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ORIGINAL ARTICLE

Resistance of *Pinus pinea* to *Bursaphelenchus xylophilus* explained by the dynamic response of phytohormones, antioxidant activity, and stress-related gene expression

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Abstract

Key message **The effects of MJ on pine trees are species-specific and trigger a resistant phenotype to the PWN. A more dynamic response of hormones and gene expression in *Pinus pinea* explains the high resistance to *Bursaphelenchus xylophilus* of this species.**

Abstract Knowledge on hormonal and genetic mechanisms of pine trees in response to the pinewood nematode (PWN; *Bursaphelenchus xylophilus*) is limited. To describe tree defence strategies against *B. xylophilus*, this study used the plant stress hormone methyl jasmonate (MJ) on four pine species with different susceptibility (*Pinus pinaster* < *P. radiata* ≈ *P. sylvestris* < *P. pinea*). Three-year-old trees were sprayed with MJ at 0, 25, and 50 mM, and 2 months later challenged with the PWN. Multiple samples were taken to assess nematode content, oxidative stress, secondary metabolites, phytohormone levels, and stress-related gene expression. Nematode infestation in trees correlated negatively with the water content of needles and phenolics of stems, and positively with the concentration of indole-3-carboxylic acid in stems. MJ spray reduced in a dose-dependent manner the nematode content in *P. pinaster* and *P. sylvestris*. The effects of MJ were species-specific, although a more pronounced impact was observed in the susceptible *P. pinaster* species, leading to a decrease of chlorophyll and water loss and to the upregulation of the gene involved in the biosynthesis of terpenoids (*AFS*). After MJ spray, increased levels of JA-Ile were observed in *P. pinea* only. Hormone profiling, predisposition to activate antioxidant response, and gene expression in *P. pinea* trees provide evidence of why this species is highly resistant to *B. xylophilus*. On the contrary, the lack of effective hormonal changes in *P. pinaster* explained the lack of defence responses to *B. xylophilus* of this susceptible species. This study is a first approach to explore biochemical, molecular, and hormonal interactions between *Pinus* species and the PWN, and presents unprecedented insights into alterations induced by exogenous MJ in regulating defence mechanisms in pine trees.

Keywords Maritime pine · Scots pine · Stone pine · Radiata pine · Jasmonic acid · OPDA · Salicylic acid · Salicyloyl glucose ester · Abscisic acid · Indole-3-acetic acid

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Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, native to North America, is responsible for causing pine wilt disease (PWD), a quarantine disease that poses a significant threat to the survival of temperate pine forests (Futai 2013; Back et al. 2024). With no effective means of control currently available, the PWN has led to the demise of millions of pine trees since the early twentieth century, spanning from East Asia to the Iberian Peninsula (Shin et al. 2008; Back et al. 2024). Adult *Monochamus* spp. beetles serve as the primary carriers responsible for transporting and transmitting *B. xylophilus* from diseased to healthy trees, hosting the nematodes within their respiratory system (Naves et al. 2007). Upon reaching adulthood and exiting the tree, *Monochamus* can introduce the nematodes to previously uninfected pine trees, thus continuing the cycle of infection and disease spread (Naves et al. 2007). During early stages of the disease, *B. xylophilus* feeds on the resin duct parenchyma cells of the host, resulting in damage to the cambium, cortex, phloem, and resin duct tissues. During advanced stages of the disease, a decrease of water potential, transpiration, and photosynthesis is observed, which ultimately result in the development of leaf chlorosis and wilting. Increased lignin production and antioxidant activity in the stem were frequently associated with PWN resistance, i.e., the ability of trees to prevent or reduce nematode reproduction and the extent of the symptoms (Hara and Takeuchi 2006; Nunes da Silva et al. 2013, 2015, 2021).

Despite decades of research, the reasons behind the tolerance of different pine species are poorly understood (Wang et al. 2023). Transcriptome analyses have been conducted to investigate changes in tree physiology associated with *B. xylophilus* infection, revealing the involvement of important gene families related to oxidative stress and flavonoid biosynthesis (Santos et al. 2012; Park et al. 2020; Modesto et al. 2021), terpenoid secondary metabolism (Zhang et al. 2020), and pathogenesis-related proteins (Lee et al. 2019a; Chen et al. 2024). Moreover, a hypersensitive reaction is triggered by *B. xylophilus* in pines (Futai 2013), and the high susceptibility of pine trees/species to PWN infection might result from an inefficient trigger of hypersensitive response (Rodrigues et al. 2021; Xing et al. 2024). A higher number of oxidative stress response genes upregulated in resistant rather than in susceptible *Pinus pinaster* trees after PWN infection (Modesto et al. 2021), suggests reactive oxygen species (ROS) to play a significant role in PWD pathogenesis and resistance. Thioredoxins, small proteins encoded by the *TRX* gene, are involved in many biological processes, including redox signalling, and are key players in the maintenance of cellular redox homeostasis. In several pine species, shortly after PWN infection, upregulation of thioredoxins has been reported (Serrato et al. 2013).

Tree hormonal regulation may also play a pivotal role in host responses against the PWN, but this issue has been scarcely explored. Plant responses to necrotrophic pathogens, including the PWN, are primarily mediated by the jasmonic acid (JA) signalling pathway following recognition of plant damage. In *P. densiflora*, *P. massoniana*, *P. pinaster,* and *P. thunbergia*, the upregulation of JA responsive genes after inoculation have been observed (references in Modesto et al. 2022). In *P. pinaster*, induction of JA and salicylic acid (SA) was reported to determine effective defence response against the PWN (Modesto et al. 2021). Since JA and SA immune responses are often antagonistic, an increase in SA levels in PWN-susceptible individuals after inoculation was related to inhibition of the JA response (Modesto et al. 2021). This observation indicates that the JA pathway might also have a central role in response to PWN in several pine species, but further research is needed to understand whether SA inhibits the JA pathway. Methyl jasmonate (MJ) is a volatile, methyl ester derivative of the phytohormone jasmonic acid, and has been used for more than 20 years to study inducible defences in conifers (Huynh et al. 2024). MJ spray has been shown to trigger strong, effective, and lasting responses against chewing insects and necrotrophic pathogens in pine trees (López-Goldar et al. 2018; Lundborg et al. 2019; Mageroy et al. 2020; Vázquez-González et al. 2022), by enhancing the accumulation of soluble phenolics, lignin, resin, and the formation of traumatic resin ducts (Moreira et al. 2012, 2015; Zas et al. 2015; Nunes da Silva et al. 2015; López-Villamor et al. 2021). Moreover, it has been reported that foliar spray of MJ significantly reduced the PWN density in *P. pinaster* and *P. koraiensis* (López-Villamor et al. 2022; Chen et al. 2024) and the feeding rates of adult *Monochamus alternatus* in *P. massoniana* (Chen et al. 2020). Evaluating the biochemistry and gene expression of pine trees elicited by MJ may help to unveil the defence mechanisms behind pine–PWN interactions.

The main objectives of this study were to evaluate the individual and combined impact of MJ spray and *B. xylophilus* infection on tree physiology, and to quantify hormone content, stress-related metabolites, and gene expression in four pine species with contrasted susceptibility to the PWD. Specific objectives were to (i) characterize constitutive components in tissues that can explain different levels of susceptibility of pine species to PWN, (ii) test if MJ spray increases resistance of four European pines to PWN, and (iii) assess if increased resistance occurs because MJ spray mimics what constitutively happens in resistant species. The susceptible *P. pinaster*, the resistant *P. pinea*, and the intermediate in susceptibility *P. sylvestris* and *P. radiata* (Nunes da Silva et al. 2015) were used, since all tree species are widely

distributed in Europe and are potential hosts for the PWN vector, according to the European Food Safety Authority (EFSA 2012). We hypothesised that in these species, there are MJ- and PWN-induced differences in the content of hormones and other signalling metabolites. The single and interactive effects of pine species, MJ spray, and *B. xylophilus* infection were explored using a multiarray analysis of PWN density, chlorophyll concentration, oxidative status, biochemical defences, and expression of four genes related to stress.

Materials and methods

Plant material

Three-year-old *P. pinaster*, *P. pinea*, *P. sylvestris,* and *P. radiata* trees, were used. The geographical origins of the seed material assayed in each species were Massif Landais (France), ES03-La Mancha (Spain), Sierra de Guadarrama (Spain), and 03-Astur-Cántabro (Spain) provenances, respectively. The seeds were sown in 2 L containers, cultivated on peat and perlite (8:2 v/v), and fertilized with Granum (Soaga SL, Vilanova de Arousa, Spain) slow release fertilizer (NPK 11:22:9).

Experimental design

In April, when the 3-year-old trees were approximately 1.6–2.2 m high, 45 trees per species were divided into three groups and subjected to MJ spray at 0 (control), 25, and 50 mM concentrations (n = 15 trees). Two months after applying the hormone, 10 trees per species and MJ concentration were inoculated with *B. xylophilus*, while the remaining 5 trees per species and MJ concentration were mock inoculated with deionised water and used as controls. The experiment consisted of 180 trees, representing four tree species × three MJ treatments × 15 replicates, and was organized as a factorial block design with five blocks.

Methyl jasmonate (MJ) treatment and *Bursaphelenchus xylophilus* inoculation

Trees were sprayed with a suspension of MJ (Sigma-Aldrich, #39,924-52-2; 2.5% ethanol v/v in deionised water) at 0, 25, or 50 mM concentrations based on previous research (Vivas et al. 2012; Zas et al. 2014; López-Villamor et al. 2021). The solutions were shaken vigorously until a uniform milky emulsion was obtained. Each tree was sprayed with *ca*. 20 mL of each solution separately. Trees from each treatment were maintained in separate cabinets of the greenhouse for 3 days and then arranged in a fully randomized design. During the culturing period, the trees were watered every two days to allow favourable conditions for plant growth. Day- and night-time temperatures from MJ spray to plant sampling were 21.6 ± 3.8 and 17.8 ± 2.6 °C, respectively, daily air relative humidity ranged from 66 to 84%, and mean diurnal global radiation was 132.5 ± 101.5 W m^{-2}.

To establish the *B. xylophilus* strain 65 GO colony, barley grains with *Botrytis cinerea* mycelium were incubated for 7 days at 25 °C in the dark. Nematodes were then extracted using the Baermann funnel technique and left overnight at room temperature (Baermann 1917). The nematode inoculum was prepared as described by Roriz et al. (2011). After 2 months of MJ application, approximately 2000 nematodes were inoculated per tree by making a gentle wound on the bark of the stem, following procedure outlined in Nunes da Silva et al. (2021).

Nematode quantification and tissue sampling

Two months after inoculation, five out of the ten inoculated trees per species and MJ concentration were destructively sampled to quantify the nematode population size in the entire stem, using the Baermann funnel technique (Baermann 1917). The remaining five inoculated and mock-inoculated trees per species and MJ concentration were destructively sampled too. A subset of stem tissue from mock-inoculated trees (i.e., 3 segments of *ca*. 1 cm from the upper, middle, and lower portion of each plant) was recovered and submitted to the Baermann funnel technique to detect any possible cross-contamination. The remaining tissues were individually collected, flash-frozen, ground with liquid nitrogen, stored at − 80 °C, and used to assess (i) chlorophyll concentration and water content in needles, (ii) plant oxidative stress through lipid peroxidation and phenolic-related antioxidant capacity in stem samples, (iii) accumulation of chemical defences by lignin and non-volatile oleoresin quantification in stem samples, and (iv) plant damage signalling and regulation through hormone profiling and expression of stress-related genes, respectively, in stem samples. The selected time intervals were chosen based on a study conducted in *Pinus* species infected by the PWN (Nunes da Silva et al. 2015).

Biochemical analyses

Leaves were used for the quantification of water status and total chlorophyll concentration. Total chlorophylls were extracted with methanol from *ca*. 500 mg (fresh weight, FW), and sample absorbance was measured at 663 and 645 nm using a spectrophotometer (NanoPhotometer, Implen GmbH, Germany). Total chlorophyll concentration was quantified as $(8.02 \times A_{663} + 20.21 \times A_{645}) \times (0.0125 \times \text{dilution factor})/\text{FW}$ (Abadia et al. 1984). A subsample of *ca*. 1 g of

leaves was lyophilized, and water content was calculated as the relative weight loss (Tan et al. 2005).

To gain insight into plant oxidative damage and antioxidative potential, lipid peroxidation and soluble phenolic compounds were assessed in the stem. Lipid peroxidation, namely the concentration of malondialdehyde (MDA), was used as a proxy of oxidative damage and determined following Li et al. (2000). Soluble phenolic compounds were determined in *ca*. 300 mg of phloem and cortex tissues, previously lyophilized. Samples were extracted with aqueous methanol (1:1, v/v), sonicated for 15 min, and the concentration of soluble phenolics was determined by the Folin–Ciocalteu procedure at 740 nm in a Biorad 650 microplate reader (Bio-Rad Laboratories Inc., Philadelphia, PA, USA), using tannic acid as a standard (Moreira et al. 2012).

To assess the activation of induced plant defence, diterpenoids and lignin, two major groups of plant defensive compounds, were evaluated in the stem. Diterpenoids were estimated gravimetrically as the content of non-volatile resin after extraction with 5 mL of hexane, as detailed by Sampedro et al. (2011). The mass of the non-volatile resin residue was determined to the nearest 0.0001 g and expressed as mg of non-volatile resin g^{-1} stem dry weight (DW). Lignin was quantified by the acetyl bromide method following four 24 h sequential extractions with methanol, water, acetone, and hexane. Samples (*ca*. 10 mg DW) were digested with 12.5% acetyl bromide (in glacial acetic acid) for 2 h with vigorous stirring and centrifuged for supernatant recovery. Following homogenization with 700 µL of glacial acetic acid, 150 µL of NaOH 0.3 M, 50 µL of hydroxylamine hydrochloride 0.5 M, absorbances were recorded at 280 nm in a NanoPhotometer, and lignin concentration was determined through a lignin calibration curve (Hatfield et al. 1999).

Phytohormones and organic acids' concentration

Hormone concentration was determined in the phloem and cortex of the stem of the most susceptible and least susceptible pine species, i.e., *P. pinaster* and *P. pinea*, respectively. Five pines from both species were previously treated with MJ at 0 or 50 mM and mock- or PWN-inoculated. The plant hormones determined were the oxylipins jasmonic acid (JA), its conjugate (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) and its precursor 12-oxo-phytodienoic acid (OPDA), salicylic acid (SA) and its non-active derivatives salicyloyl glucose ester (SGE) and 2-O-β-glucoside (SAG), abscisic acid (ABA), the auxin indole-3-acetic (IAA), and the indole-3-carboxylic (I3CA), ferulic, and caffeic acids.

Hormones were extracted with an aqueous methanol solution (90:10) containing 0.01% of formic acid and a pool of deuterated and dehydrogenated hormonal internal standards as in Gamir et al. (2012). The extraction solution was partitioned twice against diethyl ether, concentrated until dryness in a centrifugal evaporator (SpeedVac) at room temperature and finally resuspended in 1 mL of an aqueous methanol solution (90:10) with 0.01% of formic acid. Hormones were chromatographically separated in an Acquity Ultra-Performance Liquid Chromatography system (UPLC) (Waters, Mildford, MA, USA) equipped with a Kinetex C18 analytical column (Phenomenex) connected to a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK), and quantified with calibration curves of commercial standards as in Gamir et al. (2014).

Gene expression

Four genes related to plant stress responses were studied using quantitative reverse transcription-polymerase chain reaction (RT-qPCR) in each species × treatment combination. Three biological replicates were used. Primer sequences of *HSP90* (heat shock protein 90, stress signalling), *TRX* (thioredoxin, related to oxidative stress), and *SAM2* (S-adenosylmethionine synthetase, lignin biosynthetic pathway) genes were obtained from Franco et al. (2011), and *AFS* (α-farnesene synthase, terpenoids synthesis) gene from Santos et al. (2012) (Table S1). The genes were selected based on their stable expression pattern and involvement in the *Pinus* × PWN interaction (Franco et al. 2011; Santos et al. 2012; Nunes da Silva et al. 2021).

Total RNA was extracted from stem samples using the Qiagen RNeasy Mini Kit (Qiagen, Ref: #74,904), single-stranded cDNA was synthesized using the iScript cDNA Synthesis Kit (Biorad, Ref: #170–8891), and gene expression was determined through the delta CT method ($2^{-\Delta Ct}$) using ubiquitin and 18S ribosomal RNA genes as control transcripts.

Statistical analyses

To analyse the nematode population size in the stem, a mixed model was employed considering pine species, MJ spray, and their interaction as fixed factors, and blocks as random factors. For physiological traits, a linear mixed model included pine species, MJ spray, nematode inoculation, and their corresponding interactions as fixed factors, and blocks as random factors. A similar model was used for the analysis of plant hormones but limited to the two selected pine species. All analyses were performed using the Proc Mixed procedure of the SAS System Software 9.4 (SAS Institute, Inc., Cary, NC, USA) (Littell et al. 2006), fitting mixed models with restricted maximum likelihood. In cases where normality in the residuals was not met, log transformation of the dependent variable was applied to achieve normality. Variance heterogeneity models were used across MJ doses when they significantly improved the likelihood of the models, as indicated by ratio tests when homoscedastic and

heteroscedastic models were compared. Tukey's tests were used to compare least square means. To evaluate gene relative fold of expression, ratios of gene values of PWN- and mock-inoculated trees were analysed using GraphPad Prism version 9.0 (GraphPad Software, Inc., California, USA), and the same models described above were used. The degree of correlation between all physiological and biochemical parameters was evaluated using Pearson's Matrix in GraphPad Prism 9.0.

Results

Nematode population

Two months after inoculation, the number of nematodes detected in the stems of *P. pinaster* was 4.5- and 67.5-fold higher than that observed in *P. radiata* and *P. pinea*, respectively. *Pinus pinea* displayed the lowest nematode count, approximately one-fifth of the initial inoculum density (i.e., ca. 2000 nematodes).

Significant variation in nematode population size was observed among pine species in response to MJ application, with a species-specific effect exerted by MJ (Fig. 1, significant species × MJ interaction in Table S2). MJ spray at 25 and 50 mM led to a significant reduction in the nematode population in *P. pinaster* and *P. radiata* but not in *P. pinea* and *P. sylvestris*. Samples from mock-inoculated trees did not contain any nematode.

Chlorophyll and water content in leaves

Infection by *B. xylophilus* diminished chlorophyll and leaf water content in *P. pinaster* only (30% and 40%, respectively) (Fig. 2A1 and B1). Both parameters were significantly influenced by the pine species, MJ spray, inoculation, and their interactions (Fig. 2 and Table S3), mostly because in *P. pinaster* trees the adverse effects of PWN on chlorophyll concentration and water content were reduced by MJ (Fig. 2A1 and B1), whereas in *P. sylvestris*, the adverse effects of PWN were enhanced by MJ (Fig. 2A4). A strong negative correlation between leaf water content and PWN density was observed (Fig. 3).

Plant oxidative stress

After inoculation, malondialdehyde (MDA) content significantly increased in the stem of all MJ-untreated pine species except in *P. radiata* (Fig. 4). The species, MJ, and inoculation effects on MDA levels were significant, and some interactions too (Table S3). The increase of MDA concentration observed in *P. pinea*, *P. radiata*, and *P. sylvestris* when MJ and inoculation treatments were jointly applied (Fig. 4), but not in *P. pinaster*, explained the triple interaction (Table S3).

Total phenolics negatively correlated with PWN density in the stem (Fig. 3). In inoculated trees, the concentration of phenols in the stem significantly decreased by up to 65% in *P. pinaster* and *P. sylvestris* (Fig. 4B1 and 3B4). Phenolic induction by MJ was not observed in *P. radiata* and phenolic induction when MJ and inoculation treatments were jointly applied was not observed in *P. sylvestris.*

Biochemical defences

Constitutively, pines species showed different concentrations of lignin and non-volatile resin in their stems (Fig. 5A and C). P. sylvestris contained 24 and 31% more lignin than *P. pinaster* and *P. pinea*, respectively, while *P. radiata* displayed intermediate levels (Fig. 5A). Lignin and MDA concentrations were positively correlated (Fig. 3). Lignin content significantly increased in MJ-treated trees, similarly in all pine species (non-significant species × MJ interaction;

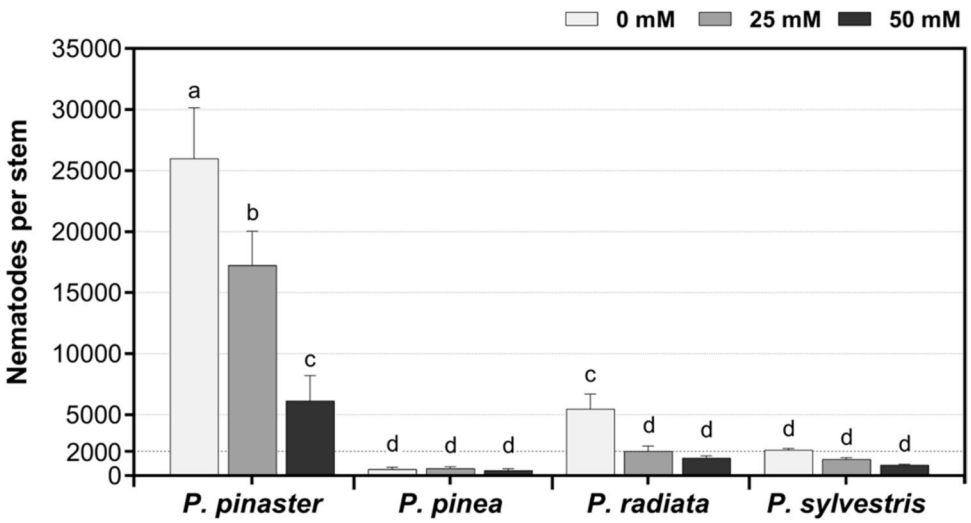


Fig. 1 Total nematode number in stems of pine trees, 4 months after methyl jasmonate spray at 0, 25, or 50 mM and 2 months after inoculation with *Bursaphelenchus xylophilus*. Trees were inoculated with ca. 2000 nematodes each. Bars represent the least square means of five biological replicates ± standard error as derived from the corresponding mixed models. Different letters indicate statistically different least square means at $p < 0.05$

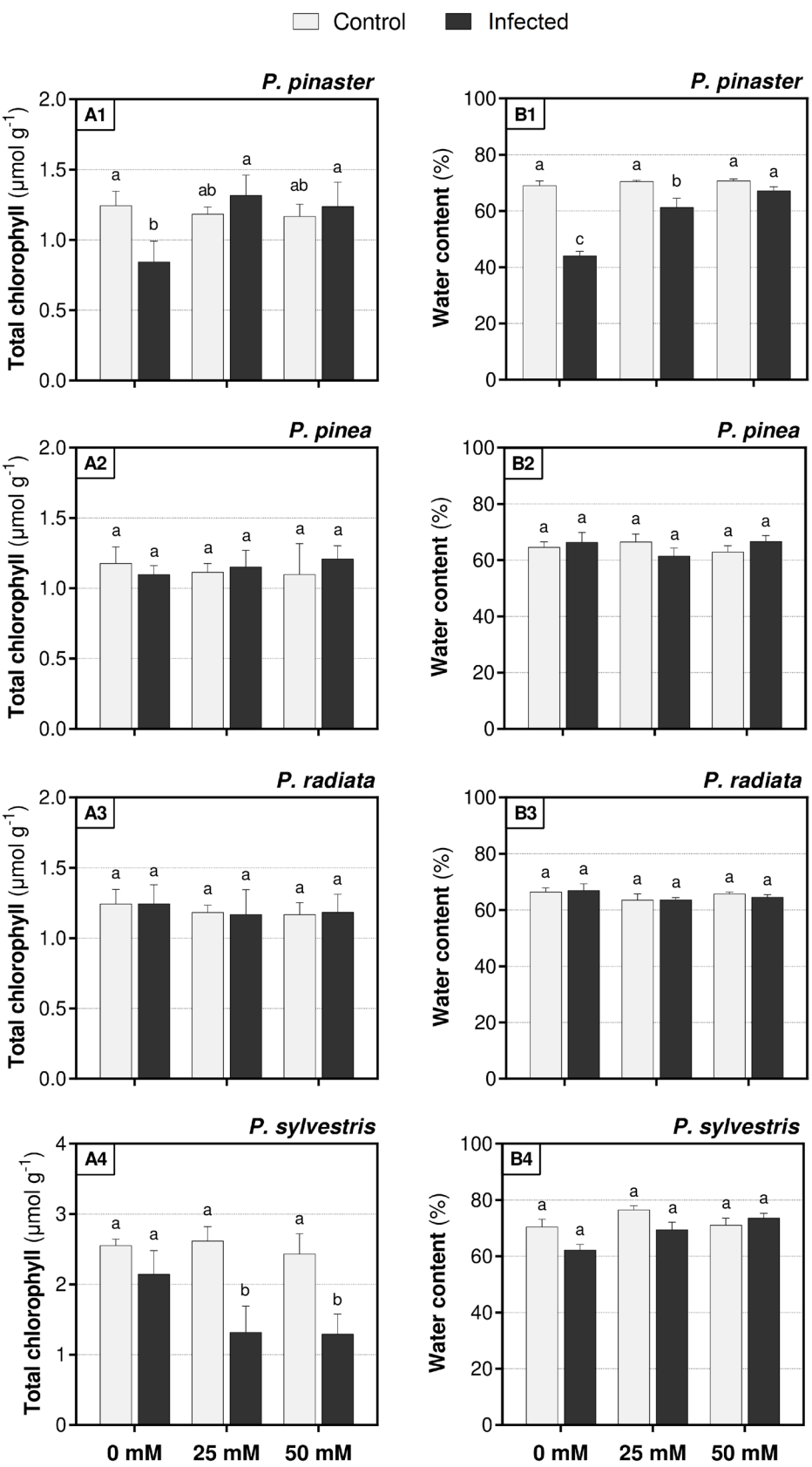


Fig. 2 Leaf chlorophyll concentration (**A**) and leaf water content (**B**) in *Pinus pinaster* (1), *P. pinea* (2), *P. radiata* (3), and *P. sylvestris* (4) after methyl jasmonate spray at 0, 25, or 50 mM and mock-inoculation with water (grey bar, control) or with *Bursaphelenchus xylophilus* (black bar, infected). Bars represent the mean of five biological replicates ± standard error of the mean and different letters indicate statistically different means at $p < 0.05$ (Tukey's tests)

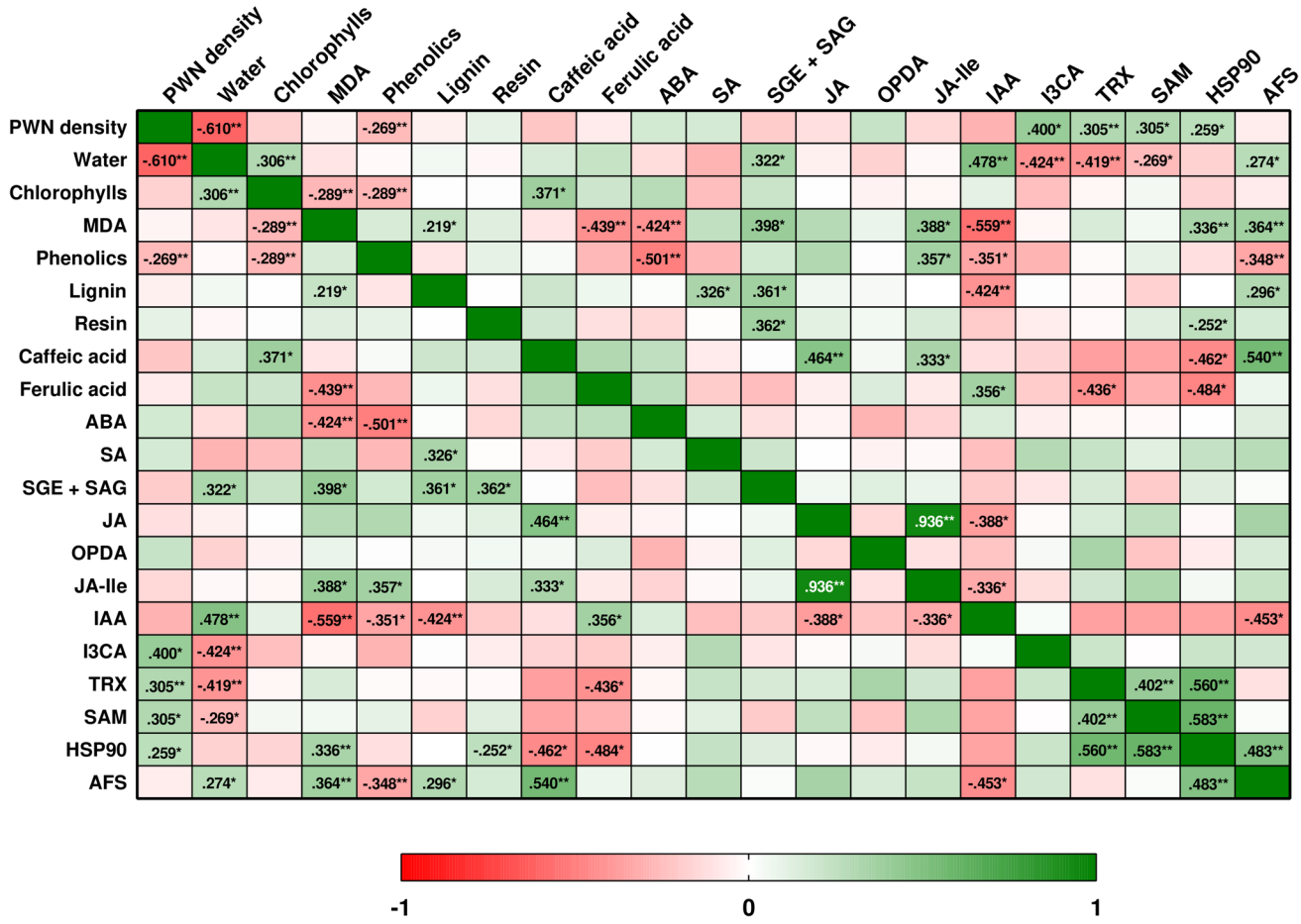


Fig. 3 Pearson's correlation matrix between the different variables analysed. In green positive correlations, in red negative correlations. Values of significant correlations are shown (*, $p < 0.05$; **, $p < 0.01$)

Table S3; results not shown), and especially if also inoculated with the PWN (significant MJ × inoculum interaction; Table S3). Averaged lignin values for the four pine species are provided in Fig. 5B. Constitutive non-volatile resins were highest in *P. pinea*, up to 50% higher than in the other species (Fig. 5C). However, non-volatile resins were not affected by MJ and PWN in any of the pine species (Table S3).

Phytohormones and organic acids

After inoculation, no changes of JA, JA-Ile, ABA, caffeic acid, and ferulic acid were observed in the stem of pines (Tables S4 and S5). However, *B. xylophilus* significantly increased SA in *P. pinea* and *P. pinaster* (Fig. 6A), SA derivatives and oxo-phytodienoic acid in *P. pinea* (Fig. 6B–C), and indole-3-carboxylic acid in *P. pinaster* (Fig. 6E). The nematode induced a significant decrease of IAA concentration in *P. pinaster* only (Fig. 6D). Indole-3-carboxylic acid was positively correlated with nematode density (Fig. 3).

Species-dependent effects of MJ were observed in most of the cases (Fig. 7; Table S4). For example, in *P. pinea* MJ significantly enhanced JA and JA-Ile, and decreased indole-3-carboxylic acid, whereas in *P. pinaster,* these hormones were not altered (Fig. 7; Table S4). When MJ and inoculation treatments were jointly applied, caffeic acid was highest if pines were MJ-sprayed, and ferulic acid was highest in *P. pinaster* (Fig. S1).

Expression of stress-related genes

PWN infection led to the upregulation of *TRX* gene in all pine species (Fig. 8). Moreover, *HSP90* and *SAM2* genes were upregulated in PWN-infected *P. pinaster* and *P. sylvestris*, *HSP90* gene in PWN-infected *P. pinea*, and *AFS* gene in PWN-infected *P. sylvestris*. The relative expression levels of *TRX*, *HSP90*, *SAM2*, and *AFS* were positively correlated with nematode density (Fig. 3).

HSP90, *SAM2*, and *AFS* expression was influenced by the pine species, MJ treatment, and their interaction (Fig. 8, Table S6). Moreover, the MJ and PWN combination led to

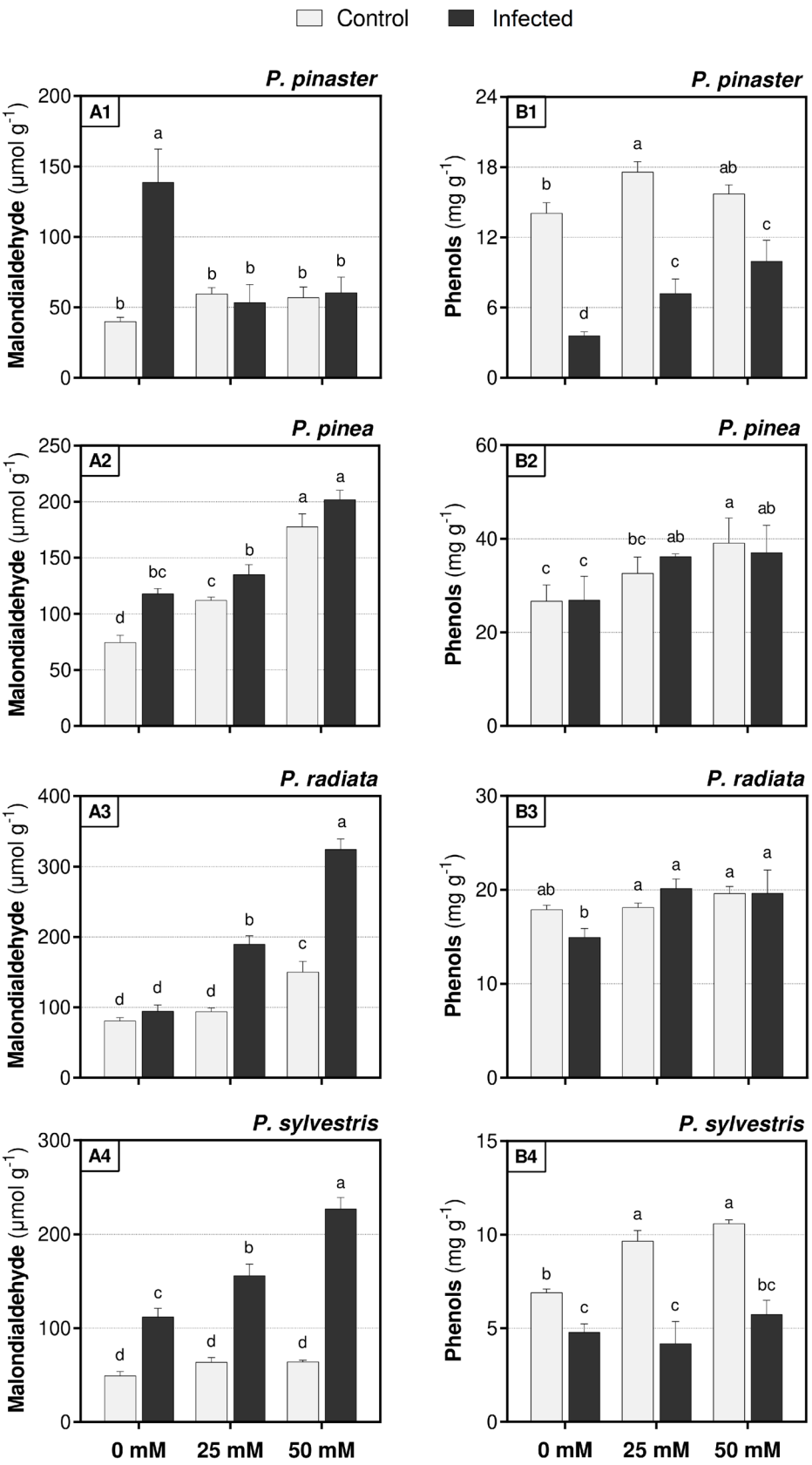


Fig. 4 Oxidative damage, expressed as concentration of malondialdehyde (MDA) (**A**) and total phenolics (**B**) in stems of *Pinus pinaster* (1), *P. pinea* (2), *P. radiata* (3), and *P. sylvestris* (4), after methyl jasmonate spray at 0, 25 or 50 mM and mock-inoculation with water (grey bar, control) or inoculation with *Bursaphelenchus xylophilus* (black bar, infected). Bars represent the mean of five biological replicates ± standard error of the mean and different letters indicate statistically different means at $p < 0.05$ (Tukey's tests)

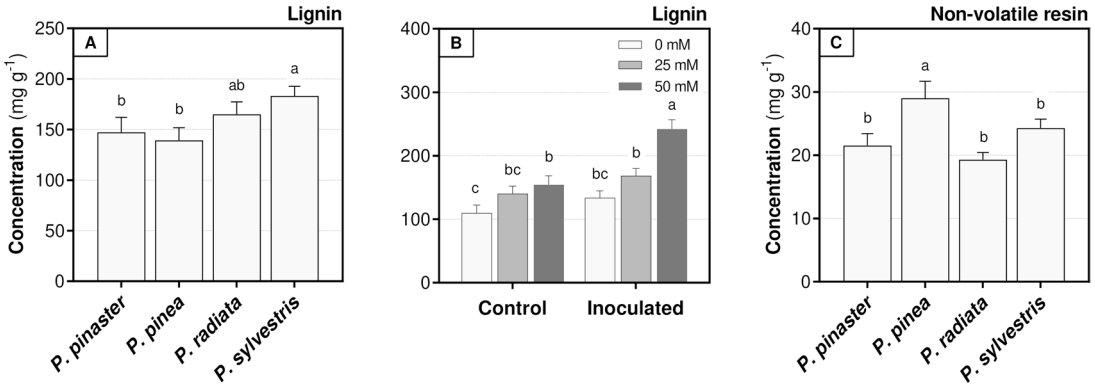


Fig. 5 Concentration in the stem of pines of constitutive lignin (**A**), averaged lignin after MJ spray at 0, 25, or 50 mM and mock-inoculation with water (control) or *Bursaphelenchus xylophilus* infection (inoculated) (**B**), and constitutive non-volatile resin (**C**). Bars represent the mean of five biological replicates ± standard error of the mean and different letters indicate statistically different means at $p < 0.05$ (Tukey's tests)

several synergistic and antagonistic effects. For instance, in PWN-infected *P. pinaster*, *AFS* no longer exhibited upregulation if trees were treated with MJ at 50 mM, while the opposite trend was observed in *P. sylvestris*; in *P. pinea*, infection following MJ at 25 mM inhibited the regulation of *TRX*, *HSP90*, and *SAM2*.

Discussion

The amount of nematodes per stem was significantly higher in *P. pinaster*, intermediate in *P. radiata* and *P. sylvestris*, and almost negligible in *P. pinea* (Fig. 1). Our findings align with prior reports of substantial variation in plant susceptibility to PWN across and within species (Nunes da Silva et al. 2015; Zas et al. 2015; Menéndez-Gutiérrez et al. 2017), suggesting the potential identification of traits of tolerance (Torres-Sánchez et al. 2023).

Infection by *B. xylophilus* induced chlorophyll and water content loss in needles, and oxidative stress and lignin accumulation in the stem

In *P. pinaster*, inoculation resulted in the loss of total chlorophyll and water content (Fig. 2). Previous studies in *P. pinaster* have also reported more negative effects on photosynthesis and water status shortly after infection (Santos et al. 2011; Nunes da Silva et al. 2021), compared to *P. pinea*, *P. radiata* and *P. sylvestris* (Nunes da Silva et al. 2015).

Lipid peroxidation was observed in all pine species following infection (Fig. 4A), indicating that PWN infestation leads to oxidative damage due to ROS generation. This is consistent with the overexpression of the stress-related *TRX* gene, observed in all species (Fig. 8). Previous studies have reported the upregulation of additional genes associated with oxidative stress in PWN-infected *P. pinaster* and *P. densiflora* (Santos et al. 2012; Lee et al. 2019a), suggesting that plant antioxidant mechanisms were activated in response to *B. xylophilus*. In our study, phenolic compounds, which have high antioxidant activity, showed a negative correlation with PWN density and total leaf chlorophyll (Fig. 3), and were lowest in the susceptible *P. pinaster* and *P. sylvestris* species (Fig. 4). Moreover, phenolic compounds showed a positive correlation with JA-Ile, suggesting that tree tolerance to the *B. xylophilus* results from the activation of the JA pathway and the increase of plant phenolic activity.

Tree infection led to the accumulation of lignin in plant tissues (Fig. 5) and was accompanied by the upregulation of *SAM2* in *P. pinaster* and *P. sylvestris* (Fig. 8). Lignin contributes to the reinforcement of plant cell walls and acts as a physical barrier against herbivores and pathogens (Vanholme et al. 2010). However, we did not find correlation between lignin and PWN density (Fig. 3).

Infection by *B. xylophilus* resulted in reduced SA derivatives and IAA in *P. pinaster* and increased SA, SA derivatives and OPDA in *P. pinea*

Regarding hormone regulation, infection by *B. xylophilus* resulted in decreased salicylic acid (SA) derivatives and indole-3-acetic acid (IAA) concentration in *P. pinaster*, and increased SA derivatives and OPDA in *P. pinea* (Fig. 6). The reduced IAA accumulation in PWN-infected *P. pinaster* may explain its susceptibility, as some pathogens can utilize this phytohormone in their colonization strategy, inducing phytostimulation and impairing basal plant defence mechanisms (Spaepen et al. 2007; González-Lamothe et al. 2012). Our results did not confirm the assumption that the SA and JA pathways acted antagonistically, as already has been mentioned for *P. pinaster*

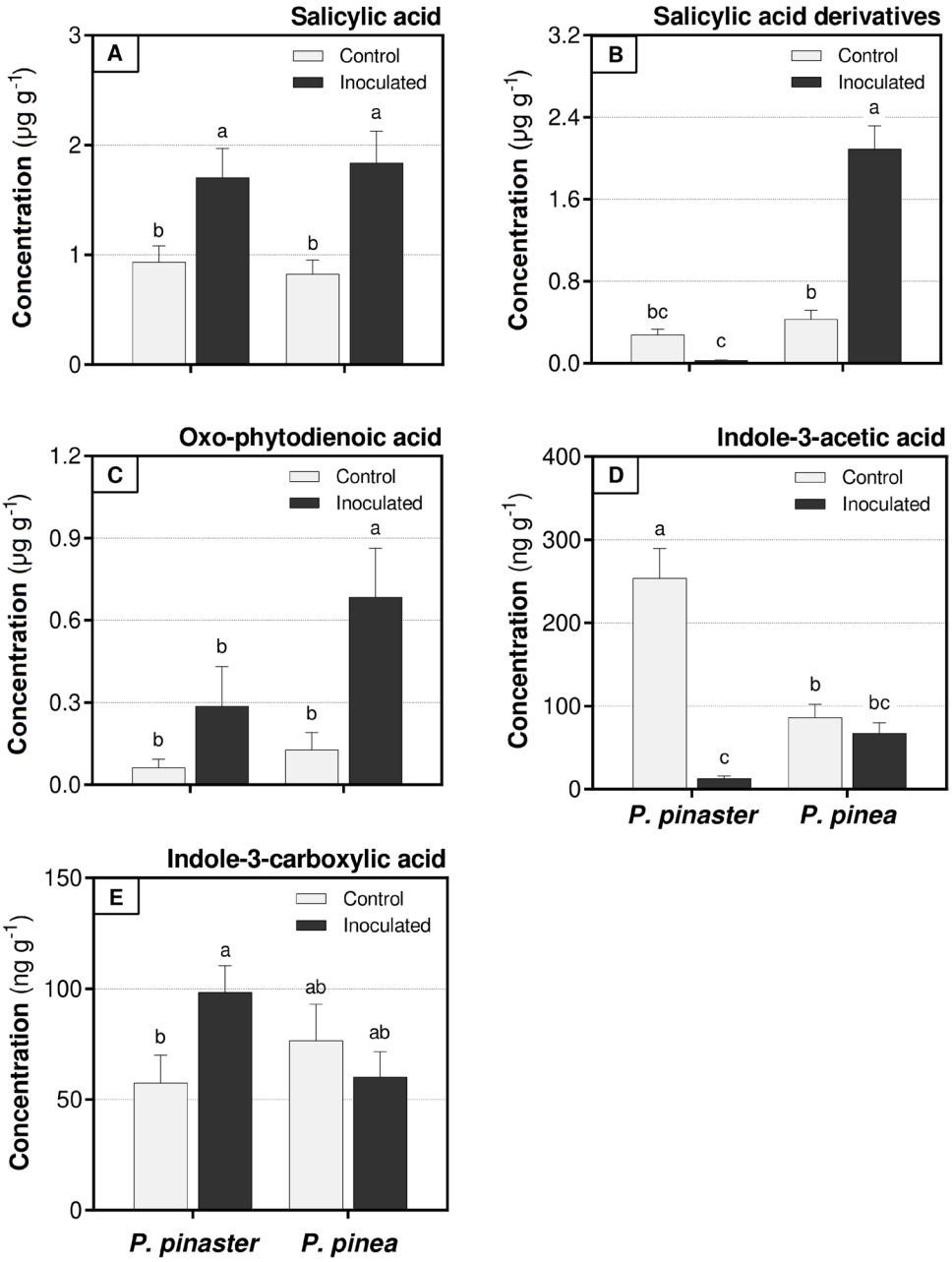


Fig. 6 In stems of *Pinus pinaster* and *P. pinea*, concentrations of salicylic acid (SA) (**A**), salicylic acid derivates salicyloyl glucose ester (SGE) and 2-O-β-glucoside (SAG) (**B**), 12-oxo-phytodienoic acid (OPDA) (**C**), indole-3-acetic acid (IAA) (**D**), and indole-3-carboxylic acid (I3CA) (**E**) after mock-inoculation with water or inoculation with *Bursaphelenchus xylophilus* (no MJ elicitation). Values represent the mean of five biological replicates ± standard errors and different letters indicate statistically different means at $p < 0.05$ (Tukey's tests)

after PWN infection (Modesto et al. 2021; Rodrigues et al. 2021). In the most resistant *P. pinea* species, after PWN infection, a concomitant increase in salicylic acid, salicylic acid derivatives, and JA precursor (OPDA) was observed, whereas in *P. pinaster*, after infection, salicylic acid derivatives and OPDA remained unchanged. The simultaneous activation of the SA and JA pathways in *P. pinea* following PWN infection may had implications in downstream stress-related regulatory mechanisms, such as plant innate immunity and efficient hypersensitive response (HR) (Ding and Ding et al. 2020). Therefore, the dual response observed in *P. pinea* may underpin the tolerance of this species to the PWN, and the lack of the dual response in *P. pinaster* may indicate the inefficient activation of HR in this pine in response to the PWN, as suggested elsewhere (Rodrigues et al. 2021).

Although the oxylipin OPDA is known as a biosynthetic precursor of JA, it is also a signalling molecule with functions related to plant growth and development independent of jasmonates (Aleman et al. 2022; Chini et al. 2023). In plant species that evolutionary do not produce JA (e.g., bryophytes and lycophytes), OPDA takes the place of JA in defence (Jiménez-Aleman et al 2022). In some plants that normally produce JA and JA-Ile, OPDA has been suggested to confer basal resistance against pathogens (e.g., *Solanum lycopersicum* against *Botrytis cinerea*; Scalschi et al. 2015).

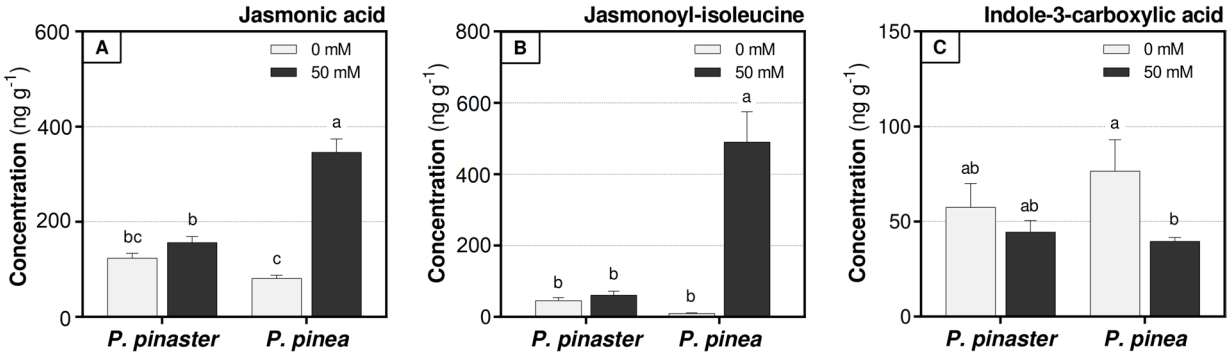


Fig. 7 In stems of *Pinus pinaster* and *P. pinea*, concentrations of jasmonic acid (JA) (**A**), (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) (**B**), and idole-3-carboxylic acid (**C**) 4 months after methyl jasmonate spray at 0 or 50 mM. Bars represent the mean of five biological replicates ± standard error of the mean and different letters indicate statistically different means at $p < 0.05$ (Tukey's tests)

Whether OPDA measured in our study lead to JA or not cannot be determined from the results.

Methyl jasmonate induces lipid peroxidation, phenolic activity, lignification, and hormone signalling in a species-specific manner in *Pinus*

When plants perceive stress, they activate a complex network of signalling pathways, often involving JA and SA phytohormones, which trigger the expression of stress-related genes resulting in the mobilization or biosynthesis of defensive molecules, e.g., phenolics, lignin, and resins (Fraser et al. 2016). The signalling pathways are initiated in response to stress or in response to damage caused by the accumulation of reactive oxygen species (ROS) in plant tissues, which, in turn, can cause oxidation of unsaturated fatty acids in membrane lipids and accumulation of lipid peroxidation products, including MDA. Previous work has shown that exogenous application of MJ induces in trees similar pathways as stress does, sometimes conferring protection against subsequent pathogen or insect attack (Vivas et al. 2012; López-Goldar et al. 2018; Lundborg et al. 2019; Martín-García et al. 2019; Chen et al. 2020; Puentes et al. 2021). Here, foliar spray of MJ in *Pinus* resulted into a complex interplay of physiological tree responses. Treated *P. pinea* and *P. radiata* showed increased concentrations of MDA (Fig. 4), similarly as observed in other conifers and in *Oryza sativa* (Wang and Wu 2005; Hung et al. 2006). MDA accumulation is primarily due to nitric oxide-induced oxidative bursts leading to H_2O_2 generation (Wang and Wu 2005; Hung et al. 2006). Following MJ application, the upregulation of *TRX* and *HSP90* was only observed in *P. pinea* (Fig. 8), probably indicating a more effective ROS-mediated stress signalling in this species. This provides evidence of a species-specific strategy in how pine species perceive and respond to stress signals like MJ, with the highest tolerance in *P. pinea* likely resulting from its predisposition to activate its antioxidant responses earlier and/or more efficiently than the PWN-susceptible species.

To counteract the potential harm caused by ROS, plants have developed intricate mechanisms involving enzymatic antioxidants and other metabolites, like phenolic compounds (Stagos, 2020). We observed an increase in phenolics following MJ, in accordance with Moreira et al. (2011). However, the lack of relation between phenols and MDA suggest that other antioxidant compounds apart from phenols in MJ-sprayed pines may be involved. These may include peroxidases, enzymes which have been reported to increase in soybeans following treatment with MJ (Mohamed and Latif 2017).

Lignin, which plays a role in plant defence to herbivory and pathogens, increased similarly in all pines with increasing MJ concentration (Table S3), supporting evidence of the ability of MJ to influence the allocation of chemical defences in pine seedlings (Moreira et al. 2012). Increased lignin content implies thicker and more rigid cell walls, which may enhance the resistance of trees to physical damage and pathogen invasion. In agreement with a previous study reporting upregulation of genes involved in lignin synthesis (Lee et al. 2019b), we observed that MJ enhanced *SAM2* expression in *P. pinea* (Fig. 8). This species-specific response also occurred with hormone accumulation after MJ spray, e.g., I3CA decreased and JA and JA-Ile increased after 50 mM in *P. pinea* only (Fig. 7). I3CA plays a role in plant defence by acting as a chemical deterrent against herbivores, inducing callose deposition in plant cells and influencing plant hormone signalling (Gamir et al. 2012). MeJA is converted to JA through the action of specific enzymes, including methyl jasmonate esterase, and JA can be further converted into its active form JA-Ile. JA-Ile acts as a signalling molecule that binds to specific JAZ proteins, releasing MYC transcription factors that activate the expression of various genes associated with plant defence, such as terpenoids and volatile organic compounds (Wang et al. 2020). Constitutive and

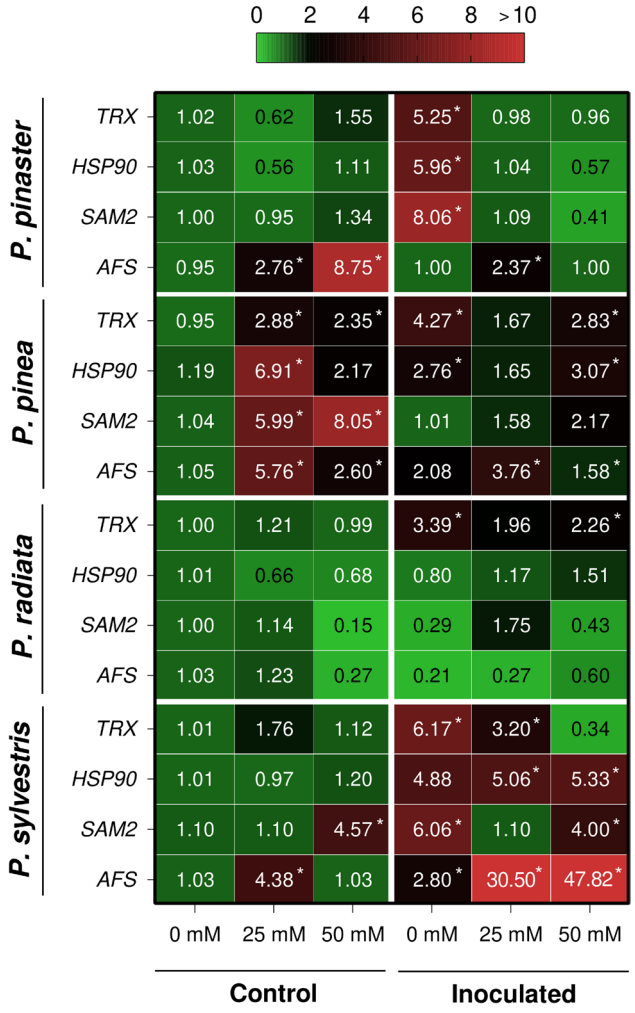


Fig. 8 Fold change of thioredoxin (TRX), S-adenosylmethionine synthetase (SAM2), heat shock protein 90 (HSP90), and α-farnesene synthase (AFS) gene expression in pine species after methyl jasmonate spray at 0, 25, or 50 mM and mock-inoculation with water or inoculation *Bursaphelenchus xylophilus*. Values represent the relative 2-ΔCt ratios in relation to the expression of housekeeping genes ($N=3$), and asterisks represent significant differences between housekeeping and target genes within each treatment ($p<0.05$). In green, downregulation of gene expression (fold changes lower than 0.5); in black and red, upregulation of gene expression (fold changes greater than 2)

induced levels of JA-Ile are only detected in resistant trees and are almost non-detectable in susceptible trees (Camisón et al. 2019). Consequently, the higher concentrations of JA and JA-Ile observed in *P. pinea* following MJ application may indicate a more dynamic response of hormonal changes in this tolerant species to the PWN.

Exogenous stimulation of plant defence by MeJA triggers a resistant phenotype to the PWN

In a previous study, the application of MJ at 25 mM to *P. pinaster* and of MJ at 5 mM to *P. koraiensis* reduced foliar symptoms and nematode density within stem tissues of PWN-infected plants compared to untreated controls (López-Villamor et al. 2022; Chen et al. 2024). This finding revealed that MJ had the potential to enhance resistance to PWN in susceptible pine species. In our study, MJ spray had the most severe impact on *P. pinaster* and, at the end of the experiment, PWN density reduction in this species was the highest. Reduction of total chlorophyll and water content loss, and upregulation of the stress-related *AFS* gene were observed in this species. Moreover, the PWN upregulated *TRX*, *HSP90*, and *SAM2*, and the upregulation of these three genes was negated by MeJA (Fig. 8), clearly pointing into as stress indicators in *P. pinaster*.

It has been suggested that terpene-based defence, induced by the JA pathway, mediated the response of *Pinus* species to the PWD (Chen et al. 2020; Hwang et al. 2021). Upregulation of *AFS*, involved in terpenoids synthesis, has been implicated in plant defence against the soybean cyst nematode (Lin et al. 2017). Here, we found that *AFS* transcription increased following inoculation of MJ-treated plants. Terpene production and expression of terpene synthesis genes have been reported to be higher in PWN-tolerant than in PWN-susceptible *P. thunbergia* trees (Wang et al. 2022). For this reason, essential oils from *Cymbopogon citratus*, rich in terpenes, have been suggested as potential biopesticides against the PWN (Faria and Barbosa 2024). Our study reports the effect of MJ in boosting JA-mediated defence in *P. pinaster*, including secondary metabolism, antioxidant activity, and stress-related gene expression as suggested in the previous work (Modesto et al. 2021). The results are a first step for considering MJ spray as part of an integrated strategy to prevent and manage the PWD in nurseries and young stands.

Overall, results showed that the effects of MJ spray were variable among the different pines, ultimately depending on the constitutive defensive capacity of each species. This confirms the hypothesis raised in the introduction. The effects of MJ application were particularly relevant when reducing the development of *B. xylophilus* in the high susceptible *P. pinaster* species. Moderate effects of MJ were observed in *P. pinea*, *P. radiata*, and *P. sylvestris*, probably due to their higher tolerance to *B. xylophilus*. This study is a first approach to explore the relevance of stress-related signals like hormones to better understand PWN resistance mechanisms in pine. For the first time in pine, the hormone content in the stem of susceptible and resistant pines exposed to MJ and PWN were described and compared. A more dynamic response of hormones and gene expression in *P. pinea* explains the resistance of this pine to *B. xylophilus*.

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Author contribution statement L.S., M.N.S., and A.S. lead the conceptualization and designed the experiment. R.Z. and L.S. performed plant elicitation. M.N.S. and C.S.S. inoculated the plants and assessed biochemistry and gene expression. J.G and V.F. run phytohormone determinations. M.N.S. and L.S. curated the data. M.W.V. and R.Z. acquired the funding and administered the project. All authors participated in data analyses, writing, and approved the final manuscript.

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Data availability The data of this study are available upon request to the first author.

Declarations

Conflict of interest All authors have no conflict of interest.

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