and Brussel sprouts (*Brassica oleracea*). The authors observed that after DIECA and phenidone treatments the attractiveness of *Cotesia glomerate* treated plants was reduced, probably because octadecanoid pathway is involved in attracting parasitoids, but may not be the only factor determining parasitoids host location.

The study of plant secondary metabolites is of great importance due to their biological activities against animals and microbes (Rodrigues *et al.*, 2000; Coleman *et al.*, 2011). Many of these substances are being incorporated in commercial insecticides/fungicides and pharmaceutical products with some degree of success (Afrin *et al.*, 2015). The further understanding the role of these compounds could provide means to manipulate induced resistance in plant, which could ultimately be a valuable tool in sustainable pest managements (Seo *et al.*, 2001; Eyles *et al.*, 2010).

1.5. Control of pinewood nematode using MeJa

As previously described, PWD is currently one of the most serious threats to pine forests all over Europe and Asia (Zhao *et al.*, 2008). Although a global effort has been made in order to control and, ultimately, eradicate this pest, limited success has been achieved.

One of the first approaches employed was the eradication of all trees positive to PWN infection, as well as all surrounding trees that showed decline symptoms. After cutting, infected material should be crushed without any other treatment, burnt or buried in the ground. Due to the low efficiency of these techniques a second approach, based on chemical control, was developed, preconizing that after cutting, chemical compounds should be applied by spraying and/or fumigation and that the infected material should be sealed with soil over a polyvinyl chloride (PVC) sheet. These chemicals were composed of chlorpyriphos-methyl, pyridaphenthion, prothiophos, or 2-sec-butylphenyl methylcarbamate, either individually or mixed together. Although these chemicals are able to kill not only the parasitic nematodes but

also the pine sawyer *M. galloprovincialis* (the insect vector), they are extremely costly and their ecologic impact is not fully understood (Kamata, 2008). Consequently, these control methodologies represent not only a negative impact in the ecosystem, but rise up a public human health concern (Rich *et al.*, 2004; Kamata, 2008).

Another chemical approach with some degree of success in controlling the PWN was tree injection with abamectin (or its common commercial formulation Avid™, Syngenta, Basil, Switzerland). This practice is very efficient and does not depend on environmental conditions; however, it fails in large scale application, such as in extensive forest stands, because it requires periodic application of three to four years to maintain its nematicide activity. This implies higher costs and labour force, which is why this technic is only used in historically important forest stands (Randall *et al.*, 2006; Kamata, 2008).

Biocontrol strategies, such as monitoring and controlling the insect vector, are extensively used in Asian countries, but their reproductivity in European forests have some limitations. For example, *M. galloprovincialis* did not respond the same way as *M. alternatus* to the pheromones used in insect traps (Kamata, 2008; Voz da Terra, 2009).

Despite all the efforts employed in the past decades, effective and sustainable methods to control the PWD have not been developed yet, and numerous environmental concerns have risen from the application of nematicides and pesticides in forest stands, being, therefore, necessary to develop a sustainable and effective alternative (Vicente, 2014).

Previous work developed in our laboratory (Nunes da Silva *et al.*, 2013) showed that two different concentrations of MeJa (25 mM and 50 mM) had a significant impact in reducing nematode population in *P. pinaster* and *P. radiata* two-year old plants. In this way, exogenous application of MeJa to *P. pinaster* plants was able to change the plant phenotype from highly susceptible to moderately tolerant to the PWN (Fig.3). Perhaps in this plant species the low tolerance to this pathogen may be due to inefficient activation of the JA pathways. Another benefit of MeJa application reported in this study was the reduction of water loss in *P. pinaster* infected plants, which is the main cause of tree mortality caused by PWD infection. In addition, the application of 25 mM MeJa showed an inductive effect on soluble phenolics biosynthesis, especially in PWN-infected plants (Nunes da Silva *et al.*, 2013).

In spite of these promising results, the role of JA pathway in *Pinus* spp. defence mechanisms and how the manipulation of this metabolic pathway could increase plants defence against economically important pests is still not known.

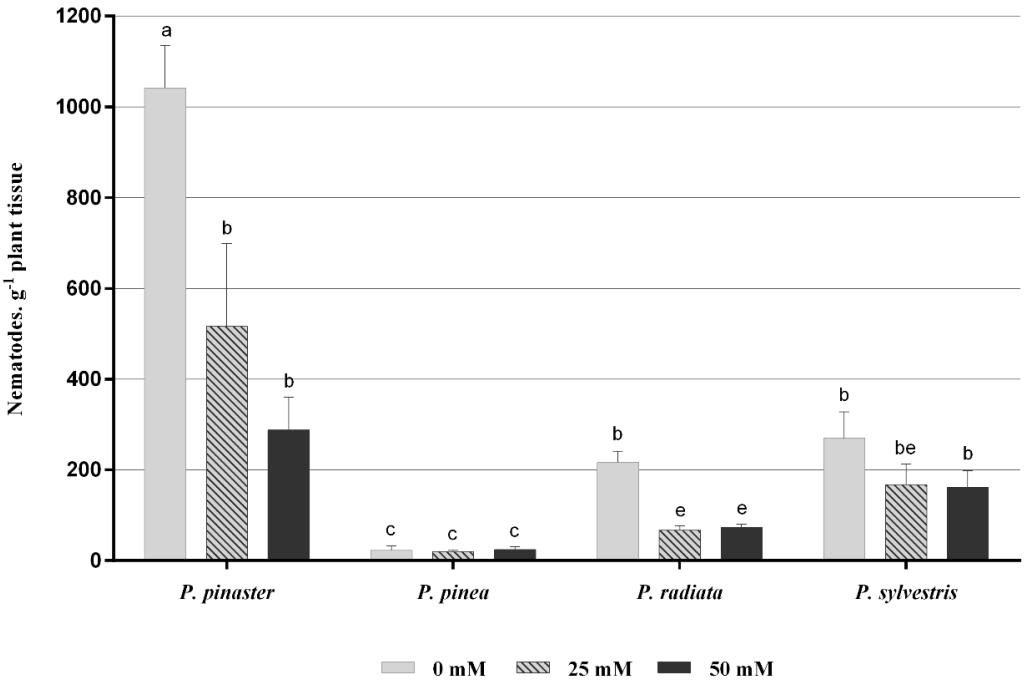

Figure 3 - Number of nematodes per gram of stem tissue in *P. pinaster*, *P. pinea*, *P. radiata* and *P. sylvestris* plants treated with 0, 25 or 50 mM MeJa, 2 months after inoculation. Vertical bars represent the standard error (n = 10). Bars with the same letter are not significantly different ($P < 0.05$, Students' T-test). *Adapted from:* Nunes da Silva *et al.*, 2013)

1.6 Objectives

The first main goal of this thesis was to understand the metabolic impact of the application of MeJa and its inhibitors DIECA and nPG on *P. pinaster* and *P. pinea* trees. The second goal was to evaluate the effect of the aforementioned elicitation on PWN infection success in both species. Specifically, the aims of this work were:

1) Evaluate how *P. pinaster* and *P. pinea* plant biomass and vigour were affected after treatment with MeJa, DIECA or nPG;
2) Assess if exogenous application of these compounds interfered with the biosynthesis of defence-related metabolites, such as soluble polyphenols, lignin, flavonoids and anthocyanins;
3) Understand how the expression of genes involved in the octadecanoic pathway and in the antioxidant response was impacted by the application of elicitors and inhibitors of the JA pathway;
4) Confirm that JA pathway elicitation in *P. pinaster* plants infected with the PWN decreased plant susceptibility to the pathogen, and
5) Verify if the application of JA pathway inhibitors (DIECA and nPG) decreased the natural tolerance of *P. pinea* plants to the PWN.

2. Materials and Methods

For the accomplishment of the aforementioned goals, a first experiment was developed to explore the phenotypic and genotypic impact of DIECA and nPG (inhibitors of the JA pathway) and MeJa (elicitor of JA pathway) application in two common pine species of the Portuguese flora, *P. pinaster* and *P. pinea*. After treatment with one of the three test compounds, plant fitness was evaluated through biomass accumulation, water content and lipid peroxidation determination. Biosynthesis of several plant metabolites (chlorophylls, soluble phenolics, lignin, flavonoids and saponins) and defence-related gene expression were also evaluated. Afterwards, a second trial was carried out to assess how the treatment with inhibitors and elicitors of the JA pathway influenced nematode multiplication after plant artificial inoculation with PWN.

2.1. Plant material

Two-year-old pine trees of two different species, *P. pinaster* and *P. pinea* originally from the Spanish National Centre of Forest Genetic Resources, El Serranillo (and gently provided by Dr. Rafael Zas and Dr. Luis Sampedro, from the Grupo de Genética y Ecología Forestal, Misión Biológica de Galicia (MBG-CSIC) were used. Throughout the experimental period of 68 days, pine trees were kept under natural environmental conditions and were watered every two days.

2.2. Solutions and treatments

A 10 mM diethyldithiocarbamic acid (DIECA, Alfa Aesar, Massachusetts, USA) solution was prepared in distilled water and 2 mM *n*-propyl gallate (nPG, MP biomedicals, LLC, California, USA) solution was prepared in 10% ethanol. Methyl jasmonate 50 mM (MeJa, Sigma-Aldrich, Missouri, USA) was prepared in 2.5% ethanol.

Plants of both species were randomly divided into four different groups according to the applied treatment: water (control), DIECA (inhibitor), nPG (inhibitor) or MeJa (elicitor). Control, DIECA and nPG treated plants were subjected to foliar spraying every two days throughout the experimental period (68 days). Approximately 40 mL of each solution were used per plant. MeJa treatment was performed at a single time in the first day of the experimental

period by foliar spaying (*ca*. 6 mL per plant). For each treatment, five biological replicates were used.

2.3. Plant sampling

Sixty-eight days after the beginning of the treatments, stem height, stem diameter and root length were recorded for all plants. Leaves, stem and roots of each plant were rapidly separated, weighted and flash frozen in liquid nitrogen. Samples were stored at -80 °C until further analysis.

2.4. Photosynthetic pigments quantification

Sims and Gamon (2002) method was used for total chlorophyll and carotenoids quantification in leaf tissues. Ten millilitres of cold acetone/Tris buffer solution at 1 M was added (80:20 vol:vol, pH= 7.8) to 0.5 g of leaf, which were incubated for 72 h at 4 °C. After the incubation period samples were centrifuged at 13 000 rpm for 5 min, and absorbances were recorded at 470, 537, 647 and 663 nm in a nanophotometer (Implen GmbH, München, Germany). The amount of pigments was calculated as (2002):

$$\text{Anthocyanin} = 0{,}08173A_{647} - 0{,}002228A_{663}$$

$$\text{Chl}_a = 0{,}01373A_{663} - 0{,}000897A_{537} - 0{,}003046A_{647}$$

$$\text{Chl}_b = 0{,}02405A_{647} - 0{,}004305A_{537} - 0{,}005507A_{663}$$

$$\text{Carotenoids} = \frac{A_{470} - (17{,}1 \times (\text{Chl}_a + \text{Chl}_b) - 9{,}479 \times \text{Anthocyanin}}{119.2}$$

2.5. Determination of water content

Leaves, stems and roots of plants subjected to each treatment were lyophilised for 3 days, after which water content was calculated as demonstrated by Tan *et al*. (2005):

$$\text{water content (\%)} = \frac{\text{sample fresh weight} - \text{sample dry weight}}{\text{sample fresh weight}} \times 100$$

2.6. Total soluble phenolics quantification

An amount of 0.2 g of lyophilized sample of each plant structure (leaves, stem, roots) was transferred to 15 mL centrifuge tubes and extracted with 5 mL of methanol at 4 °C in the dark for 24 h. The methanolic extract was recovered after centrifugation at 5000 rpm for 10 min to new centrifuge tubes and used for soluble phenolics quantification. Five millilitres of ultrapure water and 0.5 mL of Folin-Denis reagent were added to 0.1 mL of the methanolic extract of each sample. The reaction was allowed to occur for 5 min, after which 1.5 mL of 20% sodium carbonate was added. After incubation at room temperature in the dark for 2 h., 2.9 mL of ultrapure water was added. Finally, the absorbance was recorded at 760 nm using a nanophotometer (Implen GmbH, München, Germany). A quercetin calibration curve was used for soluble phenolics quantification.

2.7. Lignin quantification

Lignin concentration in leaves, stem and roots was determined by the acetyl bromide method (Hatfield *et al*., 1999) and the extraction was performed as previously described for total soluble phenolics determination, followed successive 24h incubations with water, acetone and hexane. Samples were then dried at 60 °C for 72 h, after which 1 mL of 12.5% acetyl bromide (in acetic acid, vol:vol) was added to 10 mg of dried sample. After incubation for 2 h at 50 °C with vigorous stirring, samples were centrifuged for 5 min at 13 300 rpm. One hundred microliters of the supernatant were transferred to a new microtube with 200 µL of acetic acid and 150 mL of 0.3 M sodium hydroxide. Finally, 50 µL of 0.5 M hydroxylamine hydrochloride and 500 µL acetic acid were added and mixed well. The absorbance of each sample was recorded at 280 nm in a nanophotometer (Implen GmbH, München, Germany) and a lignin calibration curve was used for lignin quantification in each sample.

2.8. Estimation of total flavonoid content

Total flavonoids content was evaluated using the aluminium chloride colorimetric method (Chang *et al.*, 2002). Ten milligrams of sample were dissolved in 0.5 mL of 80% ethanol, mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water, and were let to react at room temperature for 30 min. Absorbances were recorded at 415 nm using a nanophotometer (Implen GmbH,

München, Germany). For the calibration curve, quercetin was dissolved in 80% ethanol in a concentration range of 1 to 0.002 $mg.mL^{-1}$.

2.9. Saponins quantification

The vanillin-sulfuric acid assay was used for saponins quantification (Patel *et al.*, 2012), in which 250 mg of sample were mixed with 5 mL of 80% methanol and incubated for 12 h at 50 °C. To 100 µL of the methanolic extract of each sample were added 1 mL of 72% sulfuric acid and 100 µL of 8% vanillin solution. Samples were carefully mixed in an ice water bath, after which the mixture was warmed for 10 min at 60 °C. The absorbance of each sample was recorded at 535 nm using a nanophotometer (Implen GmbH, München, Germany). A diosgenin calibration curve was used for saponin quantification.

2.10. Lipid peroxidation

Li (2000) method was used to determinate malondialdehyde (MDA) concentration in leaves, stem and roots. Five hundred microliters of 0.1% trichloroacetic acid was added to 10 mg of plant material, which were vigorously mixed for 90 s. Afterwards, samples were centrifuged for 5 min at 9 500 rpm and 250 µL of the supernatant was mixed with 1 mL of thiobarbituric acid 0.5% in 20% trichloroacetic acid. After vigorous homogenization, samples were incubated at 100 °C for 30 min. The reaction was stopped in ice, samples were centrifuged at 9 500 rpm for 10 min and the absorbance was read at 532 and 600 nm using a nanophotometer (Implen GmbH, München, Germany). MDA concentration in each sample was calculated as:

$$MDA\ (nmol.g^{-1}\ fresh\ weight) = \frac{[(Abs_{532} - Abs_{600}) \times volume]}{\varepsilon = 155\ mM/cm \times biomass}$$

2.11. Extraction of total RNA

RNA extraction from leaves, stem and roots was performed according to an optimized method from Le Provost *et al.* (2007). Approximately 200 mg of sample was placed in a microcentrifuge tube containing 1 mL of extraction buffer (2% CTAB; 2% PVP; 100 mM Tris-HCl pH 8.0; 25 mM EDTA; 2.0 M NaCl; 0.5 $g.L^{-1}$ spermidine and 2% of β-mercaptoethanol). Samples were vigorously mixed and incubated for 10 min at 65 °C, after which 1 mL of

chloroform-isoamyl alcohol (CIA, 24:1) was added. Samples were centrifuged at 9 500 g for 10 min at room temperature and the supernatant was transferred to a new microcentrifuge tube with 800 mL of CIA. The mixture was homogenized and centrifuged at 9 500 g for 10 min at room temperature. The supernatant was transferred to a sterile microcentrifuge tube and 125 µL of 10 M lithium chloride was added. Samples were gently mixed and incubated over night at 4 °C. After the incubation period, samples were centrifuged at 9 000 g for 20 min at 4 °C, the supernatant was discarded and the pellet was dried out by inverting the tubes. The pellet was resuspended with 500 µL of Tris-NaCl-EDTA buffer (pH 8.0) and 450 µl of CIA and centrifuged at 9 500 g for 15 min at 4 °C. The supernatant was transferred to a clean microcentrifuge tube and 150 µL of STE, 100 µL of 2.0 M NaCl and 1.5 mL of cold absolute ethanol were added. Samples were incubated 30 min at -80 °C, after which they were centrifuged at 9 500 g for 20 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 400 µL of cold absolute ethanol and centrifuged at 9 500 rpm for 15 min at 4 °C. Finally, the supernatant was discarded and the pellet was dried out by inverting the tube, after which RNA was resuspended in 30 µL of sterile water. RNA quality and quantity were evaluated by UV-spectrophotometry, using a nanophotometer (Implen GmbH, München, Germany).

2.12. Synthesis of complementary deoxyribonucleic acid (cDNA)

For gene expression analysis, cDNA was synthetized using the iScript™ cDNA Synthesis Kit (Bio-Rad, California, USA) according to the manufacturers' instructions. Briefly, cDNA strand was synthesized by homogenizing 2 µL of RNA at 100 $ng.mL^{-1}$, 4 µL of iScript ReactionMix, 4 µL of reverse transcriptase and 10 µL of sterile water. This mixture was incubated in a heated lid thermal cycler (VWR DOPPIO, Radnor, Pennsylvania) at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. cDNA was diluted 1:100 and stored at -20 °C until further use.

2.13. Quantitative real-time polymerase chain reaction (qRT-PCR)

Gene expression evaluation was performed by qRT-PCR. Ten microliters of SYBR Green SuperMix (Bio-Rad, California, USA), 1 µL of each primer (forward and reverse) and 8 µL of cDNA were mixed and incubated in a StepOne™ Real-Time PCR System (Applied

Biosystems, California, USA) with the following reaction conditions: 2 min at 50 ℃, 2 min at 95 ℃ and 40 cycles with: 15 s at 95 ℃, 15 s at the appropriated annealing temperature for each primer pair (Table 1) and 1 s at 72 ℃. Melt curve profiles were analysed for each tested gene. The comparative CT method (ΔΔCT) (Livak and Schmittgen, 2001) was used for the relative quantification of gene expression values using actin (*ACT*) and 18S ribosomal RNA (*18S*) genes as control transcript. For each sample and target gene two technical replicates were analysed.

Table 1 - Primers used in gene expression analysis by *qRT-PCR.*

Gene	Accession number	Primers		Annealing temperature (°C)
		Forward	Reverse	
Actin	AY172979	TGGTGGTTCTACCATGTTTCCT	TTCGGTCTTGGCAATCCACA	59.1
18S	AH001728	GAAAGTTGGGGGCTCGAAGA	CCGGAACCCAAACACTCTGA	59.8
PAL	EU120508	GGATCCAGGAATGCAGGTCTT	TTTCATAATGGGCCAGGAGTTC	58.4
csAPX	AY485994	CCATGGTGAAGGCTTATCCC	GATGTCCAGACCGCTGTTAG	56.1
SAMS1	HE574556	ATGAGGGACACCCTGACAAA	GTCTGCTGACACGAATCCAA	56.8
ACO	FN824808	AGGAAGTGGGCTTTTTCCAG	AGCACAATGCAATCTTCCCA	57.0

2.14. Evaluation of *Pinus* spp. susceptibility to the pinewood nematode

In this experimental work, the virulent strain of pinewood nematode (*Bursaphelenchus xylophilus*) 65 GO was used to evaluate how *P. pinaster* and *P. pinea* performed against the pathogen after foliar treatment with water (control), DIECA, nPG and MeJa.

Agar plates with *Botrytis cinerea* mycelia were used to maintain nematode cultures, which were grown at 25 °C for 7 days. Nematodes were extracted from the culture medium using the Baerman funnel technic (Baerman, 1917), during 24 h at room temperature. A volume of 500 µL of nematode solution was placed in a counting dish to determinate nematode density, after which nematode inoculum was adjusted to ca. 3000 nematodes.mL^{-1}. Twenty *P. pinaster* and *P. pinea* plants were subjected to foliar treatments with water (control), DIECA, nPG or MeJa (N = 5), as described above, and nematodes were inoculated through the Asai and Futai method (2001): after peeling off a 1 cm portion of the bark at the upper third of the stem, a tissue paper swab was placed on the wound and a freshly prepared inoculum was pipetted onto the swab, after which the inoculation site was sealed with Parafilm to avoid desiccation.

Sixty-eight days post inoculation (dpi) plant stems were cut into small pieces and nematodes were extracted using the Baermann funnel technique (Baermann, 1917). The number

of nematodes in plant tissues was determined using a nematode counting dish under a transmitted light stereo microscope and calculated as nematodes.g^{-1} taking into account the fresh weight of each stem.

2.15. Statistical analysis

Statistical analysis was performed using GraphPad Prism v6.0 (GraphPad Software, California, USA), through Student's T-test with $P < 0.1$.

3. Results

3.1 Biometric and biochemical analyses

Concerning plant biometric analyses, it was possible to observe that plant height and root length were not affected in MeJa, DIECA and nPG treated plants, compared with controls, regardless of plant species (Table 2).

However, *P. pinea* trees treated with MeJa showed a 9% significant decrease in stem diameter, attaining 6.5 ± 0.1 mm, compared with the control plants, which had 7.1 ± 0.2 mm at the end of the experimental period. In *P. pinaster*, a 15% decrease in stem diameter was observed after MeJa treatment. Nevertheless, this difference was not statistically significant.

Table 2 - *P. pinaster* and *P. pinea* plant height (cm), stem diameter (mm) and root length (cm) after treatment with MeJa, DIECA, nPG or water (Control). Each value is the mean of 5 replicates ± standard error. Values showing an asterisk are significantly different from the Control at $P < 0.1$.

	P. pinaster			***P. pinea***		
	Plant height (cm)	**Stem diameter (mm)**	**Root length (cm)**	**Plant height (cm)**	**Stem diameter (mm)**	**Root length (cm)**
Control	35.0 ± 1.1	5.7 ± 0.6	39.5 ± 7.0	51.7 ± 1.7	7.1 ± 0.2	28.4 ± 5.3
MeJa	34.2 ± 0.5	4.9 ± 0.4	34.1 ± 5.5	51.5 ± 1.7	6.5 ± 0.1 *	32.8 ± 2.7
DIECA	34.2 ± 2.3	6.4 ± 0.2	38.0 ± 5.5	52.0 ± 1.6	7.0 ± 0.2	29.0 ± 5.9
nPG	32.8 ± 2.7	6.3 ± 0.6	38.8 ± 3.8	50.4 ± 1.5	7.1 ± 0.3	38.0 ± 3.1

Plant biomass was not significantly affected after treatment with neither MeJa nor the inhibitors (Table 3). Nevertheless, there seems to be a slight tendency for an increase of leaf biomass in DIECA treated *P. pinaster* plants (*ca*. 15%) and a decrease of *P. pinea* leaf biomass after MeJa treatment (*ca*. 22%).

Table 3 - *P. pinaster* and *P. pinea* leaf, stem and root biomass (g) after treatment with MeJa, DIECA, nPG or water (Control). Each value is the mean of 5 replicates ± standard error. Values showing an asterisk are significantly different from the Control at $P < 0.1$.

	P. pinaster			***P. pinea***		
	Leaf	**Stem**	**Root**	**Leaf**	**Stem**	**Root**
Control	17.78 ± 3.3	7.23 ± 0.8	12.65 ± 2.1	30.22 ± 3.1	12.34 ± 0.9	19.87 ± 2.4
MeJa	18.31 ± 3.4	6.04 ± 0.4	12.74 ± 1.4	23.67 ± 2.3	12.17 ± 1.1	17.15 ± 1.6
DIECA	20.50 ± 3.9	6.80 ± 0.7	13.52 ± 2.2	25.44 ± 5.3	10.80 ± 1.0	18.79 ± 3.7
nPG	24.84 ± 3.9	7.31 ± 1.2	13.01 ± 2.1	30.62 ± 4.1	13.86 ± 1.5	20.30 ± 2.2

Concerning plant water content, all structures analysed (leaves, stem and roots) had in average 60 to 80% of water (Table 4). After plant treatment with MeJa, leaf water content was significantly reduced by 5% in *P. pinaster* and 4% in *P. pinea*, compared with control plants.

Table 4 – Water content (%) of *P. pinaster* and *P. pinea* leaves, stem and roots after treatment with MeJa, DIECA, nPG or water (Control). Each value is the mean of 5 replicates ± standard error. Values showing an asterisk are significantly different from the Control at $P < 0.1$.

	P. pinaster			***P. pinea***		
	Leaf	**Stem**	**Root**	**Leaf**	**Stem**	**Root**
Control	70.8 ± 1.2	64.9 ± 0.2	69.9 ± 0.9	67.0 ± 0.6	61.7 ± 0.7	67.8 ± 1.6
MeJa	67.2 ± 0.9 *	61.3 ± 1.3 *	72.6 ± 2.6	64.6 ± 1.0 *	62.0 ± 0.3	70.5 ± 2.3
DIECA	68.9 ± 0.8	64.3 ± 1.4	74.1 ± 2.7	66.8 ± 1.2	59.0 ± 1.3	67.0 ± 2.0
nPG	70.3 ± 1.1	63.7 ± 0.3 *	70.8 ± 1.2	65.5 ± 0.8	60.9 ± 0.8	64.9 ± 1.8

Regarding total chlorophyll and carotenoid concentration in leaf tissues, a significant decrease of these pigments was observed in *P. pinaster* plants treated with DIECA: whereas total chlorophyll decreased about 14%, from 125.4 ± 2.2 to 107.7 ± 7.6 $\mu mol.g^{-1}$, carotenoids decreased approximately 19%, from 54.2 ± 2.0 to 43.8 ± 3.6 $\mu mol.g^{-1}$ (Table 5). A slight decrease in total chlorophyll and carotenoids was also observed in *P. pinaster* plants treated with nPG, *ca*. 20%; however, these differences were not statistically significant.

Table 5 – Total chlorophylls and carotenoids ($\mu mol.g^{-1}$) in leaves of *P. pinaster* and *P. pinea* plants treated with MeJa, DIECA, nPG or water (Control). Each value is the mean of 5 replicates ± standard deviation. Values showing an asterisk are significantly different from the Control at $P < 0.1$.

	P. pinaster		***P. pinea***	
	Total chlorophylls ($\mu mol.g^{-1}$)	**Carotenoids ($\mu mol.g^{-1}$)**	**Total chlorophylls ($\mu mol.g^{-1}$)**	**Carotenoids ($\mu mol.g^{-1}$)**
Control	125.4 ± 2.2	54.2 ± 2.0	107.9 ± 6.1	39.5 ± 3.6
MeJa	129.7 ± 12.1	63.6 ± 5.7	98.05 ± 8.7	34.8 ± 5.4
DIECA	107.7 ± 7.6 *	43.8 ± 3.6 *	113.1 ± 12.5	42.4 ± 2.5
nPG	99.7 ± 13.8	43.6 ± 5.9	112.7 ± 9.7	43.4 ± 3.1

Total soluble phenolics analysis showed that, in general, *P. pinea* had higher concentrations than *P. pinaster* in all plant tissues, independently of the treatment applied (from 30% to 140%, Fig. 4). MeJa treatment induced a significant increase in soluble polyphenols concentration in *P. pinaster* leaves, attaining 5.1 ± 0.4 $mg.g^{-1}$ (i.e. 61%), and in *P. pinea* stem and roots, which had, respectively , 3.7 ± 0.2 $mg.g^{-1}$ and 3.1 ± 0.4 $mg.g^{-1}$ (i.e. 20% and 50%),

at the end of the experimental period, comparing with control plants. Moreover, soluble polyphenols were also increased by 45% and 60% in *P. pinaster* roots after treatment with DIECA and nPG, respectively.

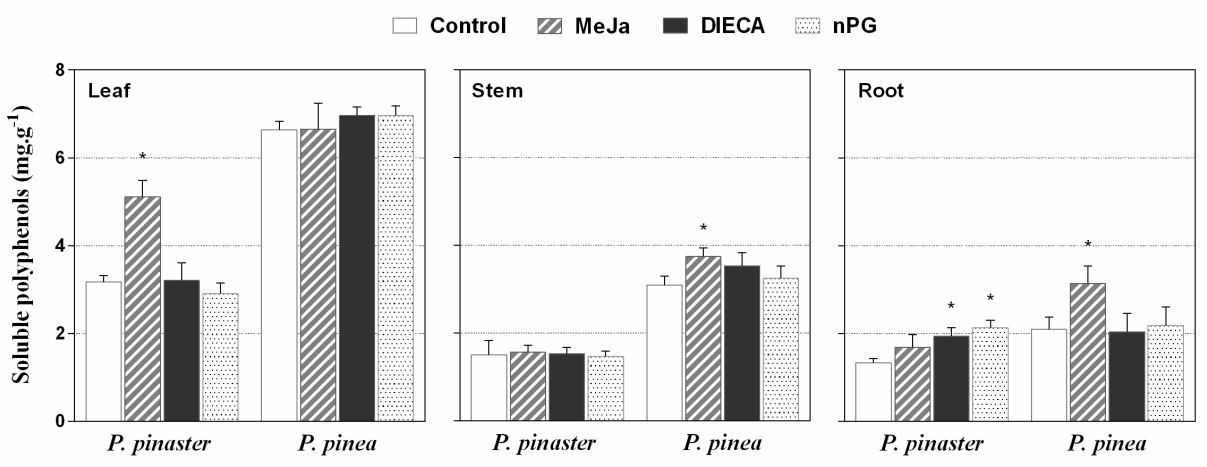

Figure 4 - Total soluble polyphenols (mg.g^{-1}) in leaves, stem and roots of *P. pinaster* and *P. pinea* plants treated with MeJa, DIECA, nPG or water (Control). Each value is the mean of 5 replicates and error bars represent the standard error. Bars showing an asterisk are significantly different from the Control at $P < 0.1$.

Flavonoids significantly decreased by 20% in *P. pinaster* leaves after treatment with both inhibitor compounds, comparing with control plants (Fig. 5). Additionally, DIECA treatment also induced a 46% decrease in flavonoids concentration in *P. pinaster* stems.

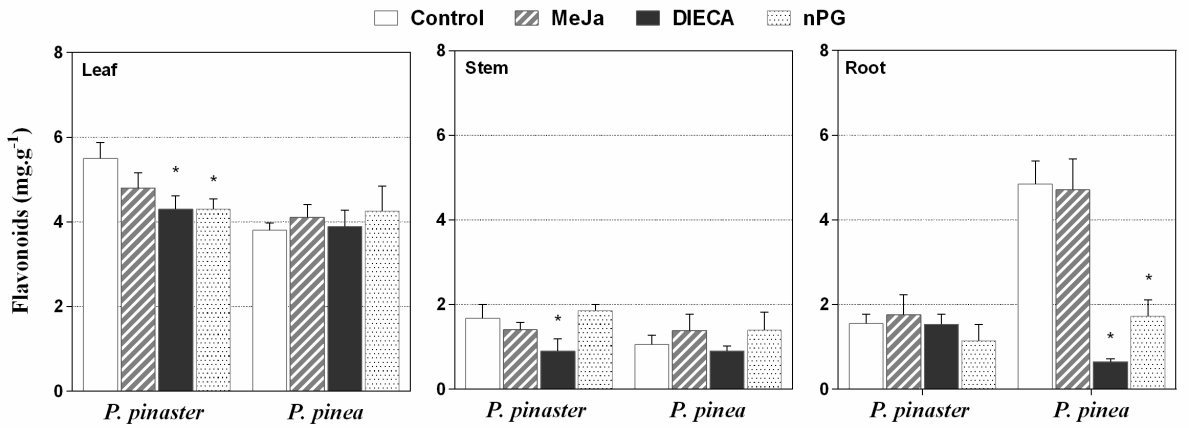

Figure 5 - Flavonoids (mg.g^{-1}) in leaves, stem and roots of *P. pinaster* and *P. pinea* plants treated with MeJa, DIECA, nPG or water (Control). Each value is the mean of 5 replicates and error bars represent the standard error. Bars showing an asterisk are significantly different from the Control at $P < 0.1$.

Concerning anthocyanin content in leaves, it was possible to observe that *P. pinaster* had always higher concentrations of anthocyanins than *P. pinea* (from 30% to 84%, Fig. 6).

MeJa treated plants had significantly higher concentration of anthocyanins. This phenomena was observed both in *P. pinaster*, in which anthocyanins increased from 88.1 ± 7.3 to 146 ± 18.6 µmol.g^{-1} (i.e. 66%), and in *P. pinea*, where an increase from 51.1 ± 5.9 to 79.2 ± 10.3 6 µmol.g^{-1} (i.e. 55%) was observed. Moreover, DIECA and nPG application to *P. pinaster* significantly decreased anthocyanins concentration by 25% and 38%, respectively, compared with control plants. In *P. pinea* plants a similar trend was observed, with plants treated with both inhibitors showing lower anthocyanin content (up to 14%). Nevertheless, these differences were not statistically significant.

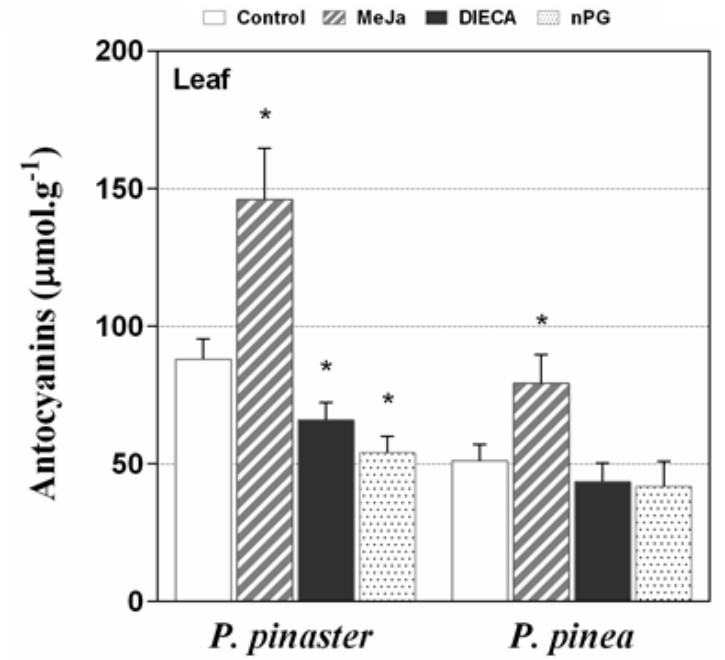

Figure 6 - Anthocyanins (µg.g^{-1}) in leaves of *P. pinaster* and *P. pinea* plants treated with MeJa, DIECA, nPG or water (Control). Each value is the mean of 5 replicates and error bars represent the standard error. Bars showing an asterisk are significantly different from the Control at $P < 0.1$.

In both species, MeJa treatment induced a significant increase in lignin concentration in all plant tissues (Fig. 7). *P. pinaster* and *P. pinea* stems achieved 151 ± 11.5 and 202.9 ± 26.1 mg.g^{-1} of lignin, respectively, which represents an increase of 50% and 70% when compared with control plants. A similar trend was observed in plant roots, where MeJa treatment significantly increased lignin concentration by 50% in *P. pinaster* and 79% in *P. pinea*. Lignin concentration was also higher in *P. pinaster* leaves by 77% after MeJa treatment. Contrastingly, in *P. pinea* plants treated with nPG, lignin content was decreased by 35% in leaves, which showed 22.2 ± 2.6 mg.g^{-1} of lignin by the end of the experimental tissue.

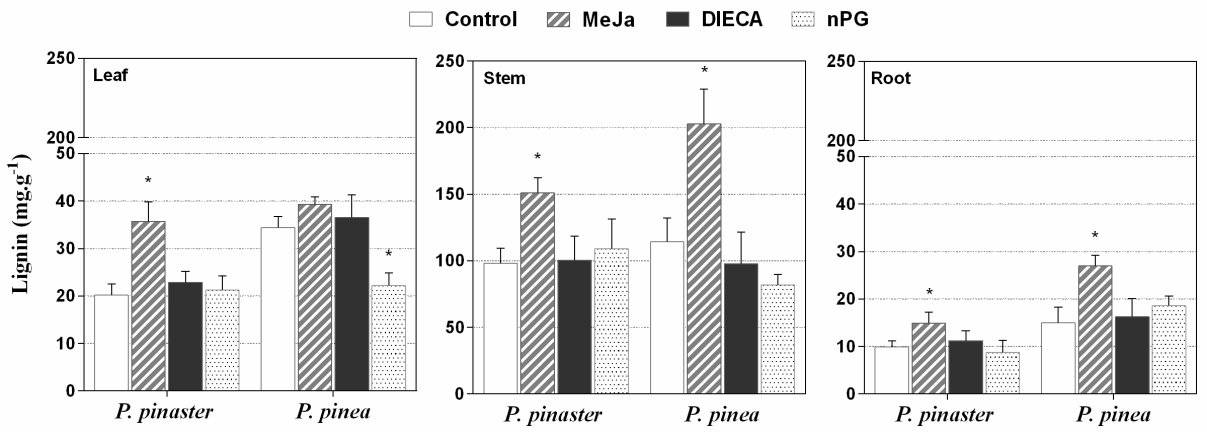

Figure 7 - Lignin (mg.g^{-1}) in leaves, stem and roots of *P. pinaster* and *P. pinea* plants treated with MeJa, DIECA, nPG or water (Control). Each value is the mean of 5 replicates and error bars represent the standard error. Bars showing an asterisk are significantly different from the Control at $P < 0.1$.

Saponins concentration analysis revealed higher concentration of this metabolite in *P. pinea* compared with *P. pinaster*: up to 50% in leaves, 6% in stems, and 40% in roots (Fig. 8). Moreover, MeJa treatment in *P. pinea* leaves induced a significant increase in saponins concentration, compared with control plants (from 15.9 ± 1.3 to 26.8 ± 2.2 mg.g^{-1}, i.e. 67%). A similar trend was observed in *P. pinaster* leaves, where saponin levels were 44% higher than control plants. However, this difference was not statistically significant.

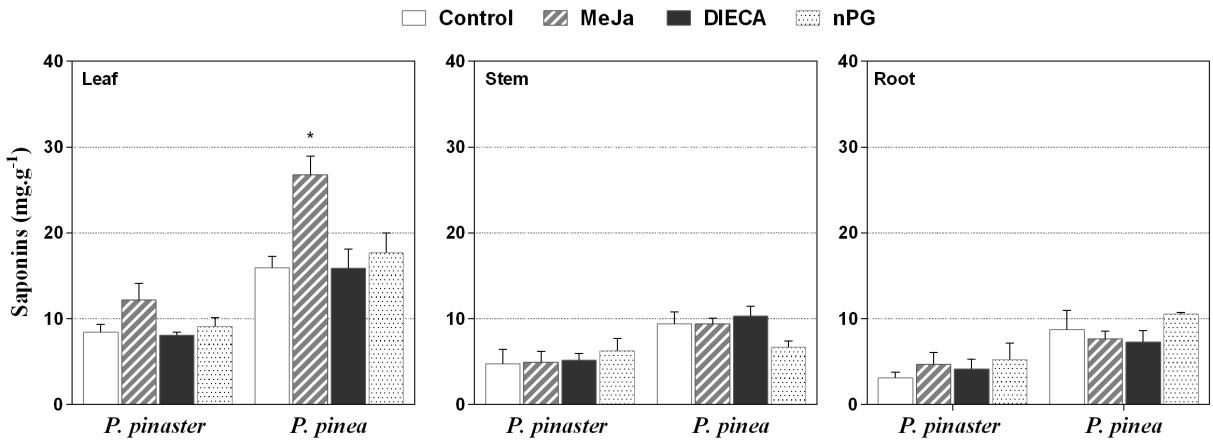

Figure 8 - Saponins (mg.g^{-1}) in leaves, stem and roots of *P. pinaster* and *P. pinea* plants treated with MeJa, DIECA, nPG or water (Control). Each value is the mean of 5 replicates and error bars represent the standard error. Bars showing an asterisk are significantly different from the Control at $P < 0.1$.

Malondialdehyde (MDA) quantification revealed significantly higher concentrations of this compound in both *P. pinaster* and *P. pinea* leaves treated with MeJa. At the end of the experimental period, these plants showed 14.7 ± 0.4 and 25.6 ± 0.24 mg.mg^{-1} of MDA, respectively, which represents an increase of 50% and 40% compared with control plants.

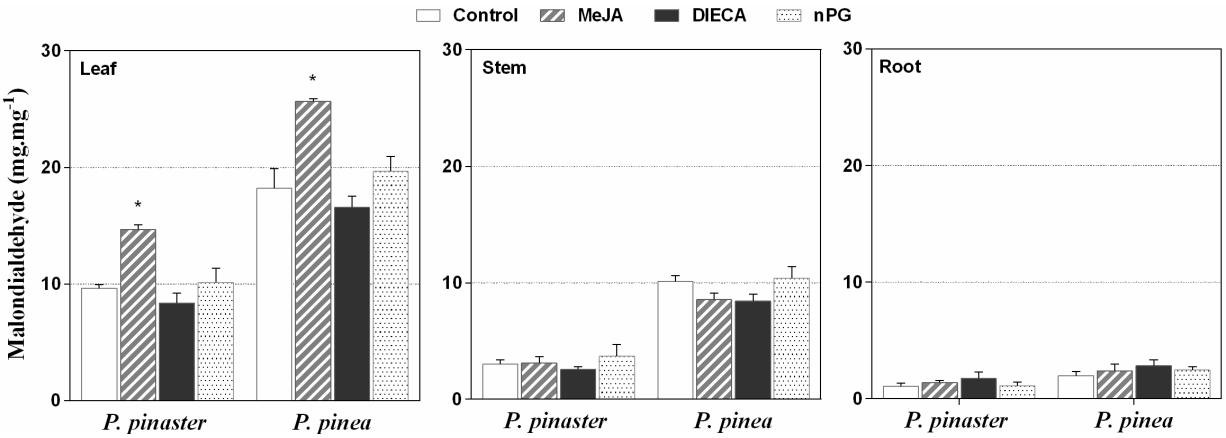

Figure 9 - Malondialdehyde (MDA) (mg. mg-1) in leaves, stem and roots of *P. pinaster* and *P. pinea* plants treated with MeJa, DIECA, nPG or water (control). Each value is the mean of 5 replicates and error bars represent the standard error. Bars showing an asterisk are significantly different from the Control at $P < 0.1$.

3.2 Gene expression analysis

Regarding gene expression, MeJa treatment significantly increased the relative expression of S-adenosylmethionine synthase 1 (*SAMS1*) and Aminocyclopropanecarboxylate oxidase (*ACO*) genes in *P. pinaster* leaves by 0.6-fold and 2.4-fold, respectively (Fig. 10), comparing with control plants. Contrastingly, Phenylalanine ammonia-lyase (*PAL*) relative expression was negatively affected up to 0,4-fold by DIECA and nPG treatments, in *P. pinaster* stem and roots.

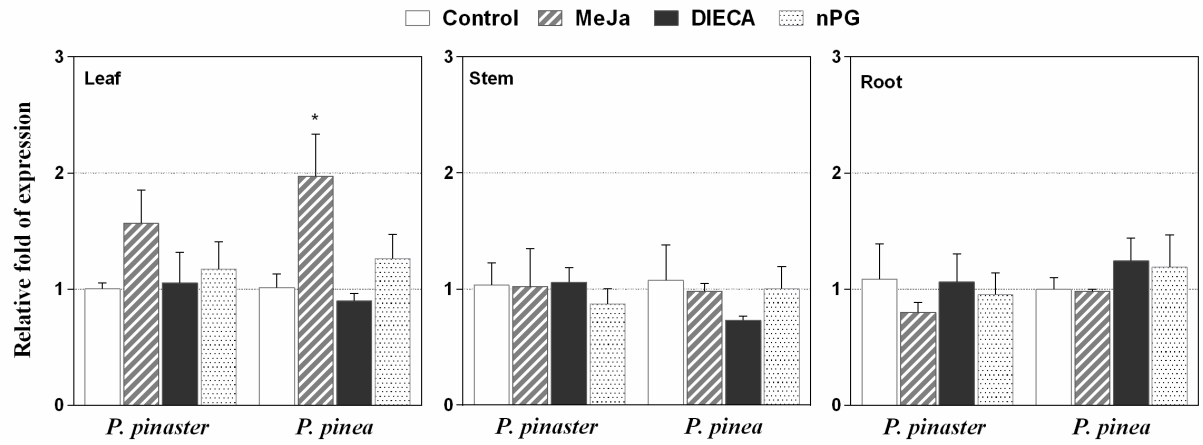

Figure 10 – Relative fold of expression of *SAMS1* in *P. pinaster* and *P. pinea* leaves, stem, and roots after treatment with MeJa, DIECA, and nPG or water (Control). Each value is a mean of 5 replicates ± standard error. Values showing an asterisk are significantly different from the Control at $P < 0.1$.

ACO relative expression was also significantly increased in MeJa treated *P. pinea* leaves, stems and roots by 2,7-, 1- and 1,5-fold, respectively (Fig. 11). Moreover, nPG had a significant impact in *ACO* relative expression in this species, which increased by 2,8- and 0,4-fold in stems and roots, respectively, in comparison with control plants.

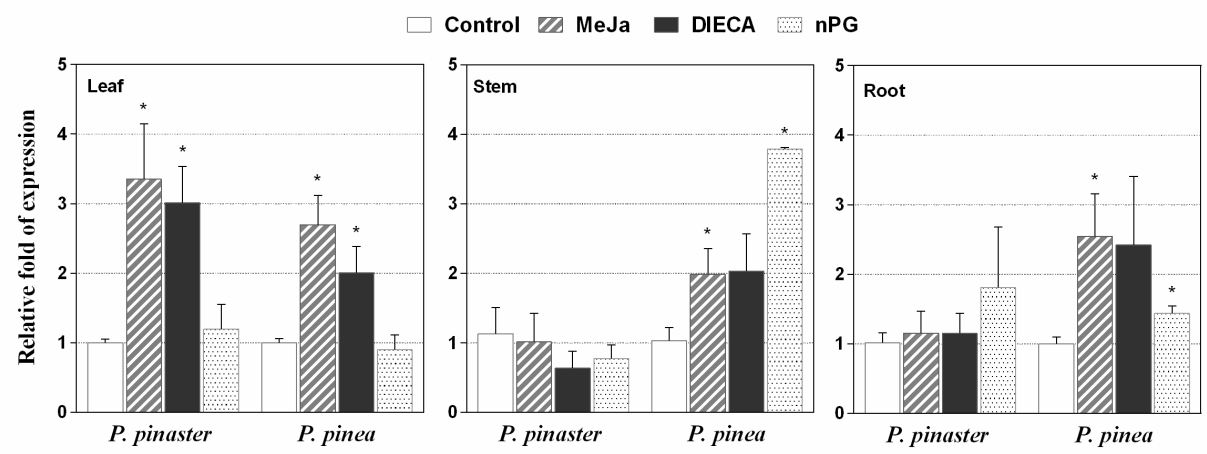

Figure 11 - Relative fold of expression of *ACO* in *P. pinaster* and *P. pinea* leaves, stem, and roots after treatment with MeJa, DIECA, and nPG or water (Control). Each value is a mean of 5 replicates ± standard error. Values showing an asterisk are significantly different from the control at $P < 0.1$.

Regarding to the expression level of ascorbate peroxidase (*csAPX*) (Fig. 12) and phenylalanine ammonia lyase (*PAL*) (Fig. 13), no significant results were found.

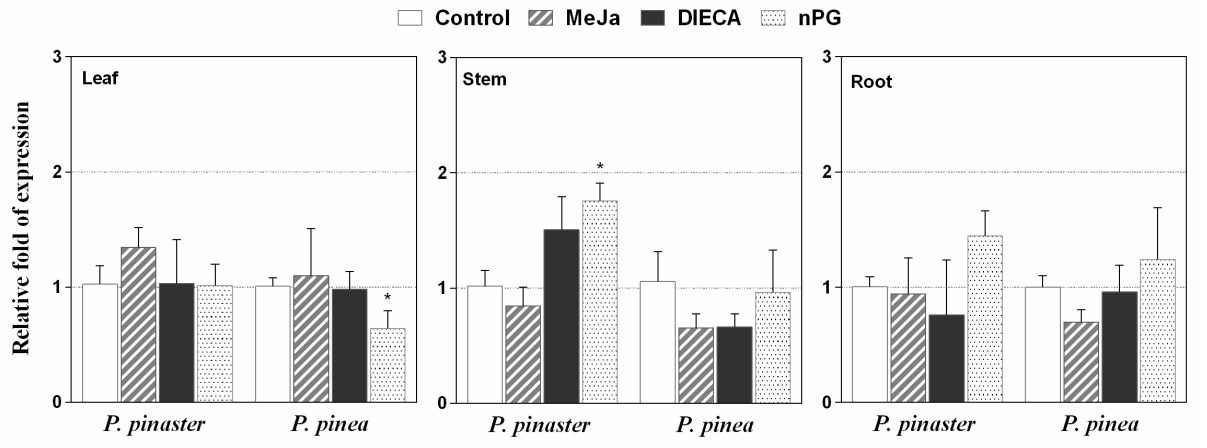

Figure 12 - Relative fold of expression of csAPX in *P. pinaster* and *P. pinea* leaves, stem, and roots after treatment with MeJa, DIECA, and nPG or water (Control). Each value is a mean of 5 replicates ± standard error. Values showing an asterisk are significantly different from the Control at $P < 0.1$.

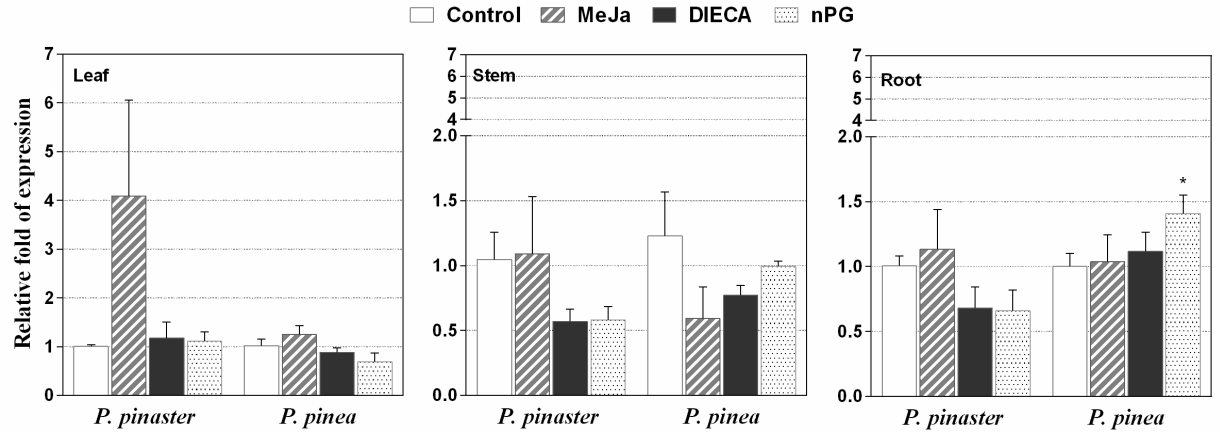

Figure 13 - Relative fold of expression of *PAL* in *P. pinaster* and *P. pinea* leaves, stem, and roots after treatment with MeJa, DIECA, and nPG or water (Control). Each value is a mean of 5 replicates ± standard error. Values showing an asterisk are significantly different from the Control at $P < 0.1$.

3.3 Impact of MeJa (elicitor) and its inhibitors DIECA and nPG on PWN population performance in *Pinus* spp. tissues

At the end of the experimental period, *P. pinaster* and *P. pinea* plants artificially inoculated with PWN and treated with water (Control), reached *ca*. 164 ± 62 and 28 ± 8 nematodes per gram of stem tissue, respectively (Fig. 14).

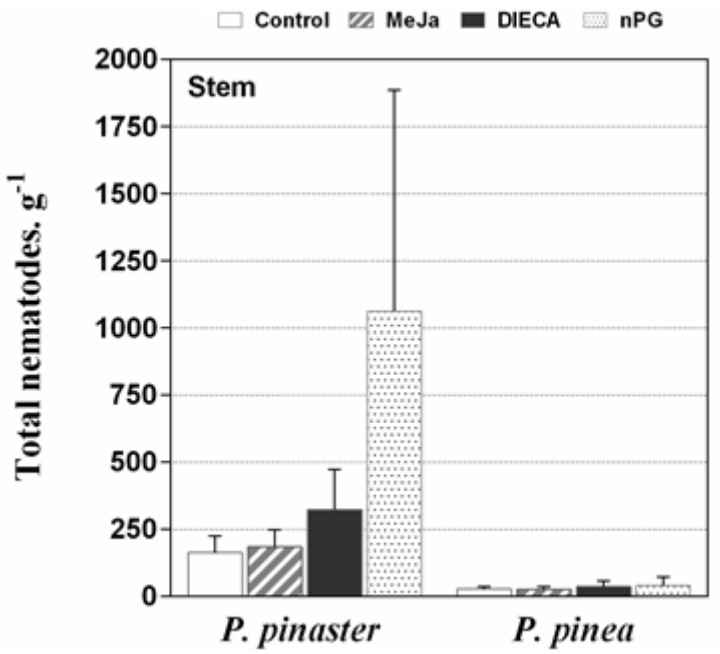

Figure 14 - Total nematodes (nematodes. g^{-1}) extract from the stems. Each value is the mean of 5 pine trees. Error bars represent standard deviation. Bars showing asterisk are significantly different from control at $P < 0.1$.

While MeJa treatment did not induce significant alterations in nematode population, there seemed to be a tendency, although not significant, for a higher nematode density after DIECA and nPG application, especially in *P. pinaster* plants. In fact, by the end of the experimental period, there were *ca*. 323 ± 150 and 1062 ± 823 nematodes.g^{-1} in DIECA and nPG treated *P. pinaster* plants, which represents more than 96% and 547% increase, respectively, compared with control plants. Nematode density in *P. pinaster* plants was always significantly higher than in *P. pinea* (up to 200%). *P. pinea* did not show any significant changes in nematode populations, during the experimental period.

4. Discussion

Forest threats have greatly increased in the past decade. Biotic impacts, in particular, pose important harmful damages to forest ecosystems, being extremely urgent to mitigate these menaces. The discovery and development of novel environmentally friendly phytosanitary compounds that increase plant resistance against pathogens and herbivores is greatly encouraged, not only to preserve forest ecosystems, but also due to the new forest policies that enforce more and more restrictions on the application of certain pesticides.

In this study, a baseline analysis of MeJa, DIECA and nPG application and their physiologic impacts on two of the most important pine species in Europe, *P. pinaster* and *P. pinea*, was performed, with the ultimate goal to improve and discover new combinations of biopesticides against the emergent challenges faced by these plant species. In addition, the impact of these compounds on PWN population performance after artificial inoculation of *P. pinaster* and *P. pinea* trees was also evaluated.

4.1 Biometric and biochemical analyses

Biometric analysis revealed that *P. pinea* plants were taller compared to *P. pinaster*, but plant height was not significantly affected by plant treatment, regardless of plant species. The same trend was observed in plant biomass, with *P. pinea* having always higher leaf, stem and roots biomass than *P. pinaster*, independently of the treatment applied. This could be due to the initial selection of plants in the nursery, because, in order to minimize intraspecific differences, a similar group of trees were selected and *P. pinea* trees could have been initially higher than *P. pinaster*. Contrarily, plant stem diameter showed a distinct behaviour, with MeJa application significantly reducing this parameter in both species. This phenomenon could be a result of MeJa toxicity to plant tissues, since there was higher contact of stems to MeJa droplets due to the spraying treatment chosen for the experiment. In fact, the same phenomena was already observed by *Sedal et al.*, (2016), which evaluated the application of two MeJa solutions (5 and 10 mM) in blueberry plants, that reported a significant reduction in stem diameter and total biomass in treated plants. Heijari *et al.* (2008), that studied the impact of MeJa application on *P. sylvestris* during an experimental period of 3 years, observed a similar reduction in stems diameter. This could be due to the fact that, as MeJa solution is prepared with a small percentage of ethanol, the solution itself could be toxic to plant cells. Alternatively, these authors also

propose that this apparently negative effect of MeJa application in stem biomass may be do to the allocation of resources from growth to defence mechanisms (Seldal *et al.*, 2016).

MeJa treatment also had a significant impact in *P. pinaster* leaves and stem and *P. pinea* leaves, inducing a reduction in water content in these structures. As leaves and stem contact directly with the solution, this reduction could be due to some toxicity induced by MeJa treatment, as the same negative effect was not verified in plant roots. Similar results were observed by Creelman and Mullet (1995), in *Glycine max* (soybean) seedlings treated with 100 and 500 µM of MeJa, in which water content decreased by 15%..

Previous studies by Heijari *et al.* (2005) showed that in *P. sylvestris* plants treated with 10 mM MeJa no significant alterations occurred in total chlorophyll content after 255 days of treatment. However, Gould *et al*. (2006) observed a reduction in photosynthetic pigments after the same period of time, demonstrating that *P. sylvestris* treatment with 100 mM MeJa solution had noxious effects in leaf pigments, possibly because the MeJa concentration used by the authors promoted leaf senescence. In the present work, plant treatment with 50 mM MeJa did not interfere with total chlorophyll concentration. However, DIECA had a negative impact in *P. pinaster* total chlorophyll and carotenoids content. Gamboa *et al*. (2009) reported that 1 mM nPG did not affect the photochemical efficiency of *Spathiphyllum wallisii* and *Chrysanthemum morifolium* under normal conditions, but when applied in stress conditions it decreased plant photochemical efficiency by 51 and 12%, respectively. These data were measured using the MINI-version of the Imaging-PAM (Heinz Walz GmbH, Effeltrich, Germany) in entire leaves after 30 min darkness. Therefore, it is clear that the impact of MeJa elicitors and inhibitors on plant photosynthetic pigments is dependent not only on the concentration used, but also on the plant species and experimental conditions. A hypothesis for further studies on this phenomenon could be the evaluation of the impact of these inhibitors on photosynthetic pigments pathways and/or in the degradation of photosynthetic proteins, such as the chlorophyll a/b-binding chloroplast proteins.

One of the major groups of constitutive defences in plants are phenolic compounds, which may serve as markers of induced plant resistance against certain diseases (Hahlbrock and Scheel, 1989; Klarzynski *et al.*, 2000). In this work, *P. pinea* had constitutively higher soluble polyphenols concentration than *P. pinaster*, especially in leaves and stem. Nunes da Silva *et al.* (2013) and Pimentel *et al.* (2017) obtained identical results concerning higher phenolics concentration in *P. pinea*, compared with *P. pinaster* and *P. radiata*. This is probably due to the fact that *P. pinea* is better adapted to poor environmental conditions, growing slower and investing more energy in constitutive defences (Endara and Coley, 2011). In fact, *P. pinaster* is

a fast growing species, adapted to rapidly occupy richer soils (Lavery and Mead, 1998; Tapias *et al.*, 2004), while *P. pinea* is considered to have evolved to occupy low-nutrient, highly drained sandy soils (Tapias *et al.*, 2004).

Inducible defences are activated by plants following pathogen or herbivore attacks or wounding (Karban and Baldwin, 1997; Franceschi *et al.*, 2005). In the past decade, several studies showed that MeJa exogenous application also induced the same effects as wounding (Seo *et al.*, 2001; Moreira *et al.*, 2009), positively affecting the phenylpropanoid pathway. Accordingly, in this work MeJa treatment significantly increased soluble polyphenols concentration in *P. pinaster* leaves and *P. pinea* stems and roots. This is an important fact to increase resilience against biotic attacks. Similar results were observed by Sampedro *et al.* (2011) in *P. pinaster* plants treated with a 22 mM MeJa, in which soluble polyphenols concentration increased by 15% in plants tissues after treatment. Therefore, MeJa application could be used to manipulate the production of polyphenols by plants, possibly allowing susceptible plant species, such as *P. pinaster*, to develop tolerant phenotypes.

Flavonoids, the major class of phenolic compounds, are known to be powerful antioxidants, playing an important role in plant protection. MeJa application has a positive impact in transcripts accumulation of chalcone synthase enzymes in leaves, which are involved in the first step of flavonoid biosynthesis in pine trees (Moreira *et al., 2012*). In this work, treatment with DIECA induced a decrease in flavonoids concentration in *P. pinaster* leaves and stem and *P. pinea* roots, whereas nPG also significantly decreased this metabolite, but only in *P. pinaster* leaves and *P. pinea* roots. These results are in accordance with previous studies, where the application of an inhibitor of the JA pathway decreased the activity of the octadecanoid pathway and, therefore, the accumulation of flavonoids. It was expected that MeJa application would increase flavonoids concentration in plant tissues; however, in this work, this was not observed. Additional evaluations, namely in time-course trials where plants are analysed in several time-points, could confirm how exogenous MeJa application impacts flavonoids synthesis and accumulation in *P. pinaster* and *P. pinea* plants.

Anthocyanins are described as one of the most important flavonoids due to their wide array of biological functions in plant tissues, being associated with cold and pathogen resistance (Sivankalyani *et al.*, 2016; Olivoto *et al.*, 2017). In this work, MeJa treatment increased anthocyanins concentration in *P. pinaster* and *P. pinea* leaves, whereas in DIECA and nPG *P. pinaster* treated plants a significant decrease in this metabolite was observed. It is possible to observe that the impact of DIECA and nPG application in certain defence-related pathways is extremely species-specific, which should be taken into consideration when using these

compounds to manipulate plant defences. Nevertheless, these inhibitors can be extremely useful for future investigations on signal-transduction pathways involving the conversion of linolenic acid, the precursor of the octadecanoid pathway that encodes the production of JA and its methylated acid, MeJa. The complexity of the interactions between environmental factors and plant responses should also be taken into consideration because this variable could directly interfere in plants cell signalling and, therefore, in their final phenotype.

Lignin, a phenolic compound responsible for cell wall rigidity and for plant defence, is known to increase in cell walls turning them impermeable to pathogens and difficult for small insects to chew (Hammerschmidt and Kuć, 1982; Xu *et al.*, 2011). It was also reported that lignin accumulation in plant cells could be a defence mechanism that allow higher resistance of *Pinus* spp. plants to PWN infection, once the concentration of this metabolite is naturally higher in resistant species (Nunes da Silva *et al*., 2013; Nunes da Silva *et al.*, 2015). In this work, lignin was constitutively more concentrated in plant stems than in leaves and roots, regardless of plant species, probably due to its primary function of cell support. Additionally, MeJa treatment generally increased lignin concentration in all structures analysed. Pauwels *et al.*, (2008) studied the transcriptional cascade induced in *Arabidopsis thaliana* by exogenous MeJa and observed an up-regulation of the monolignol biosynthesis gene set, resulting in increased production of monolignols and oligolignols, which are the building blocks of lignin. Interestingly, the application of nPG only decreased lignin concentration in *P. pinea* leaves. This was probably because these inhibitors turn off the octadecanoid pathway. Therefore, if the pathway does not encode JA or MeJa, no positive signal passes to plant cells to stimulate the biosynthesis of monolignols.

Saponins, a large and diverse group of secondary metabolites, are present in a wide range of plant species in relatively high levels in healthy plants. These compounds are generally considered to have important roles in defence against pathogens, pests and herbivores due to their antimicrobial, antifungal, antiparasitic, insecticidal and anti-feeding properties (Osbourn, 1996; Yendo *et al.*, 2010; Osbourn, Goss and Field, 2011; Moses *et al.*, 2014). Previous studies reported a six-fold increment in saponin concentration after exogenous application of MeJa in *in vitro* cultures of *Centella asiatica* (Lambert *et al.*, 2011) and in *Panax ginseng* (Ali *et al.*, 2006). In a similar manner, in the current work MeJa elicitation promoted the accumulation of saponins, in *P. pinea* leaves, potentially increasing their capacity to defend themselves from PWN attack. Moreover, inhibitor application did not have a significant impact in saponins concentration in *P. pinaster* and *P. pinea*. It is evident that the role of the JA pathway in saponin accumulation is highly dependent of plant species. In addition, saponins behaviour seems to

depend not only to the type of plant or elicitation/inhibition method used, but also on many environmental stimulus such as water stress and temperature (Szakiel and Pa, 2011), which could explain the mild effect of plant treatment in saponin accumulation in the present study.

Malondialdehyde (MDA), commonly used as a marker of oxidative stress in experimental works dealing with plants biotic and abiotic stresses, is one of the end products of polyunsaturated fatty acids peroxidation in cells, which occurs due to elevated concentrations of ROS. *P. pinaster* and *P. pinea* trees treated with MeJa showed increased MDA concentration in leaves, which was already observed in *Taxus chinensis* (Wang and Wu, 2005), *Panax ginseng* (Ali *et al.*, 2006), *Musa spp*. and *Glicine max.* Plant leaves could be more susceptible to MDA increase after MeJa treatment due to some degree of toxicity induced by the foliar treatment.

In sum, higher concentration of lignin and total soluble polyphenols was observed in both *P. pinaster* and *P. pinea* after MeJa treatment. This could be an important finding due to the fact that naturally more tolerant species to the PWN present higher levels of these two metabolites (Nunes da Silva *et al*., 2013). Contrastingly, the inhibitors DIECA and nPG impaired the accumulation of chlorophylls, carotenoids, flavonoids and anthocyanins, providing evidence on the regulatory role of the JA pathway on the biosynthesis of these metabolites.

4.2 Genetic expression analysis

To better understand how elicitors and inhibitors of the JA pathway impacted the expression of defence-related genes, *SAMS1*, *ACO*, *csAPX* and *PAL* were selected for analysis by qRT-PCR. It was hypothesised that MeJa could increase the expression of these genes, whereas DIECA and nPG would have the opposite effect.

SAMS1 is involved in the methylation of many plant cellular compounds and volatiles, and is a precursor of several metabolites, such as ethylene, polyamines, nicotianamine and biotin (Higuchi, 1981; Tabor and Tabor, 1984; Peleman *et al.*, 1989; Roeder *et al.*, 2009). In the present work, MeJa treatment increased *SAMS1* expression in *P. pinaster* and *P. pinea* leaves, more than in stems and roots. The higher exposure of leaves to MeJa fumigations could be responsible for the intense expression of *SAMS1* in this structure. A similar effect was already observed in tobacco plants treated with MeJa (Imanishi *et al.*, 1998).

According to Peleman *et al.* (1989), a strong preference for *SAMS1* expression was observed in stems and roots of *A. thaliana* plants, probably due to its essential role in

lignification. However, that was not observed in this experimental work. This can be due to the fact that gene expression analysis was performed only 68 days after the beginning of the treatments. Therefore, it is possible that a different pattern of gene expression would be present if the analyses had been done at an earlier time point, since the induction of gene expression may occur as early as shortly after the stimulus is provided to the plant.

Ascorbate peroxidase enzymes play a key role in catalysing the conversion of H_2O_2 into water, being one of the major hydrogen peroxide detoxidication systems in plant cells. In this report, cytosolic ascorbate peroxidase (*csAPX*) relative fold of expression was increased only in nPG *P. pinaster* treated plants. Maruta *et al.* (2012) demonstrated that in *A. thaliana* plants, H_2O_2 scavenging in the cytosol is essential for plant tolerance to wounding and MeJa treatment. It is possible that *csAPX* and the antioxidant enzyme it encodes could have a more active role against MeJa-induced stress shortly after its application, which could explain why the relative expression of these gene was similar in control and treated plants.

Aminocyclopropanecarboxylate oxidase (ACO) is responsible for the catalysis of the last step of ethylene biosynthesis, where 1-aminocyclopropane-1-carboxylic acid is converted to ethylene by ACC oxidase. Interestingly, ACO transcription was increased in *P. pinaster* leaves and *P. pinea* leaves, stem and roots after MeJa treatment, and also in DIECA-treated *P. pinea* leaves and nPG-treated stems and roots. ET and JA regulate a vast array of physiological processes in plants, which include the activation of specific responses to different types of stresses. However, how plants select the physiological response to a particular stress using these two hormones remains poorly understood. Nevertheless, the defence mechanisms activated in plant tissues in response to different types of stresses seem to be dependent on the type of interaction (positive or negative) between these hormonal signalling pathways and not on the independent contribution of each hormone (Rojo *et al.*, 1999; Overmyer *et al.*, 2000). In the present work, ACO overexpression in *P. pinaster* and *P. pinea* seems to occur both when JA pathways is elicited (with MeJa application) and when it is inhibited (through DIECA and nPG treatments). Is was not possible to evaluate if the higher expression of ACO is converted in actual enzymatic metabolite that ultimately induced ET biosynthesis. It would be interesting to further explore the relationship between ET and JA pathways in *P. pinaster* and *P. pinea* and how the balance of these hormones affects their defence against pathogens and herbivores.

As previously mentioned, the production of phenolic compounds together with PAL activity may serve as markers of induced plant resistance against certain diseases (Hahlbrock and Scheel, 1989; Klarzynski *et al.*, 2000). In this work, relative expression of *PAL* was highly increased in *P. pinaster* leaves after MeJa treatment and decreased in stems after DIECA and

nPG treatment. This could explain the higher anthocyanins and soluble polyphenols content observed in MeJa treated leaves, as these metabolites derive from the phenylpropanoid pathway, in which PAL enzyme plays a central role (Campos-Vargas and Saltveit, 2002; Heredia and Cisneros-Zevallos, 2009; Kepczyńska and Król, 2012) .

4.3. Impact of the elicitor (MeJa) and the inhibitors (DIECA and nPG) on PWN proliferation

The second part of this experiment aimed not only to confirm if *P. pinaster* is indeed more susceptible to the PWN when compared to *P. pinea*, but also to evaluate if MeJa elicitation would increase the resistance of *P. pinaster* to the PWN, and if the application of the DIECA and nPG would worsen the susceptibility phenotype. It was also intended to confirm, from a contrasting standpoint, if the application of DIECA and nPG to *P. pinea* (a tolerant genotype) would render it susceptible to the PWN, by inhibiting the JA pathway.

Nunes da Silva *et al*. (2015), evaluated the susceptibility of *P. pinaster, P. pinea, P. radiata* and *P. sylvestris* to the PWN and observed that 60 days after inoculation, *P. pinaster* plants had about 12 to 57 times higher PWN nematode density in stem tissues, compared to the other three species. *P. radiata* and *P. sylvestris* presented a moderate tolerance to the PWN, whereas *P. pinea* appeared to be the most resistant species to the pathogen, thanks to the small number of nematodes registered after the experimental period (Santos *et al.*, 2012; Nunes da Silva *et al.*, 2015).

Previous studies carried out in our laboratory showed that after MeJa application at 25 and 50 mM to four *Pinus* species (*P. pinaster*, *P. pinea*, *P. radiata* and *P. sylvestris*), there was a significant reduction in nematode population (Nunes da Silva *et al*., 2013). Amongst the four, *P. pinaster* and *P. radiata* were the two species that showed the greatest decrease in nematode population after elicitor treatment.

In the present assay, it would be expected that after PWN inoculation, *P. pinaster* control plants (elicited only with water) would have very low tolerance to the infection. It would also be expected that those treated with DIECA and nPG would show an even lower tolerance or more severe symptoms of the disease, since these compounds inhibit the JA defense pathway, thus weakening the plant. In contrast, with MeJa application, a significant reduction in the number of nematodes would occur, since this compound elicits this defensive pathway, potentiating the plants' defenses against the progression of the infection.

Results showed that, regardless of the treatment, PWN multiplication was higher in *P. pinaster* than in *P. pinea* (over 200%), which is in accordance to what was observed by Nunes da Silva *et al.* (2015). Contrarily to the preliminary results obtained in our laboratory, treatment of *P. pinaster* with MeJa did not induce significant changes in the species tolerance to PWN infection. While the application of both inhibitors (DIECA and nPG) seemed to increase the number of nematodes at the end of the experimental period when compared to control plants, this reduction was not statistically significant.

P. pinea plants showed no change in the PWN population after the experimental period. Regardless of the treatments, all plants had very low nematode counts, including those treated with the inhibitors. Therefore, an increase in the susceptibility of *P. pinea* was not observed after the application of DIECA and nPG.

However, these results lead to the belief that some variables may have conditioned the experimental process. In *P. pinaster* control plants, the number of nematodes reached at the end of the experiment period was relatively low, compared to other studies performed under similar conditions. In Cruz (2015), 28 days after inoculation of one-year-old *P. pinaster* plants with 2000 nematodes, 751 ± 158 nematodes per plant were observed in plant tissues. Nunes da Silva *et al*. (2015), in addition, reported that 60 after inoculation of three-year-old *P. pinaster* plants with 2 000 nematodes a count of 26 000 ± 4 147 nematodes per plant were obtained.

The low colonization by the pathogen may result from two factors. Firstly, the inoculum came from nematodes that were kept under laboratorial conditions for an extended time period, having been subcultured multiple times, and not having been in contact with its natural environment for some time. This may have weakened the nematode vigor or may have contributed to a lowering in its virulence levels (Roy *et al.*, 2014; Ravikumar *et al.*, 2016). Another factor comes from the season of the year in which the experiment was carried out: inoculation of the plants took place at the end of September, when a few days of rain and falling temperatures may have been the necessary stimulus for the entrance of the pathogen in latency (Zhao *et al.*, 2008), as it happens in natural conditions between autumn and winter.

Thus, it is concluded that this second experiment should be repeated, not only with fresh/more active inoculum, and at another time of the year which the nematodes start their reproductive and infectious activity (April/May). With this optimization, more significant results could be obtained regarding the impact of the inhibitors application.

5. Conclusions and future work

This study provided baseline data to future works that aim at studying the application of MeJa and its inhibitors, such as DIECA and nPG, on pine trees metabolism. The purpose of this integrative analysis was to give answer on a new line of approaches in pest management without the use of pesticides.

It was possible to verify a significant trend for the higher concentration of soluble polyphenols, anthocyanins and lignin in MeJa treated *P. pinaster* and *P. pinea* plants, compared with controls. Contrastingly, the inhibitors DIECA and nPG impaired the accumulation of chlorophylls, carotenoids, flavonoids and anthocyanins, providing evidence of the regulatory role of the JA pathway on the biosynthesis of these metabolites. These findings could be used for the development of MeJa-based products for the enhancement of plant defence.

P. pinaster appears to be more susceptible than *P. pinea* to the PWN, reaching higher nematodes counting at the end of experimental period. The induction of JA pathway through the exogenous application of MeJa was not clearly associated with the phenotypic change of *P. pinaster* from high to less susceptible. On the order hand, although there seems to be a slight tendency for the increase of PWN density in *P. pinaster* stems after DIECA and nPG treatment, results were not statistically significant. Similarly, inhibitor application was not effective in decreasing the resistant phenotype of *P. pinea* plants.

Therefore, further studies should be conducted to categorially prove the importance of JA pathways in *Pinus* spp. defence mechanisms. Specifically, more concentrations of the elicitors and inhibitors should be tested, as well as the frequency of their application and season of the year more suitable for their application. Additionally, the environmental conditions during the experimental period should be controlled, preventing adverse meteorological condition that could interfere with plant response and pathogen performance. More importantly, a dynamic analysis should be undertaken to evaluate the behaviour of the selected or other defence-related compounds during a designated time-period. Finally, the experimental trial should be conducted in spring or summer time, which are the seasons more favourable to nematode reproduction and infection.

6. References

Abelleira, A., Ibarra N., Aguín, O., Mosquera, P., Sorolla, A, Ares, A., Mansilla, P. (2015) 'First report of *Bursaphelenchus mucronatus kolymensis* (Nematoda: Aphelenchoididae) on *Monochamus sutor* (Coleoptera: Cerambycidae) in Spain', *Forest Pathology*, 45(1), pp. 82–85.

Afrin, S., Huang, J. J. and Luo, Z.-Y. (2015) 'JA-mediated transcriptional regulation of secondary metabolism in medicinal plants', *Science Bulletin*, 60(12), pp. 1062–1072.

Ali, M. B., Kee-Won Y., Eun-Joo H., Kee-Yoeup P. (2006) 'Methyl jasmonate and salicylic acid elicitation induces ginsenosides accumulation, enzymatic and non-enzymatic antioxidant in suspension culture *Panax ginseng* roots in bioreactors', *Plant Cell Reports*, 25(6), pp. 613–620.

Apel, K. and Hirt, H. (2004) 'Reactive oxygen species: Metabolism, Oxidative Stress, and Signal Transduction', *Annual Review of Plant Biology*, 55(1), pp. 373–399.

Apostol, I., Heinstein, P. F. and Low, P. S. (1989) 'Rapid Stimulation of an Oxidative Burst during Elicitation of Cultured Plant Cells: Role in Defense and Signal Transduction.', *Plant physiology*, 90(1), pp. 109–116.

Asai, E. and Futai, K. (2001) 'Retardation of Pine Wilt Disease Symptom Development in Japanese Black Pine Seedlings Exposed to Simulated Acid Rain and Inoculated with *Bursaphelenchus xylophilus*', *Journal of Forest Research*, 6(4), pp. 297–302.

Baker, C. J. and Orlandi, E. W. (1995) 'Active oxigen in Plant phatogensis', *Annual Review of Phytopathology*, 99, pp. 299–321.

Barnes, I., Crous, P. W., Wingfield, B. D., and Wingfield M. J. (2004) 'Multigene phylogenies reveal that red band needle blight of *Pinus* is caused by two distinct species of *Dothistroma*, *D. septosporum* and *D. pini*', *Studies in Mycology*, 50(2), pp. 551–565.

Barnes, I., van der Nest. A., Mullet M. S., Crous, P. W., Drenkhan, R., Musolin, D. L. and Wingfield, M. J. (2016) 'Neotypification of *Dothistroma septosporum* and epitypification of *D. pini*, causal agents of Dothistroma needle blight of pine', *Forest Pathology*, pp. 388–407.

Begon, M., Harper, J. L. and Townsend, C. R. (1986) 'Ecology. Individuals, populations and communities.', in *Ecology*. 2ª Ed. London: Blackwell Scientific Publ, p. 945.

Berryman, A. A. (1986) 'The European woodwasp in Australia', in *Forest Insects: Principles and Practice of Population Management*. Nova York: Plenum Press, pp. 232–236.

Bradshaw, R. E. (2004) 'Dothistroma (red-band) needle blight of pines and the dothistromin toxin: A review', *Forest Pathology*, 34(3), pp. 163–185.

Bragança, H., Diogo, E., Moniz, F. and Amaro, P. (2009) 'First Report of Pitch Canker on Pines Caused by *Fusarium circinatum* in Portugal', *Plant Disease*, 93(10), p. 1079.1.

Branco, J., Oliveira M., Ferreira, R., and Póvoa O. (2014) *Deforestation in Portugal: causes, consequences and possible solutions*. Available at: https://comum.rcaap.pt/bitstream/10400.26/5286/1/João Branco_Márcia Oliveira_Ricardo Ferreira_Orlanda Póvoa_.pdf (Last ccessed: 14 February 2018).

Brown, A., Rose, D. and Webber J. (2003) 'Red Band Needle Blight of Pine', (Figure 1), pp. 1–6.

Bruinsma, M., van Loon, J. J. A. and Dicke, M. (2010) 'Increasing insight into induced plant defense mechanisms using elicitors and inhibitors.', *Plant signaling & behavior*, 5(3), pp. 271–4.

Cabral, M. T. (1995) *Introdução à protecção integrada da floresta*. Lisboa.

Campos-Vargas, R. and Saltveit, M. E. (2002) 'Involvement of putative chemical wound signals in the induction of phenolic metabolism in wounded lettuce', *Physiologia Plantarum*, 114(1), pp. 73–84.

Chang, C. C., Yang, M. H. and Wen, H. M. (2002) 'Estimation of Total Flavonoid Content in Propolis by Two Complementary Colorimetric Methods', *Journal of Food and Drug Analysis*, 10(3), pp. 178–182.

Cheong, J. J. and Choi, Y. Do (2003) 'Methyl jasmonate as a vital substance in plants', *Trends in Genetics*, 19(7),

pp. 409–413.

Coleman, J. J., Ghosh, S., Okoli, I., and Mylonakis, E. (2011) 'Antifungal Activity of Microbial Secondary Metabolites', *PLoS ONE*. Edited by V. Chaturvedi, 6(9), p. e25321.

Cooper, W. R. and Rieske, L. K. (2011) 'Chestnut species and jasmonic acid treatment influence development and community interactions of galls produced by the Asian chestnut gall wasp, *Dryocosmus kuriphilus*.', *Journal of insect science (Online)*, 11(140), p. 140.

Creelman, R. A. and Mullet, J. E. (1995) 'Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress.', *Proceedings of the National Academy of Sciences of the United States of America*, 92(10), pp. 4114–4119.

Dempsey, D. A., Vlot, A. C., Wildermuth, M. C., and Klessig, D. F. (2011) 'Salicylic Acid Biosynthesis and Metabolism', *The Arabidopsis Book*, 9, p. e0156.

Dempsey, D. A. and Klessig, D. F. (2017) 'How does the multifaceted plant hormone salicylic acid combat disease in plants and are similar mechanisms utilized in humans?', *BMC Biology*, 15(1), p. 23.

Desikan, R., Hancock, J. and Neill, S. (2005) 'Reactive oxygen species as signalling molecules', in Smirnoff, N. (ed.) *Antioxidant and Reactive Oxygen pecies in Plants*. Blackwell Publishing Ltd., pp. 169–196.

Devoto, A. and Turner, J. G. (2003) 'Regulation of jasmonate-mediated plant responses in A*rabidopsis*', *Annals of Botany*, 92(3), pp. 329–337.

Dick, M. A. and Simpson, J. (2003) *Fusarium circinatum - an agent of damping-off disease*. Rotorua, New Zealand.

Dillon, A. B., Ward, D., Downes M. J., and GriYn, C. T. (2006) 'Suppression of the large pine weevil *Hylobius abietis* (L.) (Coleoptera: Curculionidae) in pine stumps by entomopathogenic nematodes with different foraging strategies', *Biological Control*, 38(2), pp. 217–226.

Dores, R. (2014) 'Há ouro branco em Portugal: um quilo de pinhão custa 120 euros', *Diário de Noticias* . Available at: https://www.dn.pt/portugal/interior/ha-ouro-branco-em-portugal-um-quilo-de-pinhao-custa-120-euros-4315873.html (Last accessed: 1 February 2018).

Dwinell, L. D. (1997) 'The pinewood nematode: regulation and mitigation.', *Annual review of phytopathology*, 35, pp. 153–166.

Endara, M. J. and Coley, P. D. (2011) 'The resource availability hypothesis revisited: A meta-analysis', *Functional Ecology*, 25(2), pp. 389–398.

EPPO (2017a) *A2 List of pest recommended for regulation as quarantine pests*. Available at: https://www.eppo.int/QUARANTINE/listA2.htm (Accessed: 12 February 2018).

EPPO (2017b) 'EPPO A1 AND A2 LISTS OF PESTS RECOMMENDED FOR REGULATION AS QUARANTINE PESTS', 2. Available at: https://www.eppo.int/QUARANTINE/listA2.htm.

European Commission (2013) *A new EU Forest Strategy: for forests and the forest-based sector*. Brussels. doi: COM(2015) 185 final.

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

Farmer, E. E. and Ryan, C. A. (1990) 'Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves.', *Proceedings of the National Academy of Sciences*, 87(19), pp. 7713–7716.

Farmer, E. E. and Ryan, C. A. (1992) 'Octadecanoid Precursors of Jasmonic Acid Activate the Synthesis of Wound-lnducible Proteinase lnhibitors', *The Plant Cell American Society of Plant Physiologists*, 4(2), pp. 129–134.

Fernandes, P. (2014) *Forest Context and Policies in Portugal, Forest Context and Policies in Portugal, Present and Future Challenges.* Edited by F. Reboredo. Cham: Springer International Publishing (World Forests).

Fonseca, L., Cardoso, J. M. S., Lopes, A., Pestana M., Abreu, F., Nunes, N., Mota, M. and Abrantes, I. (2012)

'The pinewood nematode, *Bursaphelenchus xylophilus*, in Madeira Island', *Helminthologia*, 49(2), pp. 96–103.

Forestry Commission (2017) *Pitch canker of Pine (Fusarium circinatum)*. Forestry Commission, 231 Corstorphine Road, Edinburgh EH12 7AT, Tel: 0131 334 0303. Available at: https://www.forestry.gov.uk/forestry/BEEH-AEMD7F (Accessed: 2 February 2018).

Franceschi, V. R., Krokene, P., Christiansen, E. and Krekling, T. (2005) 'Anatomical and chemical defenses of conifer bark against bark beetles and other pests', *New Phytologist*, 167(2), pp. 353–376.

Gamboa, J., Muñoz, R. and Quiles, M. J. (2009) 'Effects of antimycin A and n-propyl gallate on photosynthesis in sun and shade plants', *Plant Science*, 177(6), pp. 643–647.

Gershenzon, J. (1998) 'Plant defenses: surface protection and secondary metabolites.', in *Plant physiology*. Second Edi. Sunderland, MA, USA: Sinauer Associates, Inc., pp. 347–376.

Gordon, T. (2005) 'Pitch canker of pine, caused by *Fusarium circinatum*', *Phytopathology*. Forestry Commission, 231 Corstorphine Road, Edinburgh EH12 7AT, Tel: 0131 334 0303, 95(6), pp. S124–S125.

Gordon, T. R., Swett, C. L. and Wingfield, M. J. (2015) 'Management of *Fusarium* diseases affecting conifers', *Crop Protection*, 73, pp. 28–39.

Gould, N. *et al.* (2008) 'Physiological trade-offs associated with methyl jasmonate - induced resistance in *Pinus radiata*', *Canadian Journal of Forest Research*, 38(4), pp. 677–684.

Hahlbrock, K. and Scheel, D. (1989) 'Physiology and Molecular Biology of Phenylpropanoid Metabolism', *Annual Review of Plant Physiology and Plant Molecular Biology*, 40(1), pp. 347–369.

Hammerschmidt, R. and Kuć, J. (1982) 'Lignification as a mechanism for induced systemic resistance in cucumber', *Physiological Plant Pathology*, 20(1), pp. 61–71.

Heijari, J., Nerg, A. M., Kainulainen, P., Vuorinen, M. and Holopainen, J. K. (2008) 'Long-term effects of exogenous methyl jasmonate application on Scots pine (*Pinus sylvestris*) needle chemical defence and diprionid sawfly performance', *Entomologia Experimentalis et Applicata*, 128, pp. 162–171.

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

Heredia, J. B. and Cisneros-Zevallos, L. (2009) 'The effects of exogenous ethylene and methyl jasmonate on the accumulation of phenolic antioxidants in selected whole and wounded fresh produce', *Food Chemistry*. Elsevier Ltd, 115(4), pp. 1500–1508.

Higuchi, T. (1981) 'Biosynthesis of lignin', in Tanner, W. and Loewus, F. A. (eds) *Encyclopedia of Plant Physiology*. 13B edn. Berlin: Springer-Verlag, pp. 194–224.

Ichihara, Y., Fukuda, K. and Suzuki, K. (2000) 'Early Symptom Development and Histological Changes Associated with Migration of *Bursaphelenchus xylophilus* in Seedling Tissues of *Pinus thunbergii*', *Plant Disease*, 84(6), pp. 675–680.

ICNF (2013) 'IFN6 – Áreas dos usos do solo e das espécies florestais de Portugal continental em 1995, 2005 e 2010.', *6º Inventário Florestal Nacional*, p. 34.

Imanishi, S., Hashizume, K., Nakakita, M., Kojima, H., Matsubayashi, Y., Hashimoto, T., Sakagami, Y., Yamada, Y., and Nakamura, K. (1998) 'Differential induction by methyl jasmonate of genes encoding ornithine decarboxylase and other enzymes involved in nicotine biosynthesis in tobacco cell cultures', *Plant Molecular Biology*, 38(6), pp. 1101–1111.

Johnson, P. R. and Ecker, J. R. (1998) 'The ethylene gas signal transduction pathway: A molecular perspective', *Annual Review of Genetics*, 32(1), pp. 227–254.

Jones, J. T., Moens, M., Mota, M., Li, H., and Kikuchi, T. (2008) '*Bursaphelenchus xylophilus*: Opportunities in comparative genomics and molecular host-parasite interactions', *Molecular Plant Pathology*, 9(3), pp. 357–368.

De Jong, F., Hanley, S. J., Beale M. H. and Karp, A. (2015) 'Characterisation of the willow phenylalanine ammonia-lyase (PAL) gene family reveals expression differences compared with poplar',

Phytochemistry, 117, pp. 90–97.

Kamata, N. (2008) 'VI-32 Integrated Pest Management of Pine Wilt Disease in Japan -Tactics and Strategies-.pdf', in Zhao, B. G. et al. (eds) *Pine Wilt Disease*. Springer International Publishing, pp. 304–332.

Karban, R. and Baldwin, I. T. (1997) *Induced responses to herbivory (interspecific interactions)*. Illinois: University of Chicago Press.

Kaushik, P., Andújar, I., Vilanova, S., Plazas, M., Gramazio, P., Herraiz, F. J., Brar, N. S. and Prohens, J. (2015) 'Breeding vegetables with increased content in bioactive phenolic acids', *Molecules*, 20(10), pp. 18464–18481.

Kepczyńska, E. and Król, P. (2012) 'The phytohormone methyl jasmonate as an activator of induced resistance against the necrotroph *Alternaria porri f. sp. solani* in tomato plants', *Journal of Plant Interactions*, 7(4), pp. 307–315.

Kieber, J. (1998) 'Ethylene', in Taiz, L. and Zeiger, E. (eds) *Plant Physiology*. Second Edi. Sunderland, MA, USA: Sinauer Associates, Inc., pp. 652–670.

Kiyohara, T. and Tokushige, Y. (1971) 'Inoculation experiments of a nematode, *Bursaphelenchus sp.*, onto pine trees', *J Jpn For Soc*, 53, pp. 210–218.

Klarzynski, O., Plesse, B., Joubert, J. -M., Yvin, J. -C., Kopp, M., Kloareg, B. and Fritig, B. (2000) 'Linear β-1 , 3 Glucans Are Elicitors of Defense Responses in Tobacco', *Plant physiology*, 124(3), pp. 1027–1037.

Król, P., Igielski, R., Pollmann, S. and Kepczynska, E. (2015) 'Priming of seeds with methyl jasmonate induced resistance to hemi-biotroph *Fusarium oxysporum f.sp. lycopersici* in tomato via 12-oxo-phytodienoic acid, salicylic acid, and flavonol accumulation', *Journal of Plant Physiology*, 179, pp. 122–132.

Lambert, E., Faizal, A. and Geelen, D. (2011) 'Modulation of triterpene saponin production: In vitro cultures, elicitation, and metabolic engineering', *Applied Biochemistry and Biotechnology*, 164(2), pp. 220–237.

Långström, B. and Day, K. R. (2004) 'Damage, Control and Management of Weevil Pests, Especially *Hylobius Abietis*', in Lieutier, F. et al. (eds) *Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis*. First Edit. Springer International Publishing, pp. 415–444.

Lavery, P. B. and Mead, D. J. (1998) '*Pinus radiata*: a narrow endemic from North America takes on the world', in Richardson, D. M. (ed.) *Ecology and biogeography of Pinus*. Richardson. Cambridge University Press, pp. 432–449.

Lichtenthaler, H. K., Schwender, J., Disch, A. and Rohmer, M (1997) 'Biosynthesis of isoprenoids in higher plants chloroplasts proceeds via a mevalonate pathway', *FEBS Letters*, 400, pp. 271–274.

Maruta, T., Inoue, T., Noshi, M., Tamoi, M., Yabuta, Y., Yoshimura, K., Ishikawa, T. and Shigeoka, S. (2012) 'Cytosolic ascorbate peroxidase 1 protects organelles against oxidative stress by wounding- and jasmonate-induced H2O2 in *Arabidopsis* plants', *Biochimica et Biophysica Acta - General Subjects*. Elsevier B.V., 1820(12), pp. 1901–1907.

Miller, B., Madilao, L. L., Ralph, S. and Smith, M. (2005) 'Insect-Induced Conifer Defense . White Pine Weevil and Methyl Jasmonate Induce Traumatic Resinosis , de Novo Formed Volatile Emissions , and Accumulation of Terpenoid Synthase and Putative Octadecanoid Pathway Transcripts in Sitka Spruce 1 [w]', *Plant physiology*, 137(January), pp. 369–382.

Mittler, R., Vanderauwera, S., Gollery, M. and Van Breusegem, F. (2004) 'Reactive oxygen gene network of plants', *Trends in Plant Science*, 9(10), pp. 490–498.

Moreira, X., Sampedro, L. and Zas, R. (2009) 'Defensive responses of *Pinus pinaster* seedlings to exogenous application of methyl-jasmonate: Concentration effect and systemic response', *Environmental and Experimental Botany*, 67, pp. 94–100.

Moses, T., Papadopoulou, K. K. and Osbourn, A. (2014) 'Metabolic and functional diversity of saponins, biosynthetic intermediates and semi-synthetic derivatives', *Critical Reviews in Biochemistry and Molecular Biology*, 49(6), pp. 439–462.

Mota, M. M., Braasch, H., Bravo, M. A., Penas, A. C., Burgermeister, W., Metge, K., and Sousa, E. (1999) 'First report of *Bursaphelenchus xylophilus* in Portugal and in Europe', *Nematology*, 1(7), pp. 727–734.

Mota, M. M. and Vieira, P. R. (2008) *Pine Wilt Disease a Worldwide Threat*. Edited by M. M. Mota and P. R. Vieira. Dordrecht: Springer International Publishing.

Naves, P. M., Camacho, S., Sousa, E. M. and Quartau, J. A. (2007) 'Transmission of the pine wood nematode *Bursaphelenchus xylophilus* through feeding activity of *Monochamus galloprovincialis* (Col., Cerambycidae)', *Journal of Applied Entomology*, 131(1), pp. 21–25.

Niggeweg, R., Michael, A. and Martin, C. (2004) 'Engineering plant with increased levels of antioxidant chlorogenic acid', *Nature biotechnology*, 22(6), pp. 746–754.

Nunes da Silva, M., Solla, A., Sampedro, L., Zas, R. and Vasconcelos, M. W. (2015) 'Susceptibility to the pinewood nematode (PWN) of four pine species involved in potential range expansion across Europe', *Tree Physiology*, 35(9), pp. 987–999.

Nunes da Silva, M., Lima, M. R. M. and Vasconcelos, M. W. (2013) 'Susceptibility evaluation of *Picea abies* and *Cupressus lusitanica* to the pine wood nematode (*Bursaphelenchus xylophilus*)', *Plant Pathology*, 62(6), pp. 1398–1406. doi: 10.1111/ppa.12037.

Nunes da Silva, M., M., Solla, A., Sampedro, L., Zas, R. and Vasconcelos, M. W. (2013) 'The role of induced defenses in *Pinus spp*. against the pine wood nematode (*Bursaphelenchus xylophilus*)', in XIII Congresso Luso-Espanhol de Fisiologia Vegetal, 24-27 July 2013. Lisbon, Portugal, pp. 273.

Oh, W. S. *et al.* (2009) 'Identification and characterization of a dual-acting antinematodal agent against the pinewood nematode, *Bursaphelenchus xylophilus*', *PLoS ONE*, 4(11). doi: 10.1371/journal.pone.0007593.

Olivoto, T., Nardino, M., Carvalho, I. R., Follmann, D. N., Szareski, V. J., Ferrari, M., de Pelegrin, A. J., and Queiróz de Souza, V. (2017) 'Plant secondary metabolites and its dynamical systems of induction in response to environmental factors: A review', *African Journal of Agricultural Research*, 12(2), pp. 71–84.

OMAIAA (2011) *A comercialização do pinhão em Portugal*. Available at: http://www.observatorioagricola.pt/item.asp?id_item=124 (Accessed: 1 February 2018).

Osbourn, A. E. (1996) 'Preformed Antimicrobial Compounds and Plant Defense against Fungal Attack', *The Plant Cell*, 8(10), pp. 1821–1831.

Osbourn, A., Goss, R. J. M. and Field, R. A. (2011) 'The saponins – polar isoprenoids with important and diverse biological activities', *Natural Product Reports*, 28(7), p. 1261.

Overmyer, K., Overmyer, K., Tuominen, H., Kettunen, R., Betz, C., Langebartels, C., Sandermann, H. Jr., and Kangasjarvi, J. (2000) 'Ozone-sensitive *Arabidopsis* rcd1 mutant reveals opposite roles for ethylene and jasmonate signaling pathways in regulating superoxide-dependent cell death', *Plant Cell*, 12, pp. 1849–1862.

Patel, S. B., Santani, D., Shah M. B. and Patel, V. S. (2012) 'Anti-hyperglycemic and Anti-hyperlipidemic Effects of *Bryonia Laciniosa* Seed Extract and its Saponin Fraction in Streptozotocin-induced Diabetes in Rats', *Journal of Young Pharmacists*, 4(3), pp. 171–176.

Pauwels, L., Morreel, K., De Witte, E., Lammertyn, F., Van Montagu, M., Boerjan, W., Inze, D., and Goossens, A. (2008) 'Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured *Arabidopsis* cells', *Proceedings of the National Academy of Sciences*, 105(4), pp. 1380–1385.

Peleman, J., Boerjan, W., Engler, G., Seurinck, J., Botterman, J., Alliotte, T., Van Montagu, M. and Inz, D. (1989) 'Strong Cellular Preference in the Expression of a Housekeeping Gene of *Arabidopsis thaliana* Encoding', *The Plant Cell*, 1(January), pp. 81–93.

Petersson, M. and Örlander, G. (2003) 'Effectiveness of combinations of shelterwood, scarification, and feeding barriers to reduce pine weevil damage', *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere*, 33(1), pp. 64–73.

Pieterse, C. M. J., Ton, J. and van Loon, L. C. (2001) 'Cross-talk between plant defence signalling pathways: boost or burden ?', *AgBiotechNet*, 3, pp. 1–8.

Pimentel, C. S., Gonçalves, E. V., Firminoa, P. N., Calvão, T., Fonsecad, L., Abrantesd, I., Correiac, O. and C.

Máguas (2017) ‘Differences in constitutive and inducible defences in pine species determining susceptibility to pinewood nematode’, *Plant Pathology*, 66(1), pp. 131–139.

Le Provost, G., Herrera, R., Paiva, J. A., Chaumeil, P., Salin, F. and Plomion, C. (2007) ‘A micromethod for high throughput RNA extraction in forest trees’, *Biological Research*. Sociedad de Biología de Chile, 40(3), pp. 291–297.

Rajendran, S., Lin, I-W., Chen, M-J., Chen, C.-Y., and Yeh, K-W. (2014) ‘Differential activation of sporamin expression in response to abiotic mechanical wounding and biotic herbivore attack in the sweet potato’, *BMC Plant Biology*, 14(1), pp. 1–21.

Randall, J., Tisserat, N. and Todd, T. (2006) ‘Prevention of pine wilt of Scots pine (*Pinus sylvestris*) with systemic abamectin injections’, *Arboriculture and Urban Forestry*, 32(5), pp. 195–201.

Ravikumar, M. C., Singh, H. and Garampalli, R. H. (2016) ‘Comparative evaluation of long-term storage techniques on viability and virulence of *Alternaria solani*’, *Journal of Taibah University for Science*, 10(4), pp. 607–613.

Rich, J. R., Dunn, R. and Noling, J. (2004) ‘Nematicides: Past and present uses.’, in Chen, Z. X., Chen, S. Y., and Dickson, D. W. (eds) *Nematology: Advances and Perspectives Vol 2. Nematode Management and Utilization*. CABI Publishing, pp. 1041–1082.

Rodrigues, K. F., Hesse, M. and Werner, C. (2000) ‘Antimicrobial activities of secondary metabolites produced by endophytic fungi from *Spondias mombin*’, *Journal of Basic Microbiology*, 40(4), pp. 261–267.

Roeder, S., Dreschler, K., Wirtz, M., Cristescu, S. M., van Harren, F. J. M., Hell, R. and Piechulla, B. (2009) ‘SAM levels , gene expression of SAM synthetase , methionine synthase and ACC oxidase , and ethylene emission from *N . suaveolens* flowers’, *Plant Molecular Biology*, 70, pp. 535–546.

Rojo, E., Leon, J. and Sanchez-Serrano, J. . (1999) ‘Cross-talk between wound signaling pathways determines local versus systemic gene expression in *Arabidopsis thaliana*’, *Plant Journal*, 20, pp. 135–142.

Roy, C. B., Srinivas, P. and Jacob, C. K. (2014) ‘Relative Efficacy of Long-Term Storage Methods on Survival and Virulence of *Corynespora cassiicola* and *Phytophthora meadii* Pathogenic on Rubber (Hevea Brasiliensis)’, *rubber Science*, 27(2), pp. 202–214.

Sampedro, L., Moreira, X. and Zas, R. (2011) ‘Costs of constitutive and herbivore-induced chemical defences in pine trees emerge only under low nutrient availability’, *Journal of Ecology*, 99(3), pp. 818–827.

Santos, C. S., Pinheiro, M., Silva, A. I., Egas, C. and Vasconcelos, M. W. (2012) ‘Searching for resistance genes to *Bursaphelenchus xylophilus* using high throughput screening’, *BMC Genomics*, 13(1), p. 599.

Schwelm, A. and Bradshaw, R. E. (2010) ‘Genetics of dothistromin biosynthesis of *Dothistroma septosporum*: An update’, *Toxins*, 2(11), pp. 2680–2689.

Seldal, T., Hegland, S. J., Rydgren, K., Rodriguez-Saona, C., and Töpper, J. P. (2016) ‘How to induce defense responses in wild plant populations? Using bilberry (*Vaccinium myrtillus*) as example’, *Ecology and Evolution*, 7(6), pp. 1762–1769.

Seo, H. S., Song, J. T., Cheong, J.-J., Lee, Y.-H., Lee, Y.-W., Hwang, I., Lee, J. S. and Cho, Y. D. (2001) ‘Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses.’, *Proceedings of the National Academy of Sciences of the United States of America*, 98(8), pp. 4788–93.

Silva, J. (2007) ‘Pinhais e Eucaliptais. A floresta cultivada. Volume 4’, *Árvores e Florestas de Portugal, Pinhais e Eucaliptais, a floresta cultivada*, p. 283.

Smith, J. L., De Moraes, C. M. and Mescher, M. C. (2009) ‘Jasmonate- and salicylate-mediated plant defense responses to insect herbivores, pathogens and parasitic plants’, *Pest Management Science*, pp. 497–503.

Solla, A., Pérez, A., Nunes da Silva, M., Vasconcelos, M. W., Sampedro, L., and Zas, R. (unpublished) *Resin canal changes induced by exogenous methyl jasmonate in Pinus spp.: Implications for resistance of trees to pathogens*.

Soriano, I. R., Riley, I., T., Potter, M. J. and Bowers, W. S. (2004) ‘Phytoecdysteroids: A novel defense against plant-parasitic nematodes’, *Journal of Chemical Ecology*, 30(10), pp. 1885–1899. doi: 10.1023/B:JOEC.0000045584.56515.11.

Sousa, E., Bravo, M. A., Pires, J., Naves, P., Penas, A. C., Bonifácio, L. and Mota, M. M.. (2001) '*Bursaphelenchus xylophilus* (Nematoda; Aphelenchoididae) associated with *Monochamus galloprovincialis* (Coleoptera; Cerambycidae) in Portugal', *Nematology*, 3(1), pp. 89–91. doi: 10.1163/156854101300106937.

Sousa, E., Naves, P., Bonifacio, L., Bravo, M. A., Penas, A. C., Pires, J. and Serrao, M. (2002) 'Preliminary survey for insects associated with *Bursaphelenchus xylophilus* in Portugal', *EPPO Bulletin*, 32(3), pp. 499–502. doi: 10.1046/j.1365-2338.2002.00597.x.

Szakiel, A. and Pa, C. (2011) 'Influence of environmental abiotic factors on the content of saponins in plants', pp. 471–491. doi: 10.1007/s11101-010-9177-x.

Tabor, C. W. and Tabor, H. (1984) 'Methionine Adenosyltransferase (S-Adenosyltransferase Synthetase) and S-Adenosyltransferase Decarboxylase', *Advances in Enzymology and Related Areas of Molecular Biology*, 56, pp. 251–270.

Tapias, R., Climent, J., Pardos, J. A. and Gil, L. (2004) 'Life Histories of Mediterranean Pines', 171(1), pp. 53–68.

Turner, J. G., Ellis, C. and Devoto, A. (2002) 'The jasmonate signal pathway.', *The Plant cell*, 14 Suppl, pp. S153–S164. doi: 10.1105/tpc.000679.

UN (2011) *The European forest sector outlook Study II 2010-2030, United Nations publication*. Available at: http://live.unece.org/forests/outlook/welcome.html%0AUnited.

Veitch, G. E., Boyer, A. and Ley, S. V. (2008) 'The Azadirachtin Story', *Chem. Int. Ed.*, 47(49), pp. 9402–9429.

Vicente, C., Espada, M., Vieira, P. and Mota, M. (2012) 'Pine Wilt Disease: A threat to European forestry', *European Journal of Plant Pathology*, 133(1), pp. 89–99. doi: 10.1007/s10658-011-9924-x.

Wang, J. W. and Wu, J. Y. (2005) 'Nitric Oxide is Involved in Methyl Jasmonate- induced Defense Responses and Secondary Metabolism Activities of *Taxus* Cells', *Plant Cell Physiology*, 46(6), pp. 923–930. doi: 10.1093/pcp/pci098.

Wasternack, C. and Parthier, B. (1997) 'Jasmonate-signalled plant gene expression', *Trends in plant science*, 2(8), pp. 302–307. Available at: papers://ae875177-834e-4ba8-8523-120292c79891/Paper/p1724.

Wingfield, M. J. (1983) 'Transmission of pine wood nematode to cut timber and girdled trees.', *Plant Disease*, 67(1), pp. 35–37. doi: citeulike-article-id:13504120.

Xu, L., Zhu, L., Tu, L., Liu, L., Yuan, D., Jin, L., Long, L., and Xianlong Zhang (2011) 'Lignin metabolism has a central role in the resistance of cotton to the wilt fungus *Verticillium dahliae* as revealed by RNA-Seq-dependent transcriptional analysis and histochemistry', *Journal of Experimental Botany*, 62(15), pp. 5607–5621. doi: 10.1093/jxb/err245.

Yamada, T. (2008) 'Biochemical responses in pine trees affected by pine wilt disease', in *Pine Wilt Disease*, pp. 223–234. doi: 10.1007/978-4-431-75655-2_22.

Yang, S. and Hoffman, N. (1984) 'Ethylene biosynthesis and its regulation in higher plants', *Annual Review of Plant Physiology*, 35, pp. 155–89. doi: 10.1146/annurev.pp.35.060184.001103.

Yendo, A. C. A. *et al.* (2010) 'Production of plant bioactive Triterpenoid saponins: Elicitation strategies and target genes to improve yields', *Molecular Biotechnology*, 46(1), pp. 94–104. doi: 10.1007/s12033-010-9257-6.

Yu, M., Shena, L., Zhang, A. and Sheng, J. (2011) 'Methyl jasmonate-induced defense responses are associated with elevation of 1-aminocyclopropane-1-carboxylate oxidase in *Lycopersicon esculentum* fruit', *Journal of Plant Physiology*. Elsevier GmbH., 168(15), pp. 1820–1827. doi: 10.1016/j.jplph.2011.05.021.

Zas, R., Niklas, B., Göran, N., Cendána, C., Hellqvist, C. and Sampedro, L. (2014) 'Exploiting jasmonate-induced responses for field protection of conifer seedlings against a major forest pest, *Hylobius abietis*', *Forest Ecology and Management*. Elsevier B.V., 313, pp. 212–223. doi: 10.1016/j.foreco.2013.11.014.

Zehra, A., Meena, M., Dubey, M. K., Aamir, M. and Upadhyay, R. S. (2017) 'Activation of defense response in tomato against *Fusarium* wilt disease triggered by *Trichoderma harzianum* supplemented with exogenous chemical inducers (SA and MeJA)', *Revista Brasileira de Botanica*, 40(3), pp. 651–664. doi: 10.1007/s40415-017-0382-3.

Zhao, B. G., Futai, K., Sutherland, J. R. and Takeuchi, Y. (2008) *Pine wilt disease*, *Pine Wilt Disease*. doi: 10.1007/978-4-431-75655-2.