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Can Copper Oxide or Potassium Phosphonate Increase *Pinus Pinaster* Tolerance to *Bursaphelenchus xylophilus*?

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ABSTRACT

Fungicide application may improve *Pinus pinaster* (Maritime pine) defenses against *Bursaphelenchus xylophilus* (pinewood nematode, PWN). To test this hypothesis, we evaluated the effects of copper oxide (CO) and potassium phosphonate (PP) on PWN-infected *P. pinaster* plants. The overall mortality rate of infected plants was 12.5%, regardless of the treatment. PP-treated plants displayed a significant reduction in nematode densities (up to 61%) and foliar symptoms compared to untreated controls. Twenty-eight-day post-infection, CO and PP increased oxidative stress proxies, such as lipid peroxidation (1.84- and 1.77-fold increases, respectively), and PP also enhanced antioxidant defenses, particularly flavonoid concentrations, which were 1.37-fold higher than those in CO-treated plants. In addition, both CO and PP reduced zinc and phosphorus concentrations in plant tissues, compared to controls, and CO treatment led to an increase in plant endophytic bacterial diversity, while PP reduced it. These findings demonstrate that CO and PP enhance *P. pinaster* tolerance to PWN by promoting the plant oxidant system, modulating mineral uptake, and altering plant–endophyte interactions. This study also highlights the potential to enhance resource use efficiency by extending fungicide applications beyond fungal pathogens to include nematodes, such as the PWN, offering a valuable approach for the integrated management of multiple pests.

KEYWORDS

Antioxidant activity; endophytic bacteria; minerals; pinewood nematode; pine wilt disease; *Pinus pinaster*

Introduction

Forests are globally dominated by species of the *Pinus* genus, which is extensively used in commercial plantations (Mbabazi, 2011). In Western Europe, particularly in Portugal, Spain, and France, as well as in parts of North Africa, *Pinus pinaster* (Maritime pine) is fundamental for the wood and timber industries (Chupin et al., 2015). In Portugal, despite its significant economic and social importance, *P. pinaster* production has declined in recent years, driven by losses in forest area and wood volume due to wildfires and the spread of *Bursaphelenchus xylophilus* (pinewood nematode, PWN) (Abad et al., 2016). PWN causes the pine wilt disease (PWD), a highly virulent and invasive pathology that leads to rapid tree mortality, especially in Asia, including Japan, China, Korea, and Taiwan (C. Vicente et al., 2012). Effective and sustainable control measures are yet to be developed,

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making the development of novel phytosanitary strategies an urgent necessity. Current control approaches, such as pheromone traps targeting the insect vector *Monochamus galloprovincialis* (Álvarez et al., 2016; Firmino et al., 2017) and breeding for resistance, are costly, time-consuming, and ineffective in mature plantations (Carrasquinho et al., 2018; Kurinobu, 2008). Promising research avenues include biological control agents, such as ectomycorrhizal fungi and diazotrophic bacteria that enhance plant defenses (Chu et al., 2019Nakashima et al., 2016, Nunes da Silva et al., 2019), and elicitors, such as methyl jasmonate (MeJA) and salicylic acid (SA), which activate systemic resistance and increase plant tolerance to pathogens (Chu et al., 2019; López-Villamor et al., 2022; Nunes da Silva et al., 2025).

The use of fungicides to support plant resistance against pests and diseases is well-documented (Daniel & Guest, 2005; Prasad et al., 2017). Copper oxide (CO), a fungicide commonly used in organic farming to control mildew (Cabús et al., 2017), has strong antimicrobial activity, including against nematodes (Burke et al., 2016; La Torre et al., 2018). Copper is also a vital micronutrient involved in plant defense pathways and enzymatic reactions (Borgatta et al., 2018; Mir et al., 2021). It can also form complexes with nematode proteins, disrupting enzymatic activity and metabolism, ultimately leading to nematode death (Mohamed et al., 2019; Tan et al., 2013). Commercial CO-based formulations (such as Speedwave™ Syngenta, NutriSelect® 75 WG Massó Agro Department, and Red™ Ascenza) are widely used for the preventive management of fungal and bacterial diseases in horticultural crops including tomato, lettuce, and pumpkin, as well as in fruit trees such as grapevine, kiwi, and citrus. On the other hand, potassium phosphonate (PP) is a systemic fungicide known to activate systemic acquired resistance (SAR) and enhance phenylalanine ammonia-lyase (PAL) activity, a key regulator of secondary metabolite production (Astaneh et al., 2018; Yáñez-Juárez et al., 2018). By promoting the PAL-pathway, PP could potentially strengthen *P. pinaster* defenses against PWD by inducing the accumulation of secondary metabolites such as soluble phenols and lignin, which are associated with increased resilience to PWN (Nunes da Silva et al., 2021, 2025). Although PP is extensively applied across Europe, including Portugal, in horticultural and fruit crops (via commercial products such as Cuprocol® Syngenta, NORDOX® JOVAGRO, and Cuprital® epagro) its application for controlling the PWN in *P. pinaster* remains undocumented. Nevertheless, considering its systemic properties and known efficacy against diverse pathogens, including fungi and oomycetes, its deployment against nematodes in pine stands may be a valuable tool, warranting further study and practical assessment.

The current sylvicultural limitations in PWN-control are partly due to its complex life cycle, involving an initial phytophagous phase, during which nematodes invade resin ducts and cause oxidative damage to tissues, and a subsequent mycophagous phase, where nematodes feed on fungi in dead trees (Futai, 2013; Espada et al., 2016; Yazaki et al., 2018). Oxidative stress plays a central role in PWD dynamics, with malondialdehyde (MDA) having been used as a marker for lipid peroxidation and oxidative damage in infected plants (Morales & Munné-Bosch, 2019; Nunes da Silva et al., 2025; Zas et al., 2015). Plants counteract this damage through antioxidant enzymes and secondary metabolites, including flavonoids and polyphenols (Baskar et al., 2018; Nunes da Silva et al., 2021; Tuladhar et al., 2021). Therefore, fungicides, including CO and PP, may enhance *P. pinaster* tolerance to PWN by promoting these antioxidant defenses. Fungicide application also affects mineral nutrient dynamics, which are critical to plant stress responses (Bala et al., 2018; Chan et al., 2021). While these effects have been described in other species, the impact of CO and PP on *P. pinaster* mineral composition and its implications for PWD resistance remains unknown. Concomitantly, plant-associated endophytic bacteria play vital roles in mineral uptake, growth promotion, and pathogen defense (Doornbos et al., 2012). In *P. pinaster*, bacterial communities have been implicated in the progression of PWD (Proença et al., 2017; Roriz et al., 2011; C. S. L. Vicente et al., 2011). As such, fungicides like CO and PP, known for their antibacterial properties, may modulate these communities, potentially influencing disease dynamics.

This study aimed to evaluate the potential of CO and PP to increase *P. pinaster* tolerance to the PWN during its phytophagous phase through a multidisciplinary approach, focusing on: (i) foliar symptom severity (chlorosis and chlorophyll content), (ii) nematode density in stem tissues, (iii) indicators of oxidative damage and antioxidant activity (e.g., anthocyanins, flavonoids, and lipid peroxidation), (iv) mineral composition (e.g., B, Cu, Fe, Zn, K, P), and (v) plant endophytic bacterial diversity. This work provides new insights into the role of fungicides in managing the PWD, aiming to extend the potential applications of compounds traditionally used as fungicides to the control of nematodes, thereby supporting integrated pest management and sustainable sylvicultural practices.

Materials and methods

Plant material and experimental design

For this study, 132 three-year-old *P. pinaster* plants (each with an average height of 120 ± 12 cm and diameter of 0.87 ± 0.04 cm) were sourced from the French-Landes provenance region. Plants were initially grown at the Misión Biológica de Galicia-CSIC (MBG-CSIC, Pontevedra, Spain; 42.4054° N, 8.6426° W) in two-liter pots containing a 1:1 (v/v) mixture of peat and perlite. The seedlings were then transferred to the Centro de Biotecnologia e Química Fina-Universidade Católica Portuguesa (CBQF-UCP, Porto, Portugal; 41.1539° N, −8.6733° W), where they were transplanted into five-liter pots with the same peat-perlite mixture. The assay was carried out in a controlled environment chamber with 16 hours of light and 8 hours of darkness at an ambient temperature of 25°C, from April 9 to May 14, 2019.

Fungicide treatments

Seven days before PWN infection, copper oxide (CO) and potassium phosphonate (PP) were applied to the aerial parts of the plants following the protocol adapted from Zas et al. (2015). A total of 20 ± 1.5 mL of solution was sprayed on the needles and stems of each plant. The experiment included 66 fungicide-treated *P. pinaster* plants: 33 were treated with a 0.2% CO suspension (commercial product, Nordox Copper 75 WG, 75% copper oxide), and 33 were treated with a 0.4% PP suspension (commercial product, Alexin 75 LS), in accordance with the manufacturer's recommended concentrations. An additional group of 66 plants served as untreated controls and were sprayed with deionized water.

Nematode culture and plant infection

Seven days after fungicide treatment, plants were inoculated with the virulent 17AS strain of *Bursaphelenchus xylophilus*. To prepare the inoculum, *Botrytis cinerea* (Pers) was first cultivated on mycoboxes containing barley seeds for 14 days at 25°C, following the methodology described by Nunes da Silva et al. (2015). The nematodes were then harvested using the Baermann funnel technique (Baermann, 1917), and the inoculum was adjusted according to Roriz et al. (2011) to create a suspension containing 2000 nematodes in 750 μL of sterilized water. For the inoculation, a stem incision was made, adapting the technique described by Futai (2003). Each PWN-inoculated group (untreated controls, copper oxide (CO)-treated, and potassium phosphonate (PP)-treated plants) consisted of 33 seedlings infected with 2000 PWNs. Additionally, a group of 33 nontreated plants was mock-inoculated with deionized water (untreated non-inoculated control). This resulted in four distinct treatment groups: mock-inoculated nontreated control plants (niCTR), PWN-inoculated nontreated control plants (iCTR), PWN-inoculated CO-treated plants (iCO), and PWN-inoculated PP-treated plants (iPP), comprised 33 replicates each. To prevent vector-mediated transmission of the nematode between trees, plants were spaced adequately to avoid contact between needles or branches. Additionally, plant handling was conducted using separate gloves for each plant treatment to avoid the risk of cross-contamination between infected and uninfected individuals.

Scoring of foliar symptoms, sampling and nematode quantification

To assess the extent of foliar symptoms, eight plants from each treatment group were evaluated at 7, 14, 21, 28, and 35 days after infection (dai). Symptom severity was assessed based on needle chlorosis and wilting, using a scale from 0 to 4: 0 = 0–10% symptomatic needle tissue; 1 = 11–33%; 2 = 34–66%; 3 ≥ 67%; and 4 = total needle chlorosis and wilting, adapted from Cadahia et al. (1991). Plant samples were collected at the specified time points, with five randomly selected plants from each treatment group. Plant needles were separated from the stems, ground in liquid nitrogen, and analyzed for photosynthetic pigments, malondialdehyde, total soluble phenols, flavonoid content, and mineral concentrations. The stems were used for nematode quantification and endophytic bacterial analysis. For nematode quantification, five defoliated plant stems were cut into small pieces (of approximately 0.5 cm) and processed using the Baermann funnel method. Nematodes were then counted under a transmitted light stereomicroscope, following the protocol outlined by Nunes da Silva et al. (2015).

Mineral and biochemical determinations

To quantify foliar pigments (anthocyanins, carotenoids, chlorophylls a and b), 500 mg of ground needles ($n = 5$) were extracted with 5 mL of cold 1 M Tris/acetone buffer solution (20:80, v:v, pH 7.8). Absorbance measurements were recorded at 470, 537, 647, and 663 nm. Pigment concentrations were calculated based on fresh weight, following the methodology described by Sims and Gamon (2002). For the quantification of soluble phenols and flavonoids, 50 mg of lyophilized needle tissue ($n = 5$) were extracted using an 80% aqueous methanol solution (v:v). Total soluble phenols were quantified using the Folin–Denis method (Marinova et al., 2005), with absorbance readings taken at 750 nm, and a gallic acid calibration curve was used for quantification. Flavonoids were determined by the aluminum chloride method (Zhishen et al., 1999), with absorbance readings at 510 nm and a catechin calibration curve. Lipid peroxidation was assessed by measuring malondialdehyde (MDA) levels in 100 mg of needle sample ($n = 5$), following the protocol outlined by H. Li (2000). Additionally, three plants from each treatment group were randomly selected at 28 days after infection (dai), for mineral analysis. For this, 500 mg of each needle sample were homogenized with 10 mL of 65% HNO_3 in a Teflon reaction vessel and heated using a Speedwave™ MWS-3+ microwave system (Berghof, Germany). The digestion procedure followed the protocol outlined by Santos et al. (2015). After digestion, the solutions were diluted to a final volume of 50 mL with ultrapure water. Mineral determination was performed using an inductively coupled plasma optical emission spectrometer (ICP-OES), Optima 7000 DV (PerkinElmer, USA), with a radial configuration.

Identification of plant endophytic bacteria

The analysis of the endophytic bacterial population was performed at the end of the assay (35 days after infection), using three randomly selected plants from each treatment group. For each plant, six stem segments were collected, with two replicates per segment, and processed according to the method outlined by Xie and Zhao (2008). To ensure sterility, the surfaces of the stem portions were disinfected with 75% ethanol. Each segment was then placed in nutrient agar (NA; Vegitone Nutrient Agar No 2, Speedwave™ Plus) medium, with the vascular tissue facing the culture medium, and incubated for 3 days at 26°C. After incubation, pure bacterial cultures were obtained by visually assessing and isolating morphologically distinct colonies. For the molecular determination of bacterial identity, total genomic DNA was extracted from each bacterial isolate using the heat-shock method, as described by Calheiros et al. (2010). DNA concentration and integrity were assessed spectrophotometrically using a nanophotometer (Implen GmbH, München, Germany), after which the 16S rRNA gene was amplified via PCR using primers 27F (5”-GAGTTTGATCCTGGCTCA-3‘) and 1493 R (5’-TACCTTGTTACGACTT-3”). The final PCR products were analysed via electrophoresis in a 1% agarose gel in Tris-EDTA (TAE) buffer, stained with Gel NutriSelect® (Biotium, Inc., USA). The PCR products from all 22 bacterial isolates were sequenced by STAB VIDA, Lda. (Lisbon, Portugal), and identified using the Basic Local Alignment Search Tool (BLASTn, National Center for Biotechnology Information, USA).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism v.8 (GraphPad Software, USA). The effects of plant treatment (T), time point (Tp), and their interaction (T × Tp) on the number of nematodes in stem tissues, photosynthetic pigments, biochemical compounds, and mineral concentrations were evaluated considering treatment (T), time point (Tp), and their interaction as fixed factors. A mixed-effects model (REML), using the restricted likelihood method, was applied to analyze the data. A significance level of $p < .05$ was used to determine significant differences between treatments. To explore the relationships among all variables measured at 28 days after infection (dai), Pearson’s correlation matrix was employed.

Results

Mock-inoculated nontreated control plants (niCTR) exhibited no foliar symptoms throughout the entire experiment (Figure 1). Foliar damage was first observed at 21 days after inoculation (dai) in infected plants, including both treated and untreated groups. At this stage, 25% of plants treated with copper oxide (iCO) and potassium phosphonate (iPP) exhibited Stage 1 symptoms, while 75% remained asymptomatic. In contrast, 62.5% of the mock-inoculated, untreated control plants (iCTR) showed needle damage (37.5% at Stage 1 and 25% at Stage 2) leaving only 37.5% of plants healthy.

By 28 dai, needle damage remained at 62.5% in iCTR plants, with 12.5% progressing to Stage 3 symptoms. Similarly, 62.5% of iCO-treated plants showed symptoms, limited to Stages 1 (37.5%) and 2 (25%). In comparison, only 37.5% of iPP-treated plants displayed symptoms (25% at Stage 1 and 12.5% at Stage 3).

At 35 dai (the end of the assay), mortality reached 12.5% across all treatment groups (iCTR, iCO, and iPP), while 25% plants in each group remained asymptomatic. Among iCTR plants, 50% exhibited Stage 3 symptoms and 12.5% were at Stage 2. In both iCO- and iPP-treated groups, 25% of plants exhibited Stage 3 symptoms. Additionally, iCO-treated

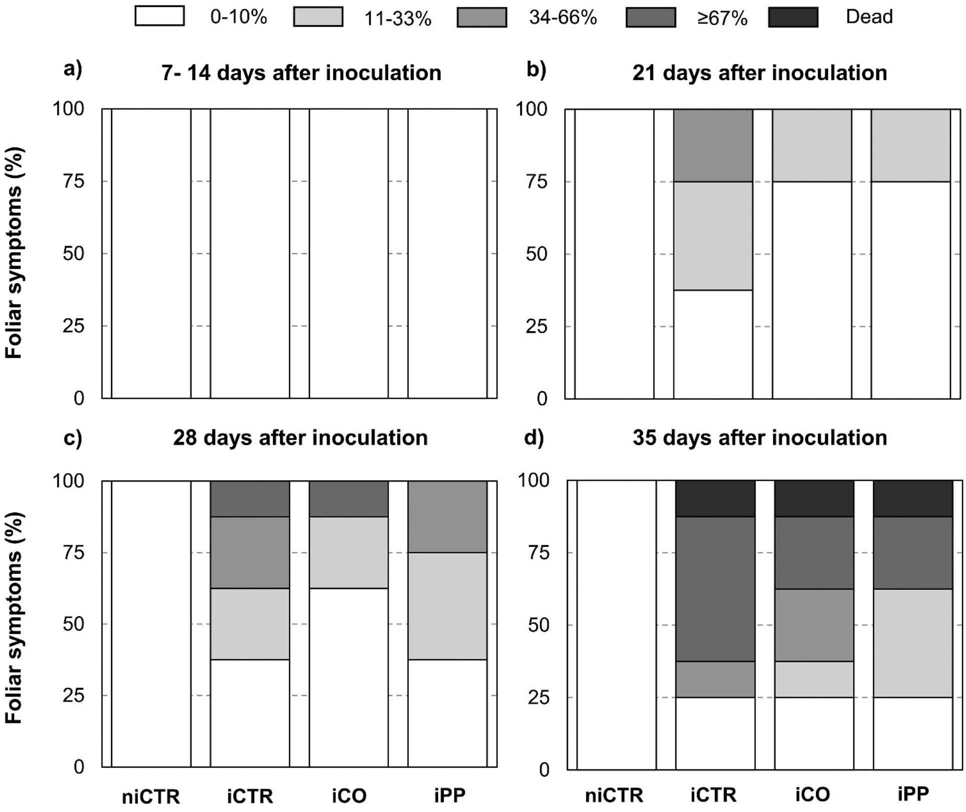


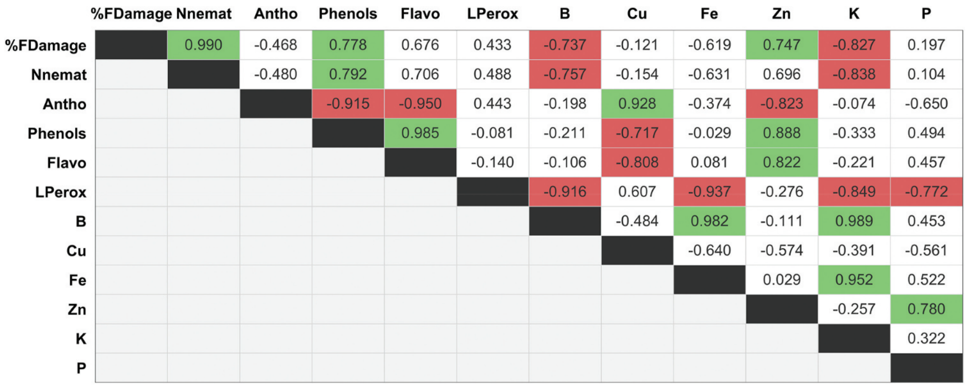
Figure 1. Degree of needle area affected by disease symptoms (%) at different time points: (a) 7–14 days, (b) 21 days, (c) 28 days, and (d) 35 days after inoculation with pinewood nematodes (PWNs). Treatments: mock-inoculated nontreated control plants (niCTR), PWN-inoculated nontreated control plants (iCTR), PWN-inoculated CO-treated plants (iCO), and PWN-inoculated PP-treated plants (iPP).

plants showed 12.5% at Stage 1 and 25% at Stage 2, whereas iPP-treated plants had 37.5% with Stage 1 symptoms and no Stage 2 damage.

Needle symptom severity was strongly correlated with nematode density in the plant stems (Figure 2).

In fungicide-treated plants, the number of nematodes was significantly lower compared to the infected plants without fungicide treatment (Figure 3, Table 1). From 7 to 35 dai, nematode count significantly increased by 18.8-fold, and by the end of the assay (35 dai), the number of nematodes in iCO (16667 ± 14612) and iPP (11227 ± 10307) plants was 0.42 and 0.61-fold lower than iCTR plants (28760 ± 3540).

Both inoculation treatment (T) and time point (Tp) had a significant impact on anthocyanin concentrations, whereas Tp and T × Tp interaction significantly influenced carotenoid content (Table 1). In PWN-inoculated CO-treated plants (iCO), anthocyanin concentration gradually increased up to 35 days after infection (dai), with a significant 1.32-fold increase observed at 28 dai (reaching 16.82 ± 4.72 μmol/g needle) (Figure 4). In inoculated PP-treated plants (iPP), the highest anthocyanin concentration (37.80 ± 10.89 μmol/g needle) was recorded at 15 dai. Conversely, in mock-inoculated nontreated control plants (niCTR), anthocyanin content progressively and significantly decreased from 7 to 28 dai (0.43-fold) and slightly increased at 35 dai (reaching 9.80 ± 1.94 μmol/g needle). Despite the significant effects of treatment (T) and time point (Tp) on anthocyanin levels, no significant differences were found in the other needle metabolites analyzed (Table 1, Figure 4, Supplementary Figure S1). Alike anthocyanin concentrations, both soluble phenol and flavonoid concentrations were significantly affected by the interactive effects of T × Tp (Table 1), with anthocyanin accumulation being negatively correlated with both soluble phenols and flavonoids (Figure 2). Mock-inoculated nontreated control plants (niCTR) showed a gradual decrease in phenol and flavonoid accumulation over time (0.70- and 0.49-fold, respectively), with a significant increase in both at 28 dai (1.65-fold increase) (Figure 4). Treatment with iCO led to a gradual decrease in phenol accumulation throughout the experimental period (0.71-fold), while flavonoid accumulation increased gradually until 21 dai (1.39-fold), followed by a decrease until the



	%FDamage	Nnemat	Antho	Phenols	Flavo	LPerox	B	Cu	Fe	Zn	K	P
%FDamage		0.990	-0.468	0.778	0.676	0.433	-0.737	-0.121	-0.619	0.747	-0.827	0.197
Nnemat			-0.480	0.792	0.706	0.488	-0.757	-0.154	-0.631	0.696	-0.838	0.104
Antho				-0.915	-0.950	0.443	-0.198	0.928	-0.374	-0.823	-0.074	-0.650
Phenols					0.985	-0.081	-0.211	-0.717	-0.029	0.888	-0.333	0.494
Flavo						-0.140	-0.106	-0.808	0.081	0.822	-0.221	0.457
LPerox							-0.916	0.607	-0.937	-0.276	-0.849	-0.772
B								-0.484	0.982	-0.111	0.989	0.453
Cu									-0.640	-0.574	-0.391	-0.561
Fe										0.029	0.952	0.522
Zn											-0.257	0.780
K												0.322
P												

Figure 2. Pearson's correlation matrix between the different variables analyzed. Significant correlations ($p < .05$) are shown as: green – positive correlations; red – negative correlations. Abbreviations: % fdamage – percentage of needle damage; nnemat – number of nematodes per plant stem; antho – anthocyanins concentrations; flavo – flavonoids concentration; LPerox – lipid peroxidation (viz. MDA concentration).

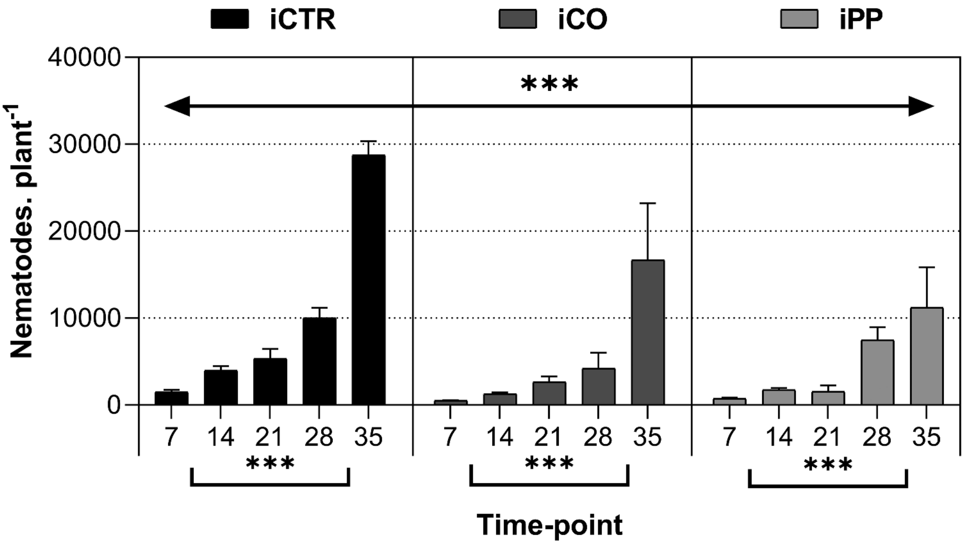


Figure 3. Number of nematodes in nontreated (iCTR), CO-treated (iCO), and PP-treated (iPP) *Pinus pinaster* plants at 7, 14, 21, 28, and 35 days after inoculation with *Bursaphelenchus xylophilus*. The values represent the means of 5 biological replicates ± standard errors of the means. Significance levels of treatments and time points: ***. $p < .001$; **. $p < .01$; *. $p < .05$; ns. not significant.

Table 1. Effect of time-point (Tp, 7, 14, 21, 28, and 35 days after inoculation) and plant treatments (T, non-treated infected control seedlings (iCTR), inoculated CO-treated seedlings (iCO), or inoculated PP-treated seedlings (iPP)) and their interaction (T x Tp) on the number of nematodes, anthocyanin, carotenoids, chlorophyll-a and chlorophyll-b, lipid peroxidation, total soluble phenols, and flavonoids. Significant *p* values (<0.05) are indicated in bold.

Response variable	Factor	*F* ratio	*P* value
Number of nematodes (nematodes.plant^{-1})	Treatment (T)	8.72	**0.0005**
	Time-point (Tp)	31.72	**<0.0001**
	T ´ Tp	2.54	**0.0188**
Anthocyanin (μmol.g^{-1} leaf)	Treatment (T)	7.11	**0.0017**
	Time-point (Tp)	15.00	**<0.0001**
	T ´ Tp	18.90	**<0.0001**
Carotenoids (μmol.g^{-1} leaf)	Treatment (T)	0.63	0.5389
	Time-point (Tp)	2.75	**0.0489**
	T ´ Tp	7.89	**<0.0001**
Chlorophyll-a (μmol.g^{-1} leaf)	Treatment (T)	1.35	0.2952
	Time-point (Tp)	7.03	**0.0005**
	T ´ Tp	2.55	**0.0211**
Chlorophyll-b (μmol.g^{-1} leaf)	Treatment (T)	1.21	0.3336
	Time-point (Tp)	1.58	0.2103
	T ´ Tp	11.16	**<0.0001**
Total soluble phenols (mg.g^{-1} leaf)	Treatment (T)	3.69	**0.0308**
	Time-point (Tp)	18.05	**<0.0001**
	T ´ Tp	5.14	**<0.0001**
Total flavonoid content (mg.g^{-1} leaf)	Treatment (T)	14.12	**<0.0001**
	Time-point (Tp)	8.70	**0.0005**
	T ´ Tp	3.91	**0.0009**
Malondialdehyde (μmol.g^{-1} leaf)	Treatment (T)	18.10	**0.0002**
	Time-point (Tp)	10.75	**0.0002**
	T ´ Tp	0.90	0.5269

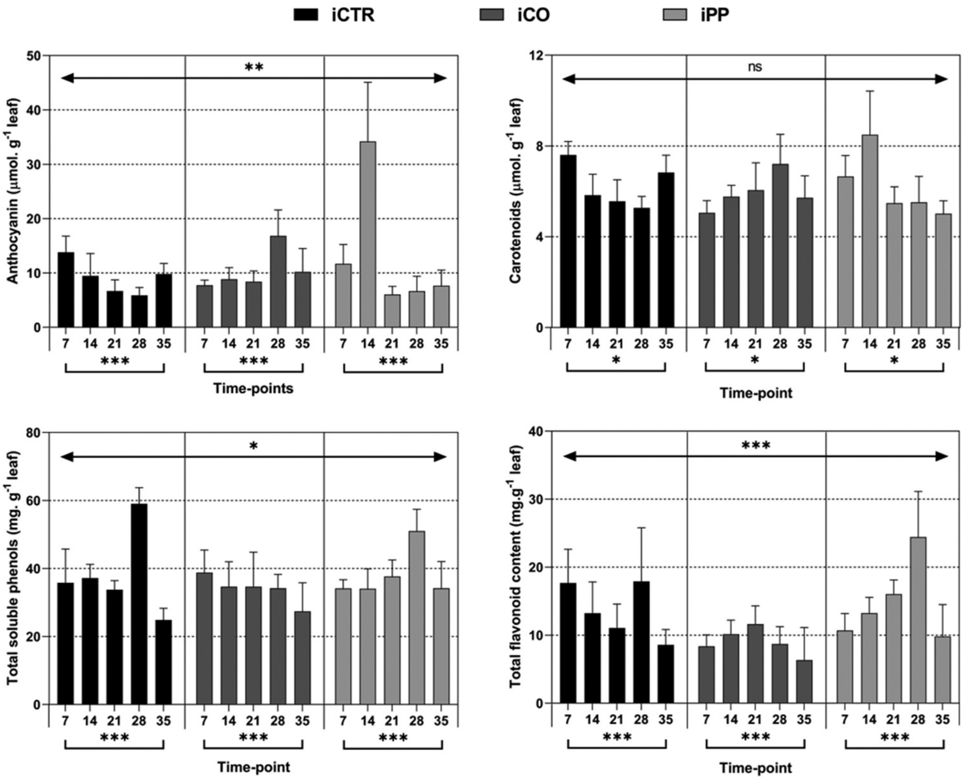


Figure 4. Anthocyanin, carotenoid, soluble phenols, and flavonoid concentrations in untreated (iCTR), CO-treated (iCO), and PP-treated (iPP) *Pinus pinaster* plants at 7, 14, 21, 28, and 35 days after inoculation with *Bursaphelenchus xylophilus*. The values represent the means of 5 biological replicates ± standard errors of the means. Significance levels of treatments and time points: ***$p < .001$; **$p < .01$; *$p < .05$; ns. not significant.

end of the experiment (0.76-fold). In contrast, iPP-treated plants showed a general increase in soluble phenol and flavonoid concentrations, peaking at 28 dai (1.49-fold and 2.28-fold increases, respectively), before decreasing at 35 dai (by 0.67- and 0.40-fold, respectively) (Figure 4). Malondialdehyde (MDA) levels at 28 dai progressively and significantly increased in infected plants (both treated and untreated), although they slightly decreased by 35 dai (Figure 5). In addition, MDA content significantly increased in all treatments throughout the experimental period: by 1.42-fold in iCTR, 1.21-fold in iCO, and 1.57-fold in iPP, compared to niCTR.

The concentrations of all analyzed minerals (B, Cu, Fe, Zn, K, and P) were significantly influenced by plant treatment (T) (Table 2), with nematode density in plant tissues being negatively correlated with B and K concentrations (Figure 2). The lowest boron (B) concentration was observed in iCO-treated plants, being 1.5-fold lower than mock-inoculated nontreated control plants (niCTR), which exhibited the highest B concentration observed (36.9 ± 3.7 µg/g) (Figure 6). The average copper (Cu) concentration was highest in iCO-treated plants (189.7 ± 39.1 µg/g), whereas niCTR, iCTR, and iPP plants showed significantly lower average concentrations, which averaged only 3 ± 0.4 µg/g (Figure 6). Iron (Fe) concentrations were lower

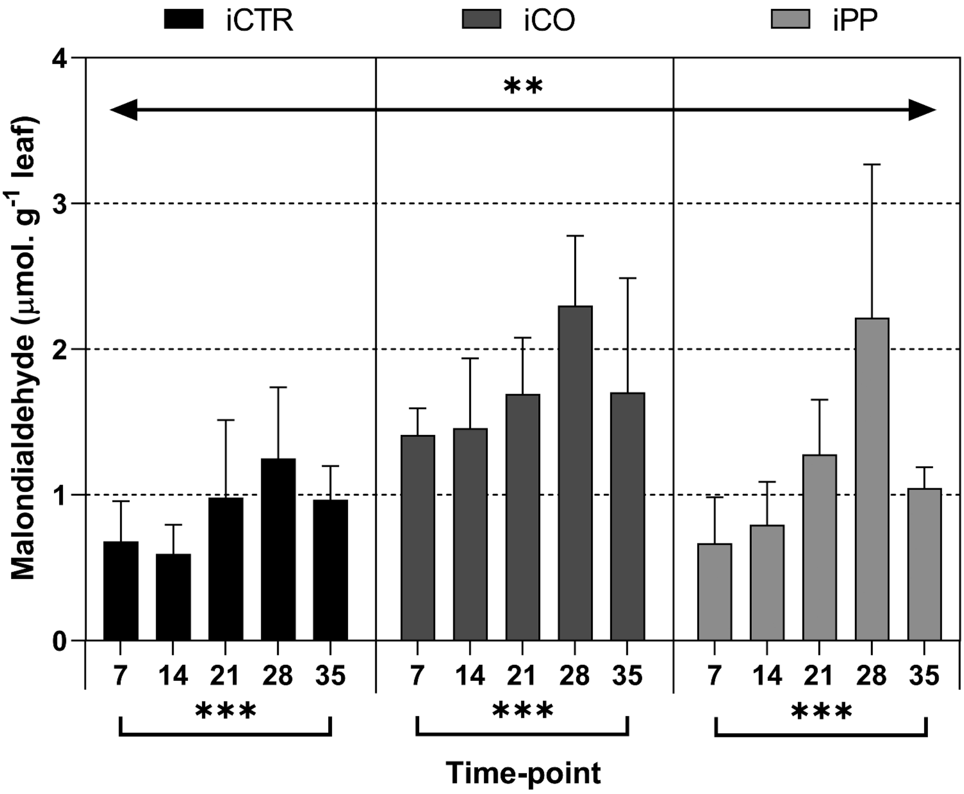


Figure 5. Malondialdehyde (MDA) concentration in untreated (iCTR), CO-treated (iCO), and PP-treated (iPP) *Pinus pinaster* plants at 7, 14, 21, 28, and 35 days after inoculation with *Bursaphelenchus xylophilus*. The values represent the means of 5 biological replicates ± standard errors of the means. Significance levels of treatments and time points: ***$p < .001$; **$p < .01$; *$p < .05$; ns. not significant.

Table 2. Effect of plant treatments at 28 days after inoculation (T, mock-inoculated non-treated control seedlings (niCTR), non-treated infected control seedlings (iCTR), inoculated CO-treated seedlings (iCO), or inoculated PP-treated seedlings (iPP)) on the concentration (µg.G-1) of micronutrients (min) and macronutrients (MaN) in leaf tissues. Significant *p* values (<0.05) are indicated in bold.

	Response variable	Factor	*F* ratio	*P* value
MiN	B (µg.g^{-1} leaf)	Treatment (T)	5.05	**0.0092**
	Cu (µg.g^{-1} leaf)	Treatment (T)	136.8	**<0.0001**
	Fe (µg.g^{-1} leaf)	Treatment (T)	6.88	**0.0023**
	Zn (µg.g^{-1} leaf)	Treatment (T)	7.917	**0.0011**
MaN	K (µg.g^{-1} leaf)	Treatment (T)	8.06	**0.0010**
	P (µg.g^{-1} leaf)	Treatment (T)	8.67	**0.0007**

in iCO-treated plants (0.11 ± 0.02 µg/g) and 1.5-fold higher in niCTR plants (Figure 6). Additionally, iCTR plants had the highest zinc (Zn) concentration (84.88 ± 11.17 µg/g), while iCO-treated plants had the lowest Zn concentration (by 0.4-fold). Potassium (K) concentrations were similar across infected plants (iCTR, iCO, and iPP), averaging around 7.6 ± 2.2 mg/g, whereas niCTR plants exhibited the highest K concentration (by 1.6-fold). Finally, iCTR-treated plants had the

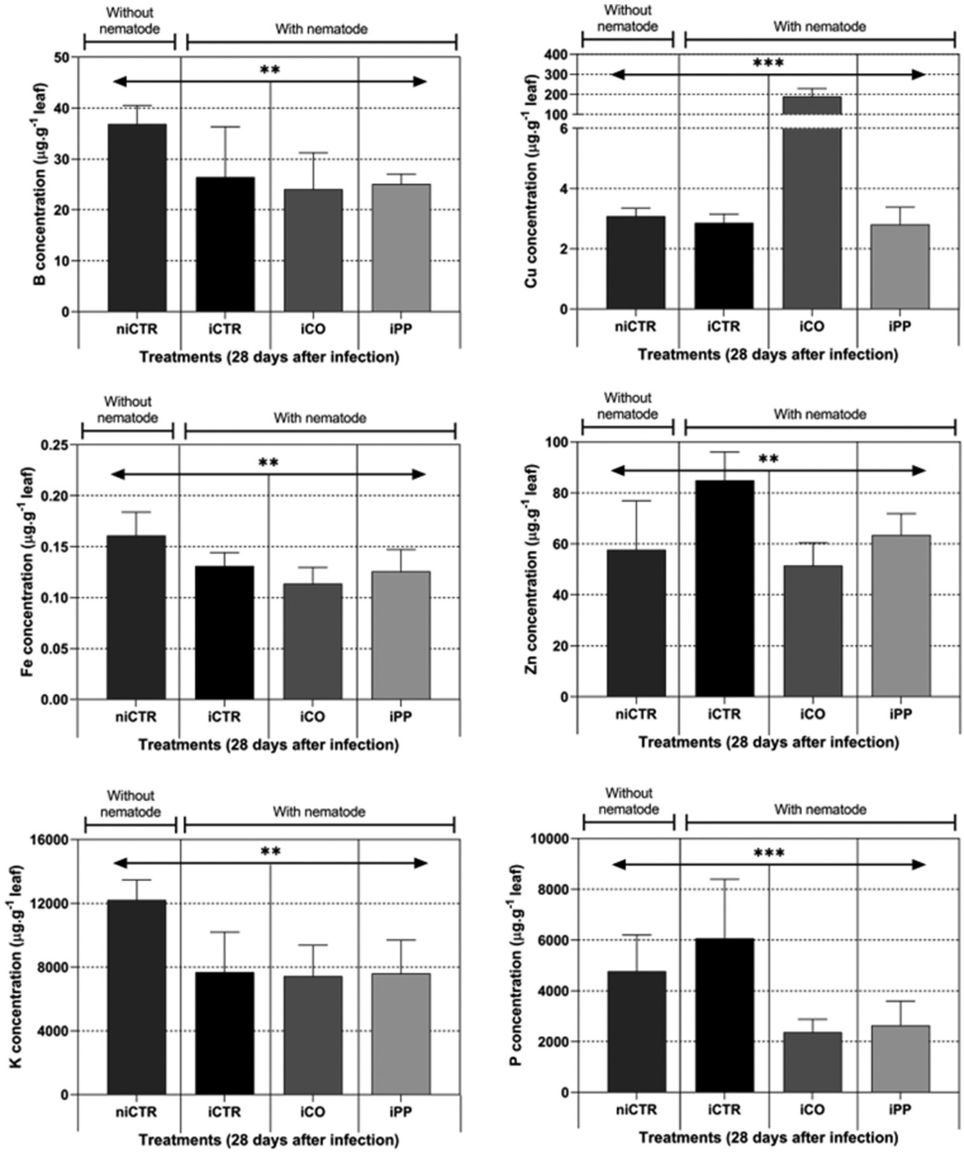


Figure 6. Boron (a), copper (b), iron (c), zinc (d), potassium (e), and phosphorus (f) concentrations in mock-inoculated nontreated *Pinus pinaster* plants (niCTR) and in nontreated (iCTR), CO-treated (iCO), and PP-treated (iPP) plants at 28 days after inoculation with *Bursaphelenchus xylophilus*. The values represent the means of 5 biological replicates ± standard errors of the means. Significance levels of treatments and time points: ***$p < .001$; **$p < .01$; *$p < .05$; ns. not significant.

highest phosphorus (P) concentration (6.1 ± 2.3 mg/g), while iCO-treated plants had the lowest (by 0.4-fold) (Figure 6).

The groups with the highest endophytic bacterial diversity were iCO, which hosted four genera, and iCTR, with three genera. In contrast, niCTR exhibited two genera, while iPP had only one (Figure 7). The predominant genera identified were *Pseudomonas*, represented by two species: *P. fluorescens*, present in all groups, and *P. monteilii*, which was exclusive to

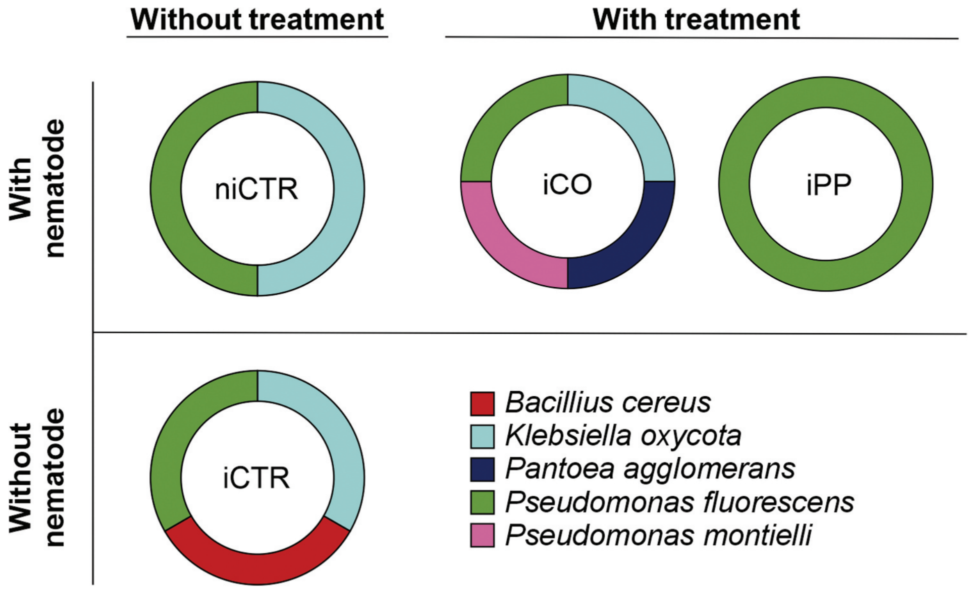


Figure 7. Bacterial populations in mock-inoculated nontreated *Pinus pinaster* plants (niCTR) and in nontreated (iCTR), CO-treated (iCO), and PP-treated (iPP) plants 35 days after inoculation with *Bursaphelenchus xylophilus*.

iCO. The second most abundant genus was Klebsiella, represented by a single species, *K. oxytoca*, found in all groups except iPP. Other identified genera included Bacillus, represented by B. cereus in iCTR, and Pantoea, represented by *P. agglomerans*, which was exclusive to iCO.

Discussion

At the end of the experimental period, fungicide treatment did not affect significantly the extent of needle chlorosis (Figure 1), but nematode density in plant stems, by up to 0.58-fold, compared to nontreated controls (Figure 3). Similar symptom-amelioration effects and reductions in PWN populations have been observed in *P. pinaster* plants treated with plant-defense elicitors such as MeJA, SA, and BTH (López-Villamor et al., 2022; Nunes da Silva et al., 2021). However, this study is the first to highlight the potential of fungicides in achieving comparable effects. Interestingly, despite CO and PP treatments reduced nematode multiplication, they did not prevent cellular damage, as MDA concentrations increased in plant tissues, particularly in fungicide-treated plants: from 7 dpi for CO and at 28 dpi for PP (Figure 5). MDA accumulation reflects damage caused by free radicals, including necrosis and the destruction of parenchyma, xylem, and phloem cells (Apel & Hirt, 2004; Yamada, 2008), but MDA accumulation has been observed to occur following PWN infection after plant elicitation with chitosan (Nunes da Silva et al., 2021) and MeJa, particularly in species with high tolerance to the pathogen, such as *P. pinea* (Nunes da Silva et al., 2025). In the present work, fungicide-treated plants also generally had higher concentrations of anthocyanins, carotenoids, and flavonoids, particularly with PP treatment. This suggests that fungicide treatment may result in a strong antioxidant response to

B. xylophilus, mitigating oxidative damage through the accumulation of molecules with antioxidant properties, such as anthocyanins and flavonoids (Bali et al., 2018; Gökbayrak & Gözel, 2022; López-Villamor et al., 2022). Interestingly, nematode density in plant tissues was positively correlated with soluble phenol concentrations (Figure 2). This pattern aligns with previous work by Zas et al. (2015) and Nunes da Silva et al. (2025), who reported a positive correlation between B. *xylophilus* migration, multiplication, and polyphenol accumulation in *P. pinaster* needles. Importantly, CO is known for its strong antimicrobial properties against nematodes (Kim et al., 2019; La Torre et al., 2018; Tan et al., 2013), while PP enhances the activity of phenylalanine-ammonium lyase (PAL), a key enzyme in the production of secondary metabolites associated with plant resistance to pathogens (Astaneh et al., 2018; Kahromi & Khara, 2021). These mechanisms likely contributed to the observed reduction in nematode density, and subsequent studies should evaluate their role in fungicide-induced PWD amelioration.

Plant defense against abiotic and biotic stresses is also benefited by the minerals absorbed by plant roots (Ahanger & Ahmad, 2019; Pathak et al., 2020). Specifically, in *Pinus massoniana* plants infected with *B. xylophilus*, needle mineral content correlated significantly with the plant's tolerance to nematodes (Zou & Sun, 2000). However, fungicides can alter mineral composition in plant tissues (dos Motta-Romero et al., 2021; Santos Silva et al., 2020; Shahid et al., 2018). In this study, inoculated plants had generally lower concentrations of B, Fe, and K, compared untreated mock-inoculated plants (niCTR, Figure 2 and 6), aligning with previous evidence on the decrease of B and Fe in *P. pinaster* needle tissues following PWN infection (López-Villamor et al., 2022). As *B. xylophilus* rapidly reproduces and migrates through the vascular system of infected trees, feeding on parenchyma and epithelial cells of the resin canals, it causes disruptions in water and transport, likely impairing the plant's ability to transport nutrients (Yazaki et al., 2018). Boron and K play a key role in plant metabolism, cell wall structure and defense mechanisms, and the lower concentrations observed in PWN-infected plants may render the plants more vulnerable to further damage (Mukherjee et al., 2019). Likewise, Fe is essential for enzymes like catalase, cytochrome oxidase, and peroxidase that play a central role in plant–pathogen interactions and, as such, reduced Fe concentrations following infection can interfere with Fe-dependent antioxidant mechanisms (Chihani-Hammas et al., 2023), which might have contributed to the negative correlation between Fe and lipid peroxidation (Figure 2). Overall, the B, P, and Fe impairments observed here may be both a cause and effect of increased plant susceptibility to the nematode infection, creating a cycle of declining plant health. These pieces of evidence underscore the importance of conducting field surveys in pine stands to identify potential nutritional deficiencies that may compromise plant growth and resilience to subsequent infections, particularly in trees that do not succumb to *B. xylophilus* infection and are retained within the stand.

Zinc and P levels were also influenced by nematode infection, but unlike the trends observed for B, P, and Fe, these effects were evident only in the absence of fungicide treatment (Figure 6). As infection progresses and oxidative stress increases in infected plants, Zn and P play a vital role in plant immunity the activation of antioxidant enzymes, such as superoxide dismutase (SOD), and cell wall strengthening (Cabot et al., 2019). This need for increased defense-related molecules and metabolites might drive the accumulation of Zn and P in infected tissues, particularly in untreated plants in which PWN colonization rates were higher, as supported by negative correlation between P and lipid peroxidation (Figure 2). Importantly, iCO resulted in extremely high Cu concentration in plant needles, exceeding the recommended concentrations essential for plant growth (Ivanov et al., 2016), underpinning the need to finetune iCO application to prevent potential phytotoxicity and ensure sustainable management practices in pine stands.

In addition to their role in modulating secondary and mineral metabolisms, CO and PP can mitigate bacterial diseases by activating various defense pathways, including SAR and LAR (INTAGRI, 2017), leading to the production of exudates that alter the plant microbiome (La Torre et al., 2018; Lebeis et al., 2015; Liu et al., 2017, 2020; Mannaa et al., 2020). Since endophytic bacteria associated with *B. xylophilus* are likely involved in infection dynamics (Alves et al., 2018; Kim et al., 2019), this study examined how fungicide treatment affects these bacterial communities, highlighting important shifts in PWD-associated bacteria (Figure 7). For example, niCTR contained only two bacterial species, *Klebsiella oxytoca* and *Pseudomonas fluorescens*, with *K. oxytoca* being found in all treatments except iPP and *P. fluorescens* being present in all treatments. *K. oxytoca* promotes plant growth through nitrogen fixation and phosphorus solubilization (de Santi Ferrara et al., 2012; Pavlova et al., 2017; Walpola et al., 2014), and it is associated with *P. pinaster* in Portugal (Roriz et al., 2011). On the other hand, *P. fluorescens* is a plant growth-promoting rhizobacterium that enhances growth and induces systemic resistance (Hol et al., 2013; Panpatte et al., 2016). iCTR also contained *Bacillus cereus*, which has nematocidal properties against *B. xylophilus* in *P. massoniana* (L. Li et al., 2020). Plants treated with iCO were colonized by *P. monteilii*, a member of the *Pseudomonas putida* group, known for promoting growth and inducing resistance to root rot (Anzai et al., 2000), as well as for its role in resistance against *B. xylophilus* (Kim et al., 2019; Pandya & Desai, 2014). Additionally, iCO plants harbored *Pantoea agglomerans*, a species with biocontrol activity and the ability to produce auxins (Dutkiewicz et al., 2016; Luziatelli et al., 2020). While previous studies have linked these endophytic bacteria with *B. xylophilus* and pine trees (López-Villamor et al., 2022; Proença et al., 2010; Roriz et al., 2011), this study is the first to identify *Pantoea agglomerans* in *B. xylophilus*-infected pine trees. Overall, CO treatment appeared to alter the diversity of the endophytic bacterial community in *P. pinaster*, favoring species that enhance resistance to *B. xylophilus*, suggesting that, aside from contributing to plant antioxidant activity, CO application fosters beneficial bacterial interactions, contributing to improved plant defense against the PWN. Despite the reduction in endophytic bacterial diversity observed in iPP plants, this effect may not necessarily indicate a disruption of beneficial microbial associations. Previous studies have reported that foliar application of PP of *Quercus robur* plants does not significantly impact endophytic bacterial communities in trees (Solla et al., 2021). Similarly, phosphite treatments have shown minimal alterations in bacterial communities within the rhizosphere of *P. radiata* treated plants (Leitão et al., 2024), suggesting that the observed reduction in culturable endophytes may result from selective antimicrobial activity or changes in host metabolite profiles rather than widespread microbial suppression. Future studies employing metagenomic approaches are needed to comprehensively assess the impacts of PP on the structure and function of microbial communities in *P. pinaster*.

Conclusions

This study highlights the promising potential of copper oxide and potassium phosphonate in modulating plant defense mechanisms and reducing nematode proliferation in *P. pinaster*. Although overall plant mortality and mean foliar symptom severity were not significantly reduced during the 35-day observation period, both treatments, particularly PP, effectively suppressed nematode multiplication within plant tissues. This reduction may help limit the availability of viable nematodes for further transmission, potentially decreasing the risk of spread to surrounding trees. The relatively short duration of the trial may have limited the detection of long-term physiological or phenotypic benefits; however, clear shifts in oxidative stress markers and enhanced accumulation of secondary metabolites, such as anthocyanins, soluble phenols, and flavonoids, suggest that early-stage biochemical responses were activated by both fungicides. Finally, although water availability was not manipulated in this study, the observed improvements in antioxidant and mineral responses, particularly under pathogen pressure, support the hypothesis that these treatments could confer additional resilience under combined stress scenarios, such as drought. Further field-based and long-term investigations will be essential to confirm the broader applicability of these findings across developmental stages and environmental contexts.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

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

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