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RESEARCH

Species-dependent wiring of jasmonate signalling determines pine defence efficiency against *Bursaphelenchus xylophilus*

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Abstract

Pine wilt disease, caused by the pinewood nematode *Bursaphelenchus xylophilus*, poses a serious threat to pine forests worldwide, yet the hormonal and metabolic mechanisms that determine whether a pine species succumbs to or tolerates infection remain poorly understood. Jasmonic acid (JA) signalling is a central defence pathway in plants, but its precise contribution to species-dependent resistance in Pinus has not been systematically evaluated. This study dissects how targeted activation and inhibition of the JA signalling pathway remodel defence responses and nematode infection dynamics in three pine species with contrasting susceptibility to the pathogen (from most to least susceptible, *Pinus pinaster*, *P. pinea*, and *P. taeda*). Seedlings were treated with methyl jasmonate (MeJA) or the jasmonate inhibitor sodium diethyldithiocarbamate (DIECA) before nematode inoculation, and effects on symptom development, nematode colonisation, primary and secondary metabolism, oxidative stress, phytohormone profiles, and defence-related gene expression were assessed. *P. pinaster* developed clear symptoms and a marked increase in nematode numbers, whereas *P. pinea* and *P. taeda* remained largely asymptomatic. In *P. pinaster*, MeJA reduced both symptom severity and nematode proliferation, while DIECA impaired early defence activation although a delayed compensatory response was detectable. Across species, nematode infection triggered significant increases in phenolic compounds, flavonoids, and carotenoids, particularly in *P. pinea*, highlighting the contribution of secondary metabolism to plant resistance to the pinewood nematode. Overall, untreated infected plants showed higher lipid peroxidation compared with MeJA-treated, and phytohormone analyses revealed species-specific regulatory patterns. Gene expression patterns confirmed activation of phenylpropanoid and terpenoid pathways, with *PAL*, *GGPPS*, and *MYC2* showing species- and treatment-specific regulation. Together, these results are consistent with the existence of distinct regulatory architectures underlying susceptibility versus tolerance, and provide hormonal, metabolic, and transcriptional profiles that may inform future breeding and priming strategies against pine wilt disease, though further functional validation will be needed to establish causation.

Keywords Gene expression · Methyl jasmonate · Phytohormones · *Pinus pinaster* · *Pinus pinea* · *Pinus taeda*

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Key messages

- MeJA priming could optimise defence in susceptible species, reducing damage without pesticides.
- DIECA-mediated JA inhibition does not compromise resistance in tolerant P. pinea or P. taeda.
- Nematode tolerance refl ects integrated defence networks, guiding resistant Pinus genotype selection.

Introduction

The pinewood nematode (*Bursaphelenchus xylophilus*, PWN) is a significant biotic threat to forest ecosystems, particularly affecting pine species and leading to the devastating disease known as pine wilt (Abad et al. 2016; Vicente et al. 2012). As one of the most economically important pests of pine trees, the infection by PWN causes rapid mortality, primarily through the damage inflicted on vascular tissues (Kuroda 2008; Jones et al. 2008). The species *P. pinaster* is highly susceptible to *B. xylophilus*, while other species like *P. taeda* and *P. pinea* exhibit greater resistance (Feng et al. 2022; Menéndez-Gutiérrez et al. 2018; Nunes da Silva et al. 2025; Trindade et al. 2022). Therefore, unravelling the molecular and biochemical pathways that underlie these varying responses to PWN is crucial for developing effective strategies for forest management and tree protection.

Recent studies have shown that pine defence involves the coordinated activation of multiple mechanisms, including modulation of secondary metabolite production, antioxidant activity, and the regulation of phytohormonal signalling pathways, responses that are often triggered by plant interactions with pathogens and herbivores (López-Villamor et al. 2022; Nunes da Silva et al. 2025). Among these pathways, jasmonates (JAs) are central phytohormones that mediate plant defence under both biotic and abiotic stress (Ali and Baek 2020; Bari and Jones 2009; Wang et al. 2019; Wasternack 2014). Perception of pathogen-induced damage activates the jasmonate signalling cascade, leading to transcriptional reprogramming, upregulation of defence-related genes and enhanced synthesis of secondary metabolites, such as phenolic compounds, flavonoids, carotenoids, and anthocyanins, which contribute to pathogen resistance (Elkhouni et al. 2018; López-Villamor et al. 2022; Nunes da Silva et al. 2015; Treutter 2006). These JA-dependent responses are tightly intertwined with reactive oxygen species dynamics, where controlled oxidative bursts contribute to signalling but excessive oxidative stress results in cellular damage. In this context, malondialdehyde (MDA), a by-product of lipid peroxidation, is widely used as a marker of oxidative injury in plant tissues (Shakya 2023; Zhang et al. 2021).

Activation of jasmonate signalling has been associated with reduced severity of *B. xylophilus* infestation in susceptible pine species (Devoto and Turner 2003; Nunes da Silva et al. 2025; Smith et al. 2009; Turner et al. 2002). Jasmonates also modulate oxidative stress responses and secondary metabolite production, both of which are crucial components of defence against nematode-induced damage (Desmedt et al. 2020; Kaur et al. 2023; Nunes da Silva et al. 2021). However, the precise molecular mechanisms by which different pine species respond to nematode infection, and how jasmonate signalling is integrated with other defence pathways, remain poorly understood. Exogenous application of methyl jasmonate (MeJA), a bioactive derivative of JA, can enhance resistance against a wide range of pathogens, including nematodes (Hudgins et al. 2003; López-Villamor et al. 2021, 2022; Nunes da Silva et al. 2025; Sampedro et al. 2011). Conversely, inhibition of the jasmonate pathway by sodium diethyldithiocarbamate (DIECA) suppresses the ability of plants to mount effective defence responses in both forest and agricultural species, increasing susceptibility to herbivores, gall forming insects, and pathogens such as nematodes (Bruinsma et al. 2010; Cooper and Rieske 2011; Rajendran et al. 2014; Xie et al. 2022). These opposing effects of MeJA and DIECA provide a powerful experimental framework to dissect the contribution of jasmonate signalling to pine-pathogen interactions and to compare defence architectures among pine species with contrasting tolerance to *B. xylophilus*.

In addition to jasmonate signalling, other phytohormones such as abscisic acid (ABA), salicylic acid (SA), and auxins (IAA) play pivotal roles in mediating plant responses to stress, and their interactions are thought to contribute to an integrated defence network (Chen et al. 2025; Nunes da Silva et al. 2025; Zhu et al. 2024). Crosstalk between JA and SA pathways, in particular, has been linked to systemic acquired resistance (SAR) and induced systemic resistance (ISR), two complementary mechanisms that enhance plant immunity against diverse pathogens (Jeon et al. 2022; Koornneef and Pieterse 2008; Park et al. 2020; Tripathi et al. 2019; Van Loon 2007). The differential expression of key defence-related genes, including those involved in secondary metabolite biosynthesis, cell cycle regulation, and oxidative stress management (e.g. amorpha-4,11-diene synthase (*AFS*), APETALA2/ethylene response factor (*AP2*), E2F transcription factor (*E2F,*) geranyl–geranyl diphosphate synthase (*GGPPS*), heat shock protein 90 (*HSP90*), basic helix–loop–helix transcription factor (*MYC2*), phenylalanine ammonia-lyase (*PAL*), S-adenosylmethionine synthase 2 (*SAM2*), and thioredoxin (*TRX*)), further supports a central role for phytohormonal signalling in shaping transcriptional

reprogramming during plant defence (Amaral 2021; Chen et al. 2018; Noir et al. 2013; Nunes da Silva et al. 2025; Ralph et al. 2006; Shin et al. 2009).

Although evidence that MeJA can prime pine defences is accumulating, the species-specific regulatory logic connecting jasmonate signalling to effective downstream defence during *B. xylophilus* infection remains poorly characterised. It is not known whether susceptible and tolerant *Pinus* species differ in the amplitude or timing of JA biosynthesis, in the efficiency of JA conjugation to JA-Ile, in the degree of JA–SA crosstalk, or in how jasmonate signals are translated into secondary metabolite production and redox regulation. To address this gap, we applied chemical perturbation of the JA pathway—activation with methyl jasmonate (MeJA) and inhibition with sodium diethyldithiocarbamate (DIECA)—across three *Pinus* species spanning contrasting susceptibility levels (*P. pinaster*, *P. pinea* and *P. taeda*), and profiled hormonal, metabolic, and transcriptional responses at multiple time points following nematode inoculation. We predict that the three species differ in the dynamics of JA-Ile accumulation and JA–SA crosstalk, and that these differences are reflected in species-specific patterns of secondary metabolite production, redox regulation, and defence gene expression that collectively determine whether nematode colonisation and symptom development are effectively limited.

Materials and methods

Plant material and experimental design

A total of 324 seedlings of *Pinus pinaster*, *P. pinea*, and *P. taeda* (approximately 15 cm in height) were used for an inoculation assay with the pinewood nematode (PWN). Seedlings were grown in forest alveoli filled with commercial peat and maintained in a growth chamber under controlled conditions (24 °C, 80% relative humidity, 16 h light, and 8 h dark photoperiod). Plants were watered once per week throughout the experiment. Symptom development was assessed by quantifying leaf damage, and samples were collected for downstream analyses at 1, 14, and 28 days post-inoculation (dpi). The experiment was conducted between 11 March and 7 April 2021. These three time points were selected to capture early (1 dpi), intermediate (14 dpi), and late (28 dpi) phases of nematode colonisation and host defence, based on previously documented temporal progression of pine wilt disease symptoms in inoculated seedlings (Nunes da Silva et al. 2015; López-Villamor et al. 2022).

Seedlings were assigned to three treatment groups (108 plants per group, 36 per pine species). For MeJA treatment, 108 seedlings (36 of each species) were foliar sprayed with 4 mL of 5 mM methyl jasmonate (MeJA, 95%, Sigma-Aldrich, Missouri, USA) prepared in deionised water containing 2.5% ethanol (v/v), following a modified version of the protocol described by Zas et al. 2019). For pathway inhibition, another 108 seedlings (36 of each species) were foliar sprayed with 4 mL of 10 mM sodium diethyldithiocarbamate (DIECA, Alfa Aesar, Massachusetts, USA) prepared in deionised water containing 10% ethanol (v/v), as described by Cruz (2018). The remaining 108 seedlings (36 per species) were used as controls and were sprayed with 4 mL of deionised water. To prevent cross contamination, treatments were applied in separate areas. Control and DIECA-treated seedlings were sprayed every other day throughout the 28-day experimental period, whereas MeJA was applied once, seven days prior to nematode inoculation. The more frequent application of DIECA was used to maintain inhibitory activity over time (Li et al. 2020), while a single MeJA treatment is sufficient to trigger jasmonate dependent signalling, including defence gene induction and secondary metabolite accumulation that can persist for days to weeks (Sohn et al. 2022). It should be noted that the three treatment groups differed in their ethanol carrier content: MeJA was dissolved in 2.5% ethanol, DIECA in 10% ethanol, and the control contained no ethanol. Although the ethanol concentrations used are below phytotoxic thresholds in comparable studies (Zas et al. 2019; Cruz 2018), the absence of ethanol-only solvent controls means that potential carrier effects on the measured variables cannot be formally excluded. This asymmetry is acknowledged as a limitation in the interpretation of between-treatment comparisons.

Nematode culturing and plant infection

For the infection assay, the virulent strain 17AS of *Bursaphelenchus xylophilus* was used. Nematodes were maintained in transparent autoclavable plastic boxes on barley seeds colonised with *Botrytis cinerea* (Pers.), incubated in the dark at 25 °C and 80% relative humidity for 14 days. Nematodes were extracted from the culture medium using the Baermann funnel technique (Baermann 1917) for 24 h at 25 °C, and the suspension was adjusted to 300 nematodes per 300 µL of sterile water. Nematodes extracted after 14 days of culture represent primarily propagative juveniles and adults (J3, J4 and adults); the 14-day culture period was selected to ensure a large, actively reproducing population prior to inoculation, consistent with previously described protocols for *B. xylophilus* bioassays (Nunes da Silva et al. 2015). Plant inoculation was performed with minor modifications to the protocol described by Futai (2003). Briefly, needles were removed from a 1 cm stem section around the midpoint of each seedling, and shallow cross sections were made using a sterile scalpel. A small piece of absorbent paper was then wrapped around the wounded area, 300 µL of the nematode suspension (300 nematodes) was pipetted onto the paper, and the inoculation site was sealed with Parafilm to prevent

desiccation and maintain nematode viability. In total, 72 untreated seedlings (24 per species) and 144 treated seedlings (48 per species) were inoculated with PWN, generating six experimental groups per species: infected untreated controls, non-infected untreated controls, infected MeJA-treated plants, non-infected MeJA-treated plants, infected DIECA-treated plants, and non-infected DIECA-treated plants.

Scoring of foliar symptoms, sampling, and nematode quantification

For symptom scoring, twelve plants per treatment per species per time point (n = 12) were randomly selected from each of the six experimental groups and used to evaluate disease progression through visual analysis of leaf damage at 1, 14, and 28 dpi. The degree of wilting was visually assessed on a 0–4 scale: 0 = 0–10% leaf damage; 1 = 11–33%; 2 = 34–66%; 3 ≥ 67%; and 4 = total leaf wilting (Sánchez et al. 2005). For biochemical analyses, five plants per treatment per species were harvested at each time point (1, 14, and 28 dpi), with five independently extracted samples per group used for each assay (n = 5 biological replicates). Needles and stems were rapidly separated, weighed, and flash-frozen in liquid nitrogen and stored at − 80 °C until analysis. For nematode quantification, another set of four infected plants per treatment per species per time point (n = 4) was used. Needles were removed and stems cut into small portions (~ 0.4 cm). Nematodes were extracted using the Baermann funnel technique for 24 h at 25 °C, and quantified using a nematode counting dish under a transmitted light stereo microscope, as described by Nunes da Silva et al. (2015).

Primary and secondary metabolites and oxidative stress

Foliar pigments (anthocyanins, carotenoids, and chlorophylls a and b) were quantified in needle tissues following Sims and Gamon (2002). Briefly, 500 mg of frozen ground needles per sample (n = 5 biological replicates, i.e. five independently extracted samples per treatment per species per time point) was extracted with 5 mL of cold acetone, Tris buffer (80:20, v:v, 1 M Tris, pH 7.8) and incubated for 72 h at 4 °C. After incubation, absorbance was recorded at 470, 537, 647, and 663 nm using a nanophotometer (Implen GmbH, München, Germany), and pigment concentrations were calculated on a fresh weight basis.

Total soluble phenols and flavonoids were quantified from stem tissues using five biological replicates per treatment per species per time point (n = 5 independently extracted samples). For each sample, 200 mg of lyophilised, finely ground stem tissue was extracted with 80% aqueous methanol (v:v) for 24 h at 4 °C in the dark. Extracts were centrifuged at 5000 rpm for 10 min, and the supernatants were used for colorimetric assays. Total soluble phenols were determined from 0.1 mL of extract using the Folin–Denis method (Marinova et al. 2005), with absorbance measured at 760 nm and quantification based on a gallic acid calibration curve. Total flavonoids were determined from 0.1 mL of extract using the aluminium chloride colorimetric method (Zhishen et al. 1999), with absorbance measured at 510 nm and quantification based on a catechin calibration curve.

Lipid peroxidation was assessed by quantifying malondialdehyde (MDA) using five biological replicates per treatment per species per time point (n = 5 independently extracted samples). Briefly, 100 mg of finely ground lyophilised stem tissue per sample was assayed following the thiobarbituric acid reaction (Heath and Packer 1968; Li 2000). Absorbance was measured at 450, 532, and 600 nm, and MDA concentration was calculated using the equation: MDA (µmol. L^{-1}) = 6.45 x ($A_{532} - A_{600}$) − 0.56 × A_{450}.

Extraction and quantification of phytohormones

Phytohormone concentrations were determined in stem phloem and cortex tissues at 28 dpi using four biological replicates per treatment per species (n = 4 plants per group). The hormones analysed included the oxylipins jasmonic acid (JA) and its active conjugate (+) 7 iso jasmonoyl L isoleucine (JA-Ile), salicylic acid (SA), and its conjugate salicylic acid 2 O β glucoside (SAG), abscisic acid (ABA), and the auxin indole-3-acetic acid (IAA). Extracts were partitioned twice with diethyl ether and concentrated to dryness at room temperature in a centrifugal evaporator (SpeedVac). The residue was reconstituted in 1 mL of aqueous methanol (90:10, v:v) containing 0.01% formic acid. Hormones were separated using an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA, USA) equipped with a Kinetex C18 analytical column (Phenomenex) and detected with a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK). Quantification was performed using calibration curves prepared from commercial standards, as described by Gamir et al. (2012). Briefly, tissue samples (~ 100 mg fresh weight) were homogenised in liquid nitrogen and extracted in 1 mL of methanol:water:formic acid (15:4:1, v:v:v) containing isotope-labelled internal standards (D6-JA, D4-SA and D6-ABA; OlChemim, Czech Republic) for absolute quantification. After centrifugation at 13,000 rpm for 5 min at 4 °C, the supernatant was collected and subjected to liquid–liquid partitioning. MRM (multiple reaction monitoring) transitions were used for each compound: JA (m/z 209 → 59), JA-Ile (m/z 322 → 130), SA (m/z 137 → 93), SAG (m/z 299 → 137), ABA (m/z 263 → 153), and IAA (m/z 174 → 130). Hormone concentrations were expressed as ppb (ng g^{-}1 fresh weight).

Gene expression analysis

For gene expression analyses, three biological replicates per treatment per species were used (n = 3 independently extracted RNA samples per group), each analysed in three technical replicates. Total RNA was extracted from stem tissues using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Massachusetts, USA) according to the manufacturer's instructions. RNA quantity and purity were assessed by UV spectrophotometry (Nanophotometer, Implen GmbH, München, Germany), and RNA was stored at − 80 °C until further use. First strand cDNA was synthesised from RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, California, USA) following the manufacturer's instructions. Gene expression was evaluated by quantitative real time PCR (qRT-PCR). Reactions were prepared using 10 µL of SYBR Green SuperMix (Bio-Rad, California, USA), 1 µL of each primer (forward and reverse), and 8 µL of diluted cDNA, and run in a StepOne™ Real-Time PCR System (Applied Biosystems, California, USA). Cycling conditions were 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, primer specific annealing temperature for 15 s (Table 1) and 72 °C for 1 s. Melting curve analysis was performed for each amplicon to confirm reaction specificity. Relative transcript levels were calculated using the comparative Ct method ($2^{\wedge-\Delta\Delta Ct}$) (Livak and Schmittgen 2001) with matrix metalloproteinase (*MMP*) and tubulin1 (*TUB1*) used as reference genes. For each sample and target gene, three technical replicates were analysed. The target genes were selected based on their established roles in pine defence against *B. xylophilus* and related pathogens: *PAL* (phenylpropanoid pathway entry point; Azevedo et al. 2010; Ralph et al. 2006), *GGPPS* (terpene biosynthesis; Chen et al. 2018), *MYC2* (master regulator of JA-mediated transcription; Dombrecht et al. 2007), *AP2* (AP2/ERF transcription factors implicated in JA- and pathogen-responsive signalling; Lin et al. 2020), and *E2F* (cell cycle and DNA-damage checkpoint regulator induced under biotic stress; Noir et al. 2013). Together, these genes span the major transcriptional modules activated during pine–nematode interactions and were previously identified as differentially expressed in *P. pinaster* or *P. pinea* under *B. xylophilus* infection (Nunes da Silva et al. 2025; Shin et al. 2009).

Statistical analysis

Data were analysed using SAS v9.4 (SAS Institute Inc., USA). For nematode counts, leaf damage and biochemical variables, analyses considered treatment (untreated control, MeJA treated, and DIECA treated), species (*P. pinaster*, *P. pinea*, and *P. taeda*), infection status (non-infected [*Ninf*] and infected [*Inf*]), Day (1, 14, and 28 dpi), and their interactions as fixed effects. Phytohormone concentrations and gene expression data were analysed using the same fixed effects, except for day, since these measurements were performed only at the end of the assay (28 dpi). Linear mixed effects models were fitted using restricted maximum likelihood (REML). Significance was assessed at $P < 0.05$, and main effects and interactions were interpreted accordingly. Plant (nested within Treatment × Species) was included as a random effect to account for individual variation. When significant effects were detected, pairwise comparisons among treatment levels were performed using Tukey's honestly significant difference (HSD) test at $P < 0.05$. All response variables were checked for normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test) prior to analysis; when assumptions were not met, data were log-transformed before fitting the model. Data in figures are presented as means ± standard error of the mean (SEM); SEM reflects the precision of the estimated group mean and is the appropriate metric to accompany the inferential tests applied; Tukey's HSD significance levels ($*P < 0.05$;$**P < 0.01$; $***P < 0.001$) are indicated directly on each figure. Although sample sizes per group (n = 4–5) are modest, REML-based linear mixed effects models are well suited to small unbalanced factorial designs because they support partial pooling across groups and provide unbiased variance component estimates compared with ordinary least squares (Bates et al. 2015).

Results

Leaf symptoms assessment and nematode quantification

Leaf damage and nematode abundance were significantly affected by the main factors treatment (Tr), species (Sp), and time point (Tp), as well as by the three-way interaction Tr × Sp × Tp (Table 2). No visible symptoms were observed at 1 day postinoculation (dpi) (data not shown). Leaf damage developed only in *P. pinaster* at 14 and 28 dpi (Fig. 1), whereas *P. pinea* and *P. taeda* remained symptom free throughout the assay. At 14 dpi, mild symptoms (stage 1) were observed in inoculated *P. pinaster* plants, affecting 25% of untreated controls and 17% of DIECA-treated plants (Fig. 1a). By 28 dpi, symptom severity increased in untreated controls, with 75% of plants showing leaf damage across multiple stages (17% at stage 4, 17% at stage 3, and 42% at stage 2, Fig. 1b). In contrast, MeJA-treated plants showed a lower incidence and severity of symptoms (17% of plants at stage 2), while DIECA-treated plants showed 17% of plants at stage 3. Treatment-related differences in nematode abundance in stem tissues (Fig. 2) were detected only in *P. pinaster*. In this species, nematode numbers increased markedly over

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

Transcript	Function	Forward sequence	Reverse sequence	Reference
Matrix metalloproteinase (*MMP*)	Housekeeping gene used to normalise qRT-PCR data	ACACCATTAACCATGGCCTC	CCTGCTCACGATCAATTCTTG	Ratnaparkhe et al. (2009)(*Pinus taeda* L.)
Tubulin 1 (*TUB1*)	Housekeeping gene used to normalise qRT-PCR data	GGCATACCGGCAGCTCTTC	AAGTTGTTGGCGGCGTCTT	Vega-Bartol et al. (2013)(*Pinus pinaster* Ait.)
Phenylalanine ammonia-lyase (*PAL*)	Involved in synthesis of molecules crucial for plant defence, such as phenylpropanoids, flavonoids, anthocyanins, lignin, lignans, condensed tannins, and salicylic acid	CCGAATCCAAACAGAACTCC	CGCAGAATGGATGTTCGAC	Amaral 2021(*Pinus radiata* D. Don., *Pinus pinea* L.)
Geranyl–geranyl diphosphate synthase (*GGPPS*)	*Encodes* an enzyme that catalyses the synthesis of GGPP predominantly in a single step reaction from farnesyl diphosphate (FPP) and IPP, originating diterpenes	TCAGGTTGTCGACGACATAC	GCTATTTGCTAGGATGTACAC ATG	Ralph et al. 2006 (*Picea sitchensis* Bong. Carr.) Chen et al. 2018 (*Pinus massoniana* Lamb.)
Apetala2 (*AP2*)	Dehydration-responsive element-binding/ERF-related proteins, important mediators of responses to various environmental stress signals, especially to drought and oxidative stress	AGCTCTACAGAGGAGTGAGAC AGA	TTCCAAGCCAGAGCCTGGTTC TAT	Shin et al. 2009 (*Pinus densiflora* Sieb. et Zucc.)
Transcription factor (*E2F*)	Transcriptional activator involved in chromatin remodelling and cell proliferation. This transcriptional factor is activated by MeJA	GCTTGCAAAGCGTGCAAAGAT TGG	AGCTGCTGATTGGAGTAGGAG CAT	Noir et al. 2013 (*Arabidopsis thaliana* L)
Basic helix–loop–helix transcription factor (*MYC2*)	Transcriptional factor involved in the jasmonate responses, mainly through defensive gene expression or root growth inhibition	ACTGACGAACCACCACATGA	AACGAGGCGGTGAACTACAT	Ralph et al. 2006 (*Picea sitchensis* Bong. Carr.)

Table 2 Effect of plant treatments (Tr, untreated control plants (control), MeJA-treated plants (MeJA) and DIECA-treated plants (DIECA), species (*P. pinaster*, *P. pinea* and *P. taeda*), nematode infection (N, 0 (without), 1 (with)), time point (Tp, 1, 14, and 28 days postinoculation) and their interactions on leaf damage, the number of nematodes, anthocyanin, chlorophyll-B, total soluble phenols, total flavonoids, and malondialdehyde

Response variable	Factor	*F* ratio	*P* value
Leaf damage (%)	Treatment (Tr)	8.02	**0.0005**
	Species (Sp)	26.74	**<.0001**
	Time point (Tp)	26.74	**<.0001**
	Tr × Sp × Tp	8.02	**0.0005**
Nº of nematodes	Treatment (Tr)	7.31	**0.0012**
	Species (Sp)	75.24	**<.0001**
	Time point (Tp)	43.26	**<.0001**
	Tr × Sp × Tp	2.87	**0.0073**
Anthocyanin (μmol. g^{-1} leaf)	Treatment (Tr)	26.87	**<.0001**
	Species (Sp)	49.60	**<.0001**
	Nematode (N)	3.33	0.0701
	Time point (Tp)	0.50	0.6096
	Tr × Sp × N × Tp	2.98	**0.0040**
Chlorophyll-B (mmol. g^{-1} leaf)	Treatment (Tr)	13.41	**<.0001**
	Species (Sp)	12.23	**<.0001**
	Nematode (N)	1.39	0.2396
	Time point (Tp)	0.22	0.8054
	Tr × Sp × N × Tp	2.72	**0.0081**
Total soluble phenols (mg. g^{-1} leaf)	Treatment (Tr)	3.06	**0.0499**
	Species (Sp)	77.67	**<.0001**
	Nematode (N)	63.01	**<.0001**
	Time point (Tp)	18.35	**<.0001**
	Sp × N × Tp	4.65	**0.0015**
Total flavonoids (mg. g^{-1} leaf)	Treatment (Tr)	0.09	0.9184
	Species (Sp)	101.89	**<.0001**
	Nematode (N)	75.09	**<.0001**
	Time point (Tp)	1.38	0.2543
	Sp × N × Tp	3.43	**0.0104**
Malondialdehyde (μmol. g^{-1} leaf)	Treatment (Tr)	1.22	0.2987
	Species (Sp)	39.28	**<.0001**
	Nematode (N)	18.14	**<.0001**
	Time point (Tp)	0.72	0.4866
	Tr × Sp × N × Tp	2.69	**0.0089**

Significant *P* values (< 0.05) are indicated in bold

time, increasing 416-fold from 1 dpi (18 ± 6 nematodes) to 28 dpi (7481 ± 212). At 28 dpi, MeJA-treated plants (914 ± 125) and DIECA-treated plants (5570 ± 187) had significantly lower nematode numbers than untreated controls, 8.2-fold (7481/914 ≈ 8.2), consistent with the values shown in Fig. 2.

Primary and secondary metabolites and oxidative stress

Treatment (Tr), species (Sp), and the four-way interaction Tr × Sp × N × Tp significantly affected anthocyanin and chlorophyll b (Chl-B) concentrations (Table 2, Fig. 3). In *P. pinea*, anthocyanin levels differed significantly between untreated controls and MeJA-treated plants (Fig. 3a). Although anthocyanins increased over time in control plants, at 28 dpi infected plants (Control *Inf*) had significantly lower anthocyanin concentrations than non-infected plants (Control *Ninf*), by 1.14-fold (Fig. 3a). In contrast, MeJA-treated *P. pinea* showed a peak anthocyanin concentration at 1 dpi (15.25 ± 2.1 μmol $g^{-}1$ leaf), followed by a progressive decrease up to 28 dpi, by 0.51-fold (Fig. 3a). Chl-B concentrations differed significantly among treatments in *P. pinaster* and *P. pinea*, but not in *P. taeda* (Table 2, Fig. 3b). At 28 dpi, infected untreated controls of both *P. pinaster* and *P. pinea* showed significantly lower Chl-B than their respective non-infected controls, by 1.15-fold and 1.23-fold, respectively. In contrast, at the same time point, infected MeJA- and DIECA-treated plants showed significantly higher Chl-B than their corresponding non-infected plants. This effect was observed for MeJA in *P. pinaster* (1.24-fold) and *P. pinea* (1.12-fold), and for DIECA in *P. pinaster* (1.18-fold) and *P. pinea* (1.34-fold) (Fig. 3b).

Carotenoid concentration was significantly affected by nematode infection (N), time point (Tp), and the N × Tp interaction (Table S1). Chlorophyll a (Chl-A) was significantly affected by N, Tr, and Sp, as well as by the Sp × N interaction (Table S1). Overall, carotenoids and Chl-A were significantly higher in infected than non-infected plants, by 2.06-fold and 1.07-fold, respectively (Fig. S1a, S1c), and carotenoids in non-infected plants increased significantly between 1 and 28 dpi, by 1.2-fold (Fig. S1b). Species comparisons showed that *P. taeda* had significantly higher Chl-A than *P. pinaster* and *P. pinea*. The Sp × N interaction further indicated species-dependent infection responses: In *P. taeda*, infected plants had significantly lower Chl-A than non-infected plants, by 1.07-fold, whereas in *P. pinaster* and *P. pinea* Chl-A differed significantly between infection statuses, with the direction of the response captured in Fig. S1d.

Total soluble phenols were significantly affected by all main factors (N, Tr, Sp, and Tp) and by the Sp × N × Tp interaction, while flavonoids were significantly affected by Sp, N, and Sp × N × Tp (Table 2). Total soluble phenols (Fig. 4a to 4c) and flavonoids (Fig. 4d to 4f) were significantly higher in infected than non-infected plants across treatments and species, with *P. pinea* showing the highest concentrations regardless of infection status (Fig. 4a, Fig. 4d). At 28 dpi, infected plants of *P. pinaster*, *P. pinea*, and *P. taeda* had significantly higher phenolic concentrations

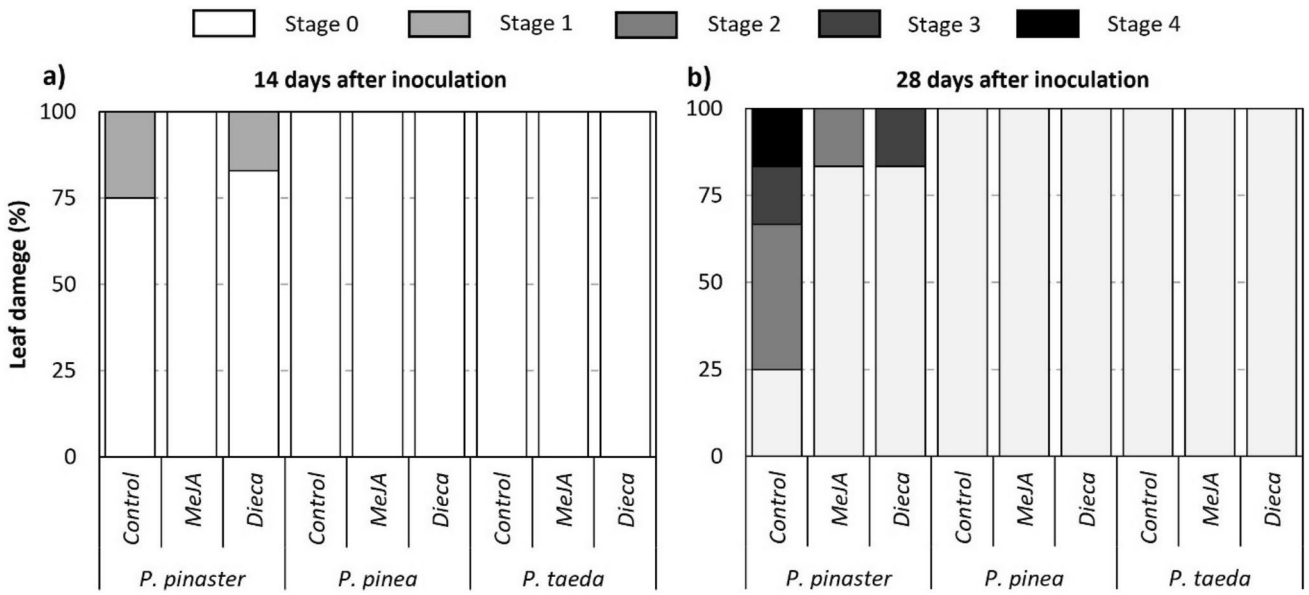


Fig. 1 Leaf damage (%) **a** 14 days postinoculation and **b** 28 days postinoculation in *P. pinaster*, *P. pinea* and *P. taeda* for the treatments (Tr, untreated control plants (control), MeJA-treated plants (MeJA), and DIECA-treated plants (DIECA). Stages of damage: Stage 0 (0–10%). Stage 1 (11–33%), Stage 2 (34–66%), Stage 3 (≥67%), and Stage 4 (dead). Significance levels of treatments within species: ∗∗∗, $P < 0.001$; ∗∗, $P < 0.01$;∗, $P < 0.05$; n.s., not significant

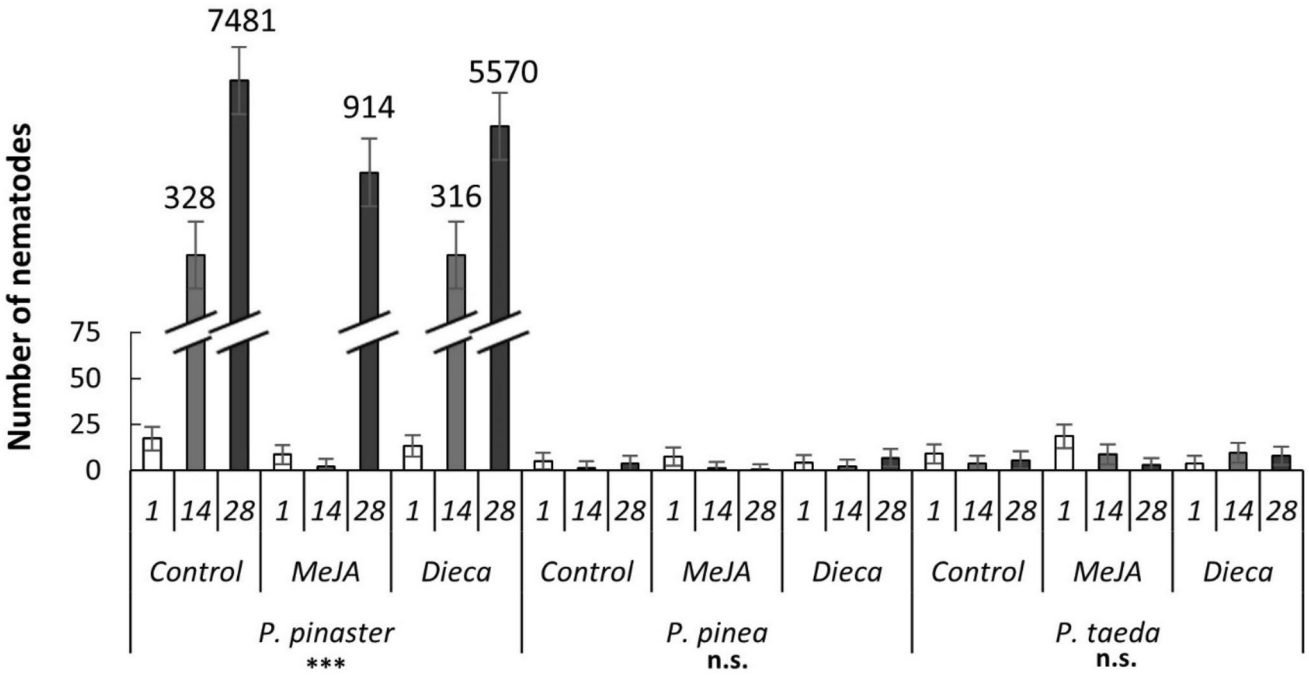


Fig. 2 Number of nematodes at 1, 14 and 28 days postinoculation in *P. pinaster*, *P. pinea*, and *P. taeda* for the treatments (Tr, untreated control plants (control), MeJA-treated plants (MeJA), and DIECA-treated plants (DIECA). Values represent the mean of four biological replicates ± standard error of the mean. Significance levels of treatments within species: ∗∗∗, $P < 0.001$; ∗∗, $P < 0.01$; ∗, $P < 0.05$; n.s., not significant

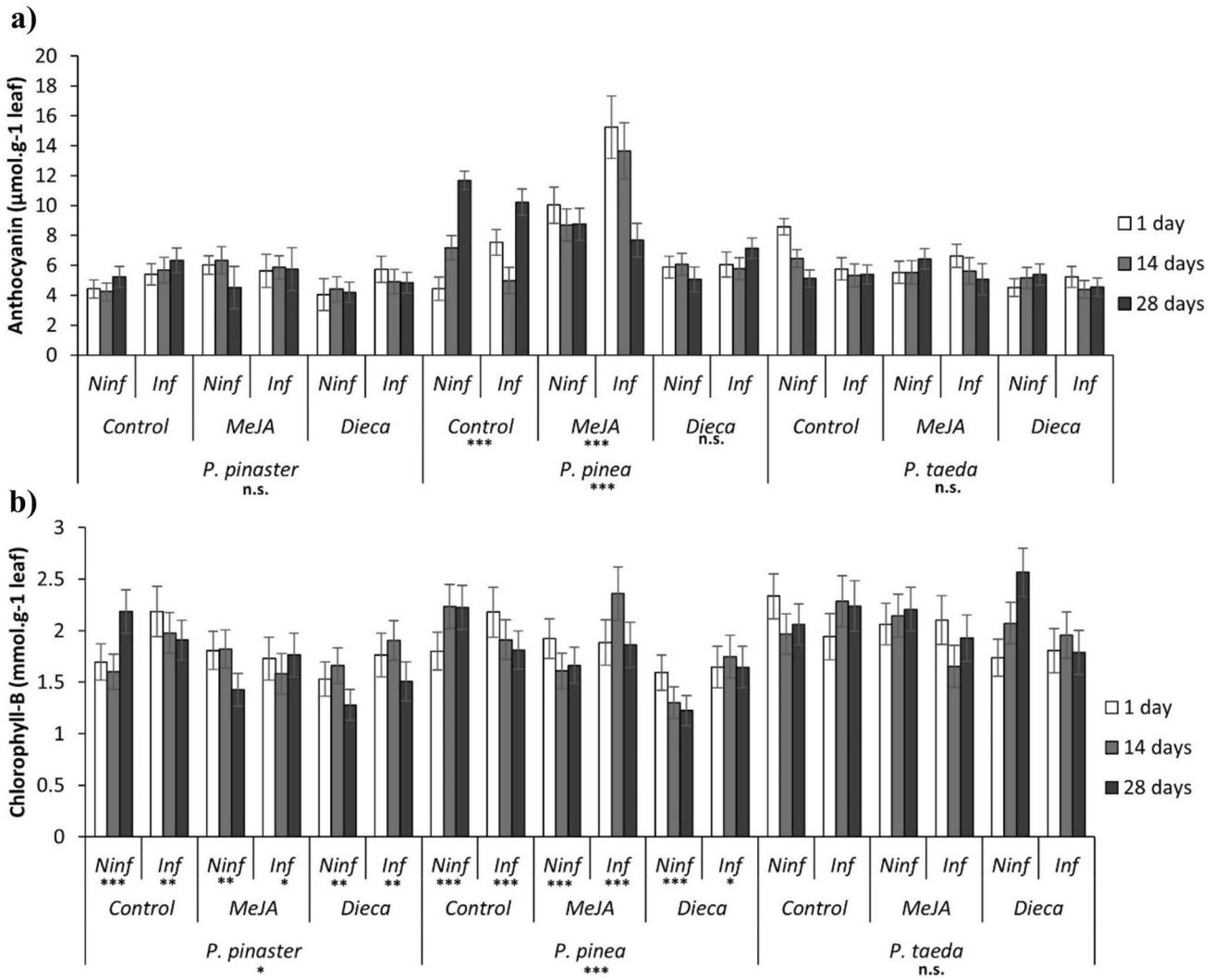


Fig. 3 **a** Anthocyanin (µmol. g^{-1} leaf) and **b** Chlorophyll-B (mmol. g^{-1} leaf) at 1, 14 and 28 days postinoculation in *P. pinaster*, *P. pinea*, and *P. taeda* for the treatments (Tr, untreated control plants (control), MeJA-treated plants (MeJA), and DIECA-treated plants (DIECA) and level of infection (non-infected (*Ninf*) and infected (*Inf*) plants). Values represent the mean of five biological replicates ± standard error of the mean. Significance levels of treatments within species: ∗∗∗, $P < 0.001$; ∗∗, $P < 0.01$; ∗, $P < 0.05$; n.s., not significant

than non-infected plants, by 1.98-fold, 1.06-fold, and 1.29-fold, respectively, and significantly higher flavonoid concentrations, by 6.44-fold, 1.10-fold, and 2.25-fold, respectively (Fig. 4c, Fig. 4f). Phenolic concentrations increased over time in infected plants in all three species (Fig. 4c). A similar temporal increase was observed for flavonoids in *P. pinaster*, whereas infected *P. pinea* and *P. taeda* showed early peaks at 1 dpi (8.74 and 4.38 mg g^{-}1 leaf, respectively) (Fig. 4d).

Malondialdehyde (MDA) concentrations were significantly affected by species (Sp), nematode infection (N), and the four-way interaction Tr × Sp × N × Tp (Table 2). The highest MDA concentrations were generally observed in infected *P. pinea* and P. *pinaster*, except in infected *P. pinea* treated with DIECA (Fig. 5). Peaks of MDA accumulation were detected at 1 dpi in infected untreated controls and in infected MeJA-treated plants of *P. pinaster* and *P. pinea*, with the highest value recorded in infected untreated *P. pinea* (0.06 µmol g^{-}1 leaf). In *P. taeda*, infected untreated controls showed significantly higher MDA than non-infected controls, whereas infected MeJA-treated plants showed significantly lower MDA than their non-infected counterparts (Fig. 5).

Dynamics of defence-related phytohormones

Plant treatment (Tr), species (Sp), nematode infection (N), and the three-way interaction Tr × Sp × N significantly affected all phytohormones analysed, except abscisic acid (ABA), for which only species and the Tr × Sp × N interaction were significant (Table 3). At 28 dpi, ABA increased

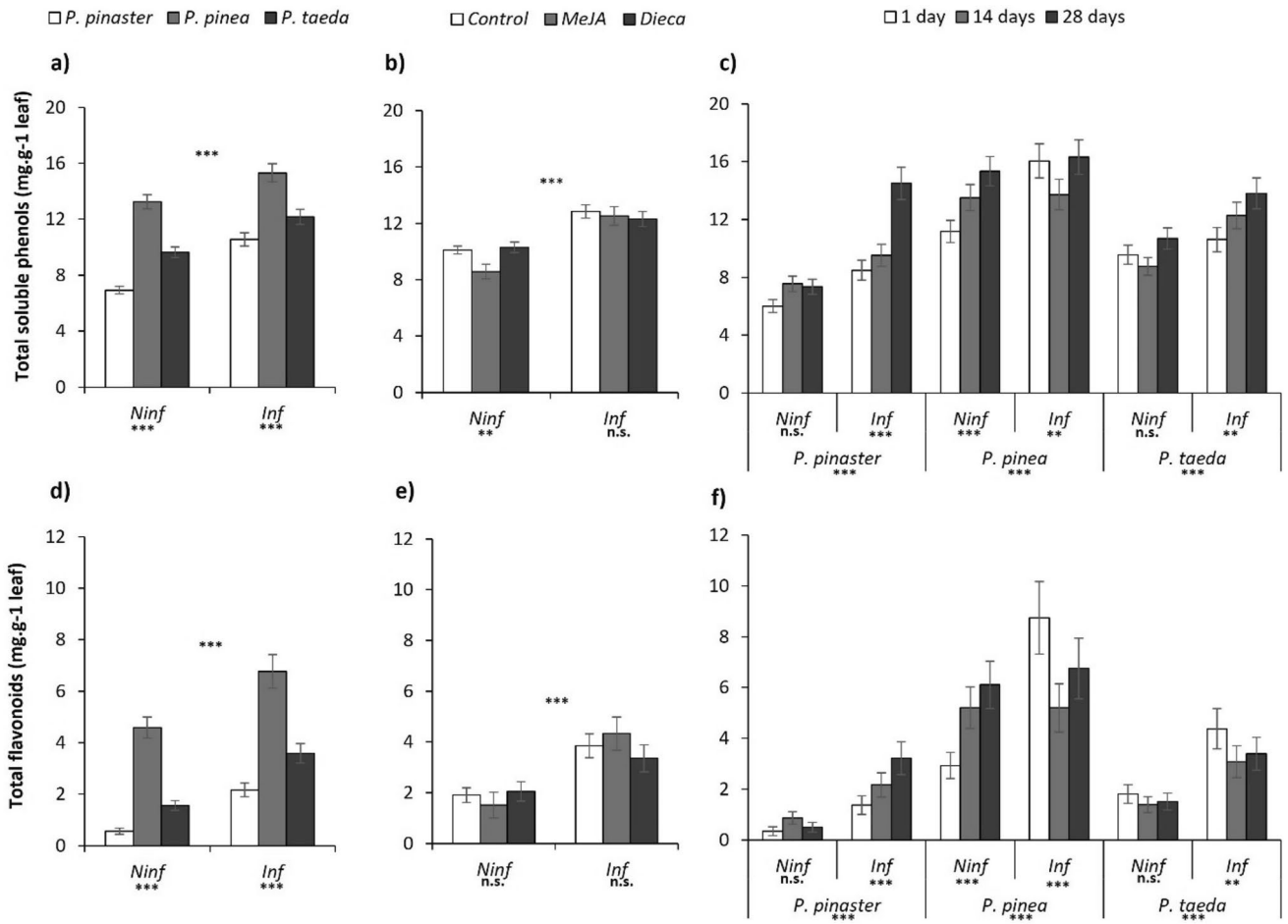


Fig. 4 Total soluble phenols (mg. g^{-1} leaf) (**a**, **b**, and **c**) and total flavonoids (mg. g^{-1} leaf) (**d**, **e,** and **f**) at 1, 14, and 28 days postinoculation in *P. pinaster*, *P. pinea*, and *P. taeda* for the treatments (Tr, untreated control plants (control), MeJA-treated plants (MeJA), and DIECA-treated plants (DIECA)and level of infection (non-infected (*Ninf*) and infected (*Inf*) plants). Values represent the mean of five biological replicates ± standard error of the mean. Significance levels of treatments within species: ∗∗∗, $P < 0.001$; ∗∗, $P < 0.01$;∗, $P < 0.05$; n.s., not significant

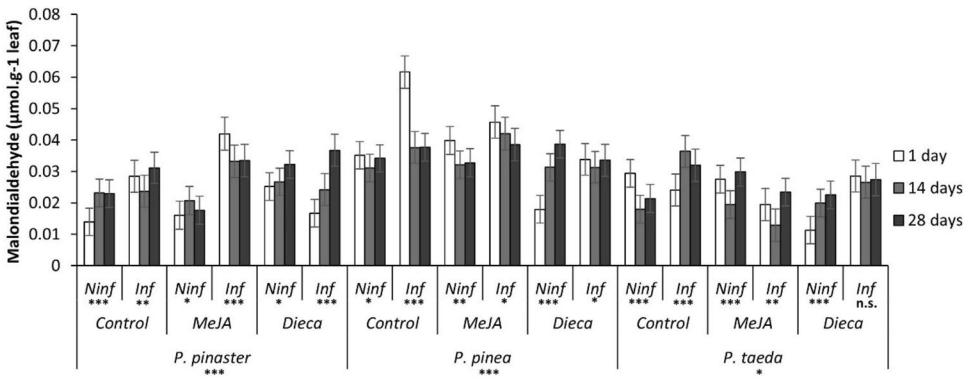


Fig. 5 Malondialdehyde (µmol. g^{-1} leaf) at 1, 14, and 28 days postinoculation in *P. pinaster*, *P. pinea*, and *P. taeda* for the treatments (Tr, untreated control plants (control), MeJA-treated plants (MeJA), and DIECA-treated plants (DIECA) and level of infection (non-infected (*Ninf*) and infected (*Inf*) plants). Values represent the mean of five biological replicates ± standard error of the mean. Significance levels of treatments within species: ∗∗∗, $P < 0.001$; ∗∗, $P < 0.01$; ∗, $P < 0.05$; n.s., not significant

Table 3 Effect of plant treatments (Tr, untreated control plants (control), MeJA-treated plants (MeJA), and DIECA-treated plants (DIECA), species (Sp, *P. pinaster*, *P. pinea*, and *P. taeda*), nematode infection (N, 0 (without), 1 (with)), and their interactions on the phytohormones and the relative gene expression at 28 days postinoculation

	Response variable	Factor	*F* ratio	*P* value
Phytohormones	Abscisic acid (ppb)	Treatment (Tr)	2.74	0.0747
		Species (Sp)	38.33	**<.0001**
		Nematode (N)	0.35	0.5563
		Tr×Sp×N	3.03	**0.0265**
	Indole-3-acetic acid (ppb)	Treatment (Tr)	8.37	**0.0007**
		Species (Sp)	7.76	**0.0012**
		Nematode (N)	24.31	**<.0001**
		Tr×Sp×N	4.29	**0.0047**
	Jasmonic acid (ppb)	Treatment (Tr)	335.56	**<.0001**
		Species (Sp)	43.1	**<.0001**
		Nematode (N)	40.13	**<.0001**
		Tr×Sp×N	10.92	**<.0001**
	JA-isoleucine (ppb)	Treatment (Tr)	18.33	**<.0001**
		Species (Sp)	87.64	**<.0001**
		Nematode (N)	92.88	**<.0001**
		Tr×Sp×N	32.4	**<.0001**
	Salicylic acid (ppb)	Treatment (Tr)	17.66	**<.0001**
		Species (Sp)	20.3	**<.0001**
		Nematode (N)	124.59	**<.0001**
		Tr×Sp×N	4.75	**0.0027**
	SA-glucoside (ppb)	Treatment (Tr)	4.59	**0.015**
		Species (Sp)	43.72	**<.0001**
		Nematode (N)	698.49	**<.0001**
		Tr×Sp×N	28.44	**<.0001**

	Response variable	Factor	*F* ratio	*P* value
Relative gene expression	*AP2*	Treatment (Tr)	8.56	**0.0009**
		Species (Sp)	33.09	**<.0001**
		Nematode (N)	0.11	0.7477
		Tr×Sp×N	7.05	**0.0003**
	E2F	Treatment (Tr)	2.19	0.1245
		Species (Sp)	5.1	**0.0103**
		Nematode (N)	18.56	**<.0001**
		Tr×Sp	3.24	**0.0207**
		Sp×N	7.38	**0.0018**
	GGPPS	Treatment (Tr)	6.86	**0.0027**
		Species (Sp)	79.2	**<.0001**
		Nematode (N)	9.81	**0.0032**
		Tr×Sp×N	7.06	**0.0002**
	MYC2	Treatment (Tr)	2.16	0.1279
		Species (Sp)	0.54	0.5868
		Nematode (N)	10.31	**0.0026**
		Tr×Sp×N	3.99	**0.008**
	PAL	Treatment (Tr)	5.22	**0.0156**
		Species (Sp)	48.2	**<.0001**
		Nematode (N)	28.69	**<.0001**
		Tr×Sp×N	3.02	**0.03**

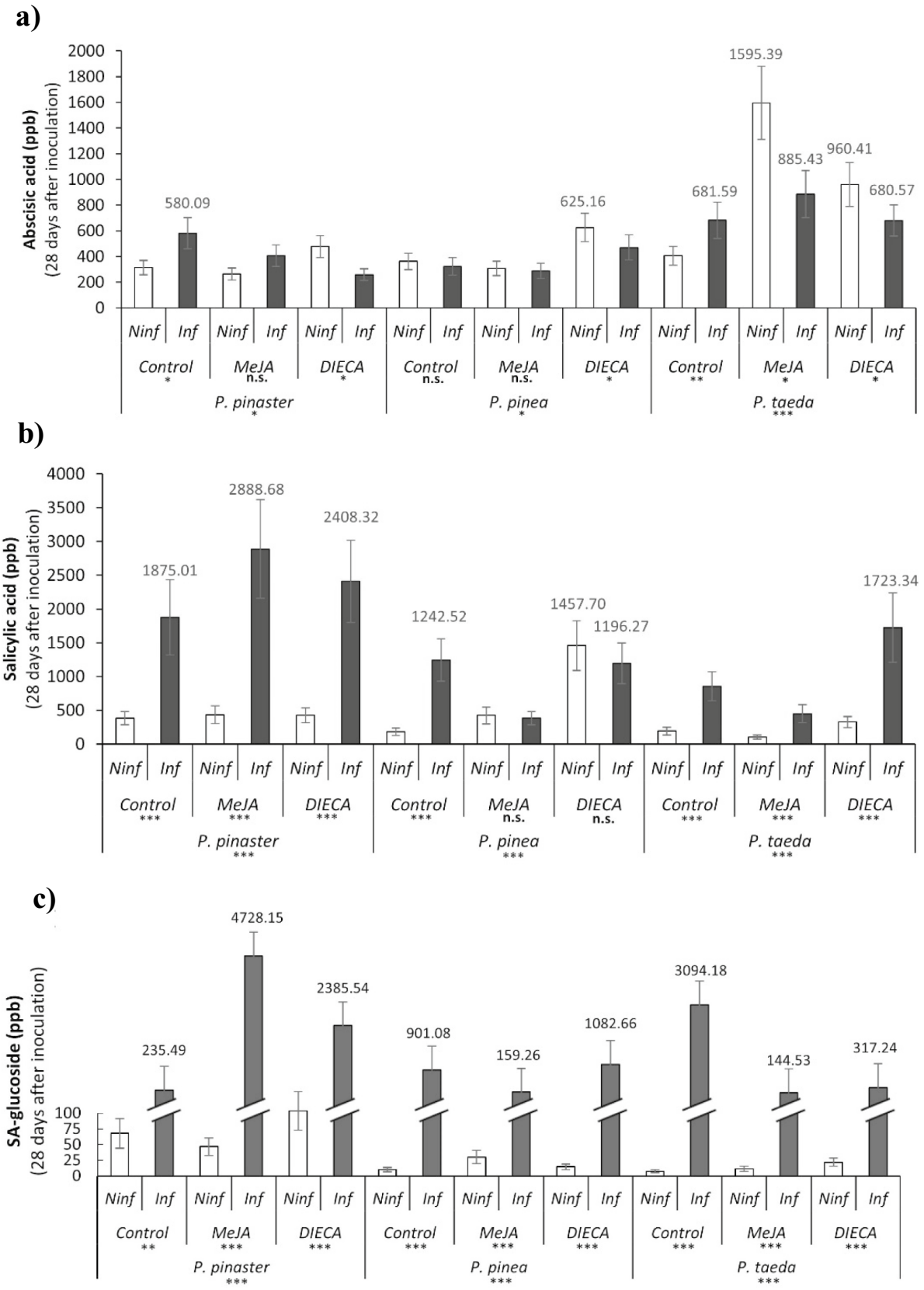
Significant *P* values (< 0.05) are indicated in bold

significantly in infected untreated *P. pinaster* (Control *Inf*), by 1.85-fold relative to non-infected untreated controls (Control *Ninf*) (Fig. 6a). In contrast, within DIECA-treated *P. pinaster*, ABA was significantly higher in non-infected plants (DIECA *Ninf*) than in infected plants (DIECA *Inf*), also by 1.85-fold (Fig. 6a). In *P. pinea*, DIECA-treated plants were the only group showing a significant decrease in ABA upon infection, by 1.33-fold (Fig. 6a). Across treatments, *P. taeda* displayed the highest ABA concentrations. Within *P. taeda*, ABA was highest in non-infected MeJA-treated plants and decreased significantly following infection, by 1.81-fold. A similar pattern was observed in DIECA-treated plants, with ABA decreasing by 1.41-fold in infected plants. By contrast, untreated controls showed a significant increase in ABA upon infection, by 1.68-fold (Fig. 6a).

Salicylic acid (SA) increased significantly in infected *P. pinaster* and *P. pinea* across treatments relative to their non-infected counterparts (Fig. 6b). The largest increases were observed in MeJA-treated *P. pinaster* (6.65-fold) and in DIECA-treated *P. pinea* (5.29-fold) (Fig. 6b). Finally, salicylic acid glucoside (SAG) was significantly higher in infected than in non-infected plants across all species and treatments (Fig. 6c), with particularly high increases in MeJA-treated *P. pinaster* (100-fold) and in untreated *P. taeda* (23.0-fold) (Fig. 6c).

Like ABA (Fig. 6a), indole-3-acetic acid (IAA) concentrations were highest overall in *P. taeda*, but showed no significant differences between non-infected and infected plants under any treatment (control, MeJA, or DIECA) (Fig. 7a). In *P. pinea*, MeJA-treated plants showed a significant 2.03-fold reduction in IAA following infection, while in *P. pinaster*, infection significantly decreased IAA across all treatments (control, 5.38-fold; MeJA, 1.70-fold; DIECA, 2.13-fold) (Fig. 7a).

Jasmonic acid (JA) concentrations were highest in MeJA-treated plants across all species (Fig. 7b). In *P. pinaster*, infection induced strong JA accumulation, with significant increases in infected untreated controls (72.1-fold) and in


a)
Abscisic acid (ppb)
(28 days after inoculation)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
580.09
625.16
681.59
1595.39
885.43
960.41
680.57
Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf
Control MeJA DIECA Control MeJA DIECA Control MeJA DIECA
*
n.s.
*
n.s.
n.s.
*
**
*
*
P. pinaster P. pinea P. taeda
*
*
***
b)
Salicylic acid (ppb)
(28 days after inoculation)
4000
3500
3000
2500
2000
1500
1000
500
0
1875.01
2888.68
2408.32
1242.52
1457.70
1196.27
1723.34
Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf
Control MeJA DIECA Control MeJA DIECA Control MeJA DIECA
***
***
***
***
n.s.
n.s.
***
***
***
P. pinaster P. pinea P. taeda
***
***
***
c)
SA-glucoside (ppb)
(28 days after inoculation)
100
75
50
25
0
235.49
4728.15
2385.54
901.08
159.26
1082.66
3094.18
144.53
317.24
Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf Ninf Inf
Control MeJA DIECA Control MeJA DIECA Control MeJA DIECA
**
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P. pinaster P. pinea P. taeda
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***

◂**Fig. 6** **a** Abscisic acid (ppb), **b** salicylic acid (ppb), and **c** SA-glucoside (ppb) at 28 days after infection in *P. pinaster*, *P. pinea*, and *P. taeda* for the treatments (Tr, untreated control plants (control), MeJA-treated plants (MeJA), and DIECA-treated plants (DIECA) and level of infection (non-infected (*Ninf*) and infected (*Inf*) plants). Values represent the mean of four biological replicates ± standard error of the mean. Significance levels of treatments within species: ∗∗∗, $P < 0.001$; ∗∗, $P < 0.01$; ∗, $P < 0.05$; n.s., not significant

infected MeJA-treated plants (3.60-fold) relative to their respective non-infected plants. In *P. pinea*, infected MeJA-treated plants also showed a significant infection-associated increase in JA, by 1.68-fold, relative to MeJA-treated non-infected plants (Fig. 7b). In *P. taeda*, MeJA treatment resulted in significantly higher JA than in untreated controls and DIECA-treated plants, but no significant differences were detected between infected and non-infected MeJA-treated plants (Fig. 7b). At 28 dpi, infected *P. pinaster* accumulated the highest concentrations of JA-Ile across treatments, with the largest increase observed in infected untreated controls (196-fold relative to non-infected plants) (Fig. 7c), while in *P. pinea*, absolute JA-Ile concentrations were comparatively low. In this species, infected untreated controls contained significantly less JA-Ile than non-infected controls, by 2.65-fold, whereas MeJA-treated plants showed a significant infection-associated increase, by 3.16-fold (Fig. 7c). In *P. taeda*, infected MeJA-treated plants accumulated significantly less JA-Ile than non-infected plants, by 3.41-fold (Fig. 7c).

Transcriptional profiling of antioxidant and defence pathways

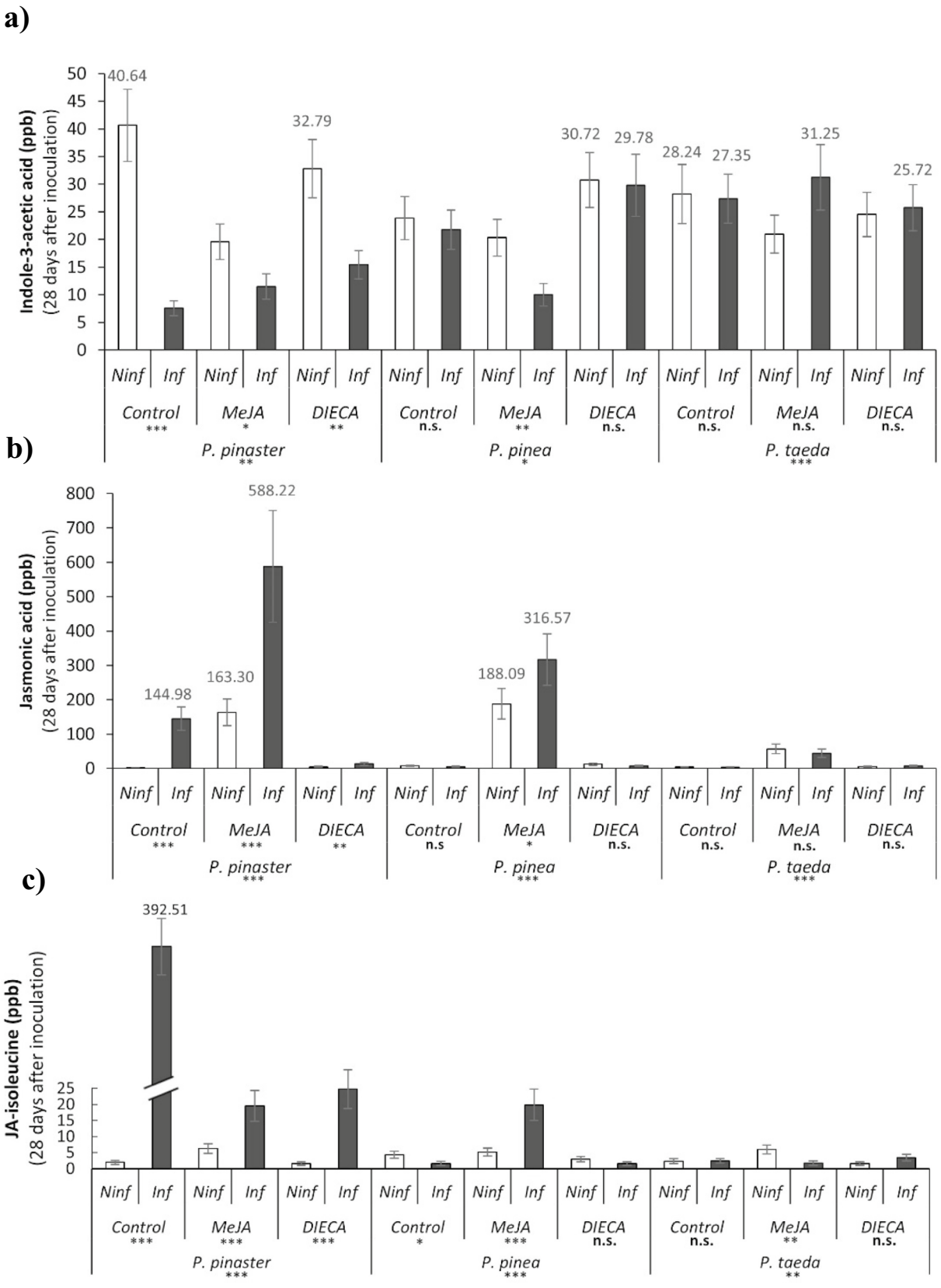
Relative expression of *GGPPS* and *PAL* was significantly influenced by Treatment (Tr), Species (Sp), and nematode infection (N), whereas *AP2* was not significantly affected by infection, *E2F* was not significantly affected by treatment, and *MYC2* was significantly affected only by infection (Table 3). In addition, *AP2*, *GGPPS*, *MYC2*, and *PAL* showed significant Tr × Sp × N effects. For *E2F*, the Tr × Sp and Sp × N interactions were significant, while the three-way interaction was not (Table 3).

In *P. pinaster*, PWN infection upregulated *PAL* in both treated and untreated plants, whereas *AP2* and *GGPPS* were induced only in MeJA- and DIECA-treated plants following infection. In non-infected plants, MeJA treatment upregulated *GGPPS* and *MYC2*, while DIECA treatment upregulated *PAL* (Fig. 8). In *P. pinea*, MeJA and DIECA treatments upregulated *E2F* in non-infected plants, while *GGPPS* was induced only by MeJA. In infected plants, MeJA and DIECA treatments upregulated *MYC2*, whereas *E2F* was specifically upregulated by DIECA. In *P. taeda*, infection upregulated *PAL*, while the combination of infection and MeJA treatment upregulated all analysed genes except *E2F*. Moreover, *PAL* was also upregulated in non-infected plants under both control and MeJA treatments (Fig. 8).

Discussion

Leaf damage development and the marked increase in nematode abundance were largely restricted to *P. pinaster*, reinforcing its higher susceptibility to pinewood nematode relative to *P. pinea* and *P. taeda*. By 28 days postinfection, most untreated *P. pinaster* plants displayed symptoms, consistent with earlier reports describing rapid disease progression and high PWN multiplication in this species (López-Villamor et al. 2022; Nunes da Silva et al. 2025). In this context, MeJA pretreatment reduced symptom incidence and severity and strongly constrained nematode proliferation in stem tissues, in line with evidence that jasmonates can limit nematode performance by altering host suitability, including effects on feeding behaviour, reproduction, and the induction of defence-related metabolites (Chen et al. 2025; López-Villamor et al. 2022; Nunes da Silva et al. 2025). Importantly, the phenotype observed under DIECA, with a weaker reduction in nematode numbers and milder symptom development than in untreated controls at the end of the trial, suggests that *P. pinaster* may retain some capacity for defence even when JA biosynthesis is partially inhibited by DIECA, though the mechanisms underlying this response remain unclear and direct evidence of JA-independent pathway activation is lacking in the present dataset. We note that DIECA could have partial or incomplete inhibitory effects under the conditions used, and the unexpected reduction in nematode numbers relative to untreated controls warrants cautious interpretation. Future studies including direct measurement of JA and JA-Ile levels following DIECA application, and assessment of JA-pathway marker genes such as LOX2 or VSP2, would be needed to confirm the extent of pathway inhibition and to distinguish between compensatory defence recruitment and incomplete JA suppression (Desmedt et al. 2020; Nunes da Silva et al. 2025).

The limited impact of MeJA or DIECA on symptom expression in the two tolerant species (*P. pinea* and *P. taeda*) does not imply that jasmonate signalling is unimportant. Indeed, as demonstrated by the strong MeJA effect in the susceptible *P. pinaster*, JA signalling is a central determinant of pine defence against *B. xylophilus*. However, for species that are already tolerant, perturbation of JA signalling alone does not abolish or dramatically alter disease outcome, suggesting that tolerance may reflect a broader defence architecture in which hormonal coordination, secondary metabolism, and redox homeostasis are deployed in a way that prevents the establishment of a permissive environment for PWN, even when JA signalling is chemically perturbed. A consistent response across species was the induction of


a)
Indole-3-acetic acid (ppb)
(28 days after inoculation)
40.64
32.79
30.72
29.78
28.24
27.35
31.25
25.72
Ninf Inf
Control MeJA DIECA
P. pinaster P. pinea P. taeda
b)
Jasmonic acid (ppb)
(28 days after inoculation)
144.98
163.30
588.22
188.09
316.57
c)
JA-isoleucine (ppb)
(28 days after inoculation)
392.51

◂**Fig. 7** **a** Indole-3-acetic acid (ppb), **b** jasmonic acid (ppb), and **c** JA-isoleucine (ppb) at 28 days postinfection in *P. pinaster*, *P. pinea*, and *P. taeda* for the treatments (Tr, untreated control plants (control), MeJA-treated plants (MeJA), and DIECA-treated plants (DIECA) and level of infection (non-infected (*Ninf*) and infected (*Inf*) plants). Values represent the mean of four biological replicates ± standard error of the mean. Significance levels of treatments within species: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., not significant

secondary metabolism following infection, particularly the accumulation of total soluble phenols and flavonoids, with *P. pinea* showing the highest concentrations overall. Phenolics and flavonoids are frequently associated with improved resistance to pathogens and nematodes due to antioxidant capacity, antimicrobial activity, and contributions to structural reinforcement through cell wall associated phenylpropanoid products (Elkhouni et al. 2018; Falcone Ferreyra et al. 2012; López-Villamor et al. 2022; Treutter 2006). The strong metabolite response in *P. pinea*, combined with minimal symptoms and low nematode establishment, supports a model where secondary metabolism is not merely a stress marker but part of an effective, early deployed defence output. The observation that MeJA and DIECA treatments modulated some of these metabolite pools, particularly in *P. pinea* and *P. pinaster*, further indicates that both activation and inhibition of jasmonate associated signalling can reshape downstream biochemical outputs, likely through pathway crosstalk and compensatory signalling (Falcone Ferreyra et al. 2012).

Changes in photosynthetic and photoprotective pigments provide additional context for how pines balance defence with physiological maintenance. Anthocyanins in *P. pinea* peaked early after MeJA treatment and then declined towards 28 dpi, which is compatible with anthocyanins acting as part of an early stress response. Infection-associated shifts in Chl-B, and the overall infection-associated changes in carotenoids and Chl-A, point to treatment and species-dependent regulation of photosynthetic function under biotic stress. Because carotenoids contribute to photoprotection and reactive oxygen species scavenging, their increase in infected plants is consistent with an oxidative challenge triggered by PWN and with the need to protect chloroplast integrity during defence activation (Fatma et al. 2021; Strzałka et al. 2003; Yu et al. 2019; Young and Lowe 2018).

The MDA patterns further support oxidative stress as a major component of the host response. Infection increased lipid peroxidation, particularly in *P. pinaster* and *P. pinea*, indicating that PWN challenge imposes substantial oxidative pressure (Apel and Hirt 2004; Tikoria et al. 2023; Yamada 2008). Notably, MeJA was associated with lower MDA in specific contexts, including in *P. taeda*, suggesting that jasmonate driven priming can enhance oxidative stress containment, likely by promoting antioxidant capacity and by coordinating the timing of defence activation to avoid runaway damage (Desmedt et al. 2020; Gaspar et al. 2017; Kawaguchi 2006; Kuroda et al. 2011). The combination of strong secondary metabolite accumulation and moderated oxidative damage is consistent with a defence strategy that couples signalling to protective biochemical outputs rather than sustaining damaging oxidative states.

Phytohormone profiles revealed species-dependent patterns of hormone accumulation that are consistent with, though not proof of, differences in hormonal coordination among species. These correlative associations suggest that resistance may involve multi-hormone integration rather than accumulation of any single hormone, but causal relationships cannot be established from the present observational data alone. ABA responses differed markedly among species and treatments, consistent with its role as a context-dependent modulator of stress responses and defence outcomes, though the direction of causation between ABA levels and resistance outcomes remains to be demonstrated (Muhammad Aslam et al. 2022; Verma et al. 2016). Infected control *P. pinaster* accumulated ABA, whereas patterns in treated plants were more variable, suggesting interaction between ABA and jasmonate-related signalling during PWN challenge (Beneventi et al. 2013; Chen et al. 2025; Nunes da Silva et al. 2025; Sun et al. 2024). At the same time, the consistent reduction of IAA in infected *P. pinaster* across treatments suggests a growth defence shift and potential disruption of auxin regulated processes during susceptibility, consistent with evidence that pathogens and herbivores can manipulate or indirectly alter auxin homeostasis (Gomes and Scortecci 2021; Nag et al. 2023; Nunes da Silva et al. 2025; Zhang et al. 2022).

Jasmonate-related metabolites distinguished susceptible versus tolerant responses in a way that goes beyond the known protective effect of MeJA. MeJA increased JA and JA-Ile in all species, but the infection induced amplification of these signals, and especially the relationship between JA and JA-Ile, differed strongly among species. In *P. pinaster*, infection triggered very large increases in JA and JA-Ile, particularly in untreated controls, yet this hyperaccumulation coincided with high nematode multiplication and symptom development. This pattern suggests that strong jasmonate accumulation alone is not sufficient, and that susceptibility may involve inefficient coupling between signalling and effective downstream outputs, or mistiming that allows colonisation before defences are fully deployed (Roychowdhury et al. 2025; Song et al. 2022). In *P. pinea*, JA-Ile remained low and was reduced in infected controls, while MeJA-treated plants showed an infection-associated rise, pointing to tight regulation of JA conjugation. Because JA-Ile is the bioactive ligand for COI1-dependent signalling, controlled JA-Ile dynamics may reflect a strategy that avoids excessive, costly responses while still enabling targeted defence activation when needed (Li et al. 2022a, b). In *P. taeda*, infection

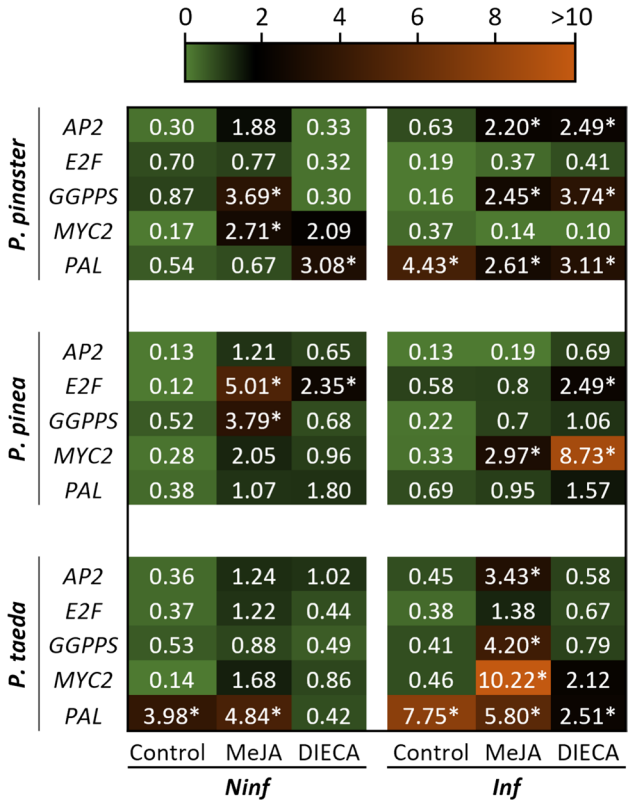


Fig. 8 Fold change of *AP2*, *E2F*, *GGPPS*, *MYC2*, and *PAL* gene expression, at 28 days after infection in *P. pinaster*, *P. pinea*, and *P. taeda* for the treatments (Tr, untreated control plants (control), MeJA-treated plants (MeJA), and DIECA-treated plants (DIECA) and level of infection (non-infected (*Ninf*) and infected (*Inf*) plants). 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 orange, upregulation of gene expression (fold changes greater than 2)

did not further enhance JA under MeJA treatment and JA-Ile declined in infected MeJA-treated plants, together with pronounced ABA patterns, suggesting active attenuation or rerouting of jasmonate signalling in favour of alternative regulatory modes (Chen et al. 2025; Sharma and Sharma 2023; Xing et al. 2024).

SA and SAG accumulated strongly upon infection across species, underscoring that SA-related signalling is a conserved component of the response to PWN challenge. The magnitude and treatment sensitivity of SA and SAG nevertheless differed, suggesting distinct modes of JA-SA integration. In *P. pinaster*, strong SA increases in infected MeJA-treated plants are consistent with jasmonate salicylate crosstalk, but excessive co-activation can also generate signalling interference rather than functional synergy, depending on timing and regulatory context (Hou and Tsuda 2022; Kuromori et al. 2022). In *P. pinea*, robust SAG accumulation, including under DIECA, may reflect efficient conjugation and storage of SA, a feature often associated with controlled mobilisation of defence signalling (Modesto et al. 2022). In *P. taeda*, strong SAG induction in infected controls without further amplification under MeJA suggests that SA-dominant responses may contribute to tolerance, consistent with the broader concept that resistant pines rely on balanced, well-coordinated JA and SA deployment rather than extreme activation of a single pathway (Nunes da Silva et al. 2025).

Transcriptional patterns support these mechanistic interpretations and further emphasise species-dependent defence architectures. Infection consistently upregulated *PAL* across species, corroborating the central role of phenylpropanoid metabolism in pine defence (Bagal et al. 2012; Ralph et al. 2006; Li et al. 2022a, b). Differences in the conditions required to induce *PAL* suggest that tolerant species may activate phenylpropanoid outputs more readily, while *P. pinaster* shows a more infection-dependent, and potentially delayed, activation. *GGPPS* regulation was strongly species and treatment dependent, consistent with the importance of terpene biosynthesis in conifer defence and the possibility that resin-related barriers contribute differentially among species, consitent with broader patterns of conifer defence against insects and pathogens (Coman et al. 2014; Kolosova and Bohlmann 2012; Trindade et al. 2016). The infection responsiveness of *MYC2*, a major regulator of jasmonate signalling, indicates that PWN challenge engages core JA transcriptional control, but the persistence of susceptibility in *P. pinaster* despite *MYC2* induction reinforces that activation of a single regulator is not necessarily predictive of outcome without coordinated engagement of additional transcriptional networks (Dombrecht et al. 2007; Du et al. 2017). The species-dependent behaviour of *AP2* and *E2F* further supports divergent regulatory programmes, where *AP2*-related regulation appears more inducible and broadly engaged in *P. taeda*, while *E2F* responses in *P. pinea* align more closely with hormonal or redox perturbation than with infection per se, consistent with roles in cell cycle and metabolic reprogramming under stress (Azevedo et al. 2010; Li et al. 2022a, b; Lin et al. 2020; Modesto et al. 2022; Song et al. 2022; Ye et al. 2024).

Overall, the data support a model in which PWN tolerance is associated with tighter coordination among jasmonate signalling, JA-Ile formation, SA conjugation, secondary metabolite deployment, and oxidative stress containment, whereas susceptibility involves strong but poorly integrated hormonal activation and less effective coupling to protective biochemical and transcriptional outputs. By combining jasmonate activation and inhibition with multi-layer phenotyping, this study moves beyond confirming MeJA efficacy and instead identifies species-specific regulatory signatures that help explain why closely related pines differ

so markedly in outcome following *B. xylophilus* infection. Several limitations of the present study should be acknowledged when interpreting these results. First, the asymmetric application schedule—MeJA applied once seven days before inoculation versus DIECA applied every two days—means that the two chemical treatments are not directly comparable in terms of treatment duration and intensity, which complicates the direct juxtaposition of "activation" versus "inhibition" responses. Second, while DIECA is an established inhibitor of JA biosynthesis, the absence of JA-pathway-specific marker gene measurements (e.g. LOX2, JAZ1, VSP2) immediately following MeJA and DIECA application means that the extent of pathway activation or inhibition was not directly verified in the present material. Future work should include such molecular confirmation to strengthen mechanistic interpretations. Third, as noted in the Materials and Methods, the differing ethanol content of the three treatment solutions (0%, 2.5%, and 10%) represents a potential confound that could not be fully controlled in the current experimental design. Finally, many of the correlative relationships discussed here, including those involving hormone-to-resistance associations, should be treated as hypothesis-generating rather than mechanistically established, pending functional validation.

Conclusion

This study shows that resistance to *B. xylophilus* emerges from a species-dependent, dynamically coordinated interplay among jasmonate signalling, additional hormonal pathways, and oxidative stress regulation, rather than from any single defence route. Across species, MeJA pretreatment enhanced defensive capacity, reducing nematode proliferation in stem tissues, attenuating foliar damage, and strengthening antioxidant and phenylpropanoid related responses, with the clearest phenotypic impact in the susceptible *Pinus pinaster*. Rather than simply confirming a priming effect, the multi-layer dataset indicates that improved outcomes are associated with tighter coupling between jasmonate signalling, secondary metabolism, and redox control.

Inhibition of jasmonate biosynthesis with DIECA did not universally compromise resistance. In *P. pinaster*, DIECA-treated infected plants showed reduced nematode numbers and milder symptom severity relative to untreated controls at 28 days postinoculation, while the more tolerant *P. pinea* and *P. taeda* maintained low disease expression irrespective of JA suppression. These patterns indicate that, when the JA pathway is constrained, pines can recruit compensatory defence modules, likely involving ROS-associated processes and broader hormonal adjustments. Consistent with this, DIECA altered pigment stability and secondary metabolite pools and modulated transcriptional regulators such as *AP2* and *E2F* in a species-dependent manner. Transcriptional responses further highlighted distinct defence architectures: *P. taeda* showed the most robust MeJA associated activation in infected plants, *P. pinea* displayed flexible, treatment-dependent regulation, and *P. pinaster* appeared to rely more strongly on exogenous priming to compensate for weaker endogenous coordination. Together with the strong infection-associated accumulation of SA and SAG and the context-dependent changes in ABA, these results support a flexible rerouting among JA, SA, and ABA pathways during PWN challenge.

Overall, our findings position jasmonate signalling as a major, but not exclusive, determinant of effective defence against pinewood nematode, and show that resistance outcomes depend on how JA integrates with SA- and ABA-linked responses and oxidative stress management. By identifying species-dependent hormonal, metabolic, and transcriptional signatures associated with tolerance versus susceptibility, this work provides a mechanistic framework to guide the development of priming strategies and the selection of resilient Pinus genotypes for pine wilt disease management.

Author contributions

ALV, MNS and MWV conceived and designed research. ALV and MNS conducted the experiment. VP analysed phytohormone concentrations. ALV analysed data. ALV wrote the manuscript. All authors read, corrected and approved the final manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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